

Requirements for Empirical Immunogenicity Trials, Rather than Structure-Based Design, for Developing an Effective HIV Vaccine



Introduction

Human immunodeficiency virus type 1 (HIV-1) is the virus we know most about (Klatt et al. 2010) and its antigenic structure has been analyzed in considerable detail (Norrby 1993; Wyatt et al. 1998; Zolla-Pazner 2004; Montero et al. 2008; Hoxie 2010). Many studies have been devoted to the HIV-1 envelope glycoprotein (Env) present as spikes embedded in the viral membrane. Each functional spike consists of trimers of three gp120 surface glycoproteins non-covalently attached to three gp41 transmembrane glycoproteins. In addition to functional trimers, the viral membrane may also display gp120-gp41 monomers, single gp41 molecules and other non-functional fragments (Poignard et al. 2003; Moore et al. 2006; Crooks et al. 2007). The antigenicity of the spikes has been studied extensively and numerous Env epitopes have been shown to induce a wide range of neutralizing antibodies in HIV-1 infected individuals (Zolla-Pazner 2004; Javaherian et al. 1989; Muster et al. 1993; Zwick et al. 2001c; Poignard et al. 2001; Gorny et al. 2002; Dhillon et al. 2007; Li et al. 2007; Hioe et al. 2010). As a result, the antigenic sites of the Env protein have been considered promising candidates for developing a preventive HIV vaccine able to provide prophylactic immunity (Zwick 2005; Zwick and Burton 2007; Willey and Aasa-Chapman 2008; Binley 2009; Stamatatos et al. 2009).

The strategy of trying to develop a vaccine by studying the interaction of human Mabs with HIV-1 Env epitopes has been called reverse vaccinology because it attempts to generate a vaccine from the known structure of neutralizing antibodies rather than by following the reverse, more common approach of generating protective

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antibodies by immunization (Burton 2002, 2010). When crystallographers study the structure of an Env epitope recognized by a neutralizing Mab, they often claim that their data will help the rational design of vaccine immunogens able to elicit antibodies endowed with the same neutralizing capacity as the Mab used to define the epitope (Schief et al. 2009; Saphire et al. 2001; Karlsson-Hedestam et al. 2008).

In vaccine development, the term design usually means selecting a vaccine candidate and finding appropriate vaccine formulations, schedules, adjuvants and routes of administration. The strategy of rational, structure-based design discussed here is more circumscribed since it refers to the development and optimization of a vaccine immunogen within the restricted context of a single Mab-antigen pair. This approach assumes that if a viral antigen can be modified so that it binds better to a neutralizing Mab, it will have acquired the immunogenic capacity of eliciting neutralizing polyclonal antibodies (Van Regenmortel 2011a).

It will be argued here that the claims of reverse vaccinology are based (1) on misconceptions regarding the nature of protein epitopes and of immunological specificity, (2) on the assumption that a discontinuous epitope defined by X-ray crystallography can act as an effective vaccine immunogen on its own without being embedded in a multi-epitopic protein that always induces many different types of antibodies, (3) on the belief that effective vaccine immunogens can be predicted from the antigenic structure of viral antigens bound to neutralizing Mabs.

Research carried out over the last two decades has demonstrated that reverse vaccinology has not been able to guide the design of an HIV vaccine. The present review expands on an earlier analysis of the limitations of reverse vaccinology (Van Regenmortel 2011b) and shows that antibody polyspecificity explains why elucidating the structure of viral epitopes recognized by a neutralizing Mab is of little use for discovering immunogens capable of inducing a protective immune response. It may be advisable, therefore, to shift the current emphasis in HIV vaccine research from a rational design approach based on the structural analysis of Env epitopes to a more empirical approach that investigates possible therapeutic benefits that may result from the administration of a variety of HIV-1 vaccine immunogens (Caputo et al. 2009).

The Nature of Protein Epitopes

The regions of antigen molecules recognized by antibody molecules are called epitopes while the regions of Antibodies that bind to epitopes are called paratopes. Both regions are identified by solving the 3D structure of antigen-antibody complexes and determining which amino acid residues in the two partners make contact with each other.

Discontinuous Epitopes

The vast majority of protein epitopes have been found to be discontinuous (Atassi and Smith 1978; Barlow et al. 1986; Sundberg and Mariuzza 2002) and to consist of two to five short stretches of residues that are distant in the protein sequence but are brought together by the folding of the peptide chain. Most HIV-1 epitopes that have been characterized are discontinuous epitopes (Thali et al. 1991; Moore and Ho 1993; Van Cott et al. 1995b; Zwick et al. 2003b). The atomic groups forming a discontinuous epitope are not held together by internal chemical bonds and the epitope is a 3D entity that emerges only because the chain acts as a scaffold. If the scaffold is perturbed, for instance by a change in chain conformation or by denaturation of the protein, the epitope ceases to exist (Laver et al. 1990; Van Regenmortel 2009b). Discontinuous epitopes cannot be extracted and isolated in active form from the protein antigen and they possess binding activity only within the context of the 3D protein structure in which they are embedded (Fig. 1). In spite of claims to the contrary, it is not possible to predict the exact structure of a discontinuous epitope from nucleotide or amino acid sequences (Hopp 1993, 1994; Van Regenmortel and Pellequer 1994; Ponomarenko and Van Regenmortel 2009).

Continuous Epitopes

A second type of protein epitope known as a continuous epitope is defined as any short peptide fragment of the protein, usually five to eight residues long, that is able to cross-react with antibodies raised against the protein. When linear peptides are given the status of continuous epitopes of a protein, the impression is created that these epitopes actually exist as discrete binding and immunogenic sites in the native protein. In most cases, however, only some of the residues of a continuous epitope are located at the surface of the native protein where they are usually part of a more complex discontinuous epitope (Fig. 1). Although epitope databases list thousands of continuous epitopes presumed to exist in individual proteins (Greenbaum et al. 2007; Zhang et al. 2008), these linear peptides are actually poor mimics of the larger antigenic structures present in native proteins and they mostly cross-react only weakly with antiprotein antibodies (Van Regenmortel 1999a). When the structure of continuous epitopes bound to anti-peptide antibodies is compared to the structure of the corresponding regions in the cognate native protein, the two structures are usually found to differ considerably, with the result that most antibodies raised against the peptides are unable to bind to the parent protein because bumps and clashes prevent antibody binding (Chen et al. 2009).

Continuous epitopes always have ill-defined, fuzzy boundaries because they are only defined by their binding activity rather than by showing that all the residues in the peptide interact with antiprotein antibodies (Van Regenmortel 1999a). Attempts are sometimes made to assess the boundaries of continuous epitopes by measuring

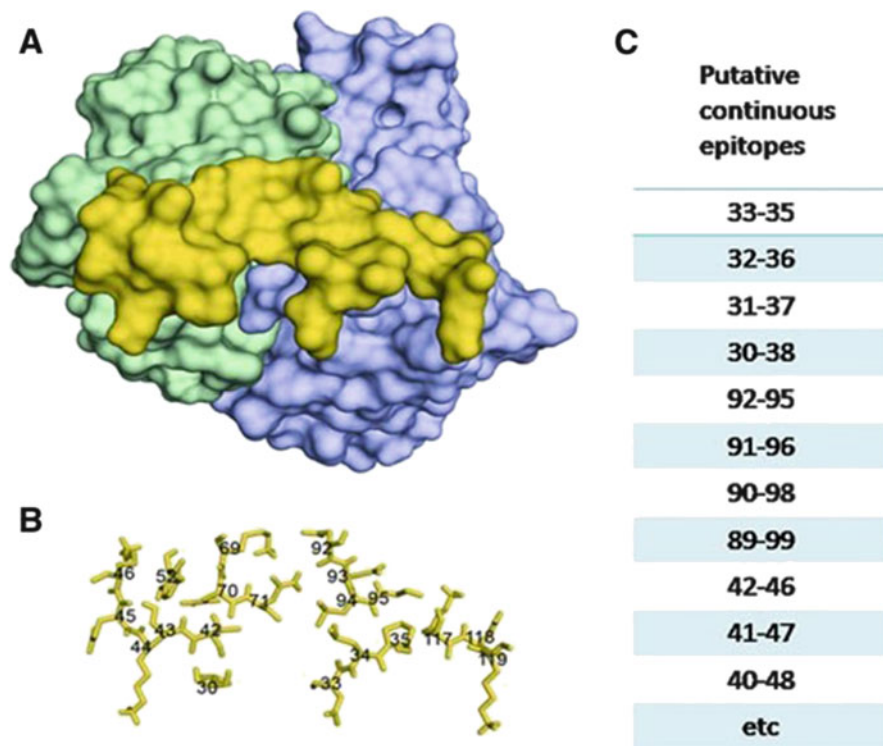


Fig. 1 Discontinuous epitope of the outer surface protein A of the spirochete *Borrelia burgdorferi* elucidated by X-ray crystallography from a complex with Mab 184.1. (a) Outline of the epitope in yellow, (b) Position in space of the residues comprising the epitope. This set of residues cannot be isolated as such from the protein to demonstrate that it possesses binding activity in its own. (c) Parts of the discontinuous epitope and other peptide segments of the protein that may be able to bind Mab 184.1, in which case they would be called continuous epitopes (courtesy of Pernille Haste-Andersen, Danish Technical University)

the binding activity of peptides of decreasing size and giving the status of epitope to the smallest peptide that retains detectable activity. However, the results of such analyses depend on the immunoassay used because the peptide conformation usually differs in different types of assay (Muller et al. 1986). In some assays, tripeptides can be shown to possess significant binding activity (Trifilieff et al. 1991). The contribution of individual residues to the epitope activity can also be evaluated by measuring the binding capacity of sets of peptide analogs presenting single residue replacements. When this is done, certain residues are found to be essential for binding while others, called replaceable residues, can be replaced by any of the other 19 amino acids without impairing antigenic reactivity (Getzoff et al. 1988; Geysen et al. 1988). When a residue substitution is found to affect the epitope activity, it could mean that the residue is in contact with the paratope or that the

substitution induced a conformational change which affects the reactivity of the peptide.

It has been suggested that most continuous epitopes are not genuine epitopes of native proteins but correspond to unfolded regions in denatured proteins that are recognized by antibodies directed to denatured molecules (Laver et al. 1990). It can, indeed, never be excluded that the antiprotein antiserum used for detecting continuous epitopes contains antibodies specific for denatured protein molecules because some of the protein molecules used for immunization became denatured before or after being injected in the animal (Jemmerson 1987). In the reciprocal situation where antibodies raised to peptides are allowed to react with the cognate protein, it also cannot be excluded that the antibodies recognize denatured protein molecules present in the immunoassay format that was used (Spangler 1991). It is now accepted, for instance, that earlier claims that immunization with peptides always elicits high levels of antibodies that cross-react with the native cognate protein (Lerner 1984) arose because it was not realized at the time that the protein used in solid-phase immunoassays became denatured by adsorption to plastic (Van Regenmortel 1999a; Darst et al. 1988). The classification of epitopes as either continuous or discontinuous may give the impression that the fundamental units of antigenic recognition are amino acid residues, although epitope-paratope interactions involve only a few atoms in each residue which may be side chain or main chain atoms. It should also be appreciated that crystallographers often use different criteria for identifying which contact residues are involved in an interface (Getzoff et al. 1988). For instance, contact residues in the epitope have been defined as those that have any atom less than 0.5 nm away from any atom in the antibody (Hafenstein et al. 2009). Other distance thresholds have been used for identifying different types of contact, for instance 0.35 nm for hydrogen bonds and salt bridges, 0.45 nm for van der Waals interactions and 0.45 nm for π cation interactions (Chen et al. 2009). The use of different thresholds may lead to different residues being attributed to the binding interface.

Neotopes and Cryptotopes

The quaternary structure of the protein subunits assembled in virus particles gives rise to additional types of epitopes known as neotopes and cryptotopes. The term neotope was introduced in 1966 (Van Regenmortel 1966) to refer to epitopes that arise either from the juxtaposition of residues in neighbouring proteins or subunits that are recognized by antibodies as a single epitope, or from conformational changes induced in the proteins by intersubunit interactions (Van Regenmortel 1992a). Neotopes have been found in the capsid and membrane proteins of most viruses (Neurath and Rubin 1971; Van Regenmortel and Neurath 1985) and are also present in HIV-1 (Broder et al. 1994; Cho et al. 2000; Gorny et al. 2005). Since the quaternary structure of virions is dynamic rather than static (Bothner et al. 1998;

Yuan et al. 2006), neotopes tend to be transient epitopes that can assume different conformations (Zolla-Pazner 2004; Binley 2009).

When the glycoproteins in HIV-1 spikes associate to form trimers, a part of their surface is no longer accessible to antibodies. These masked regions harbour epitopes known as cryptotopes (Jerne 1960) that are active only when the subunits are dissociated (Broder et al. 1994).

There is evidence that differences in the quaternary structure of gp120 between primary HIV-1 isolates, i.e. viruses that have been minimally passaged in cell lines, and T-cell line adapted (TCLA) isolates are responsible for their differential sensitivity to neutralization. It seems that a more open gp 120 conformation in TCLA viruses allows neutralizing antibodies to access more easily the binding sites for CD4 and for the coreceptors CCR5 and CXCR4 as well as the V3 loop (Poignard et al. 2001; Bou-Habib et al. 1994; Poignard et al. 1996).

Mimotopes

Another type of linear peptide epitope was recognized in 1986 and given the label mimotope (Geysen et al. 1986). Mimotopes are detected when peptides from combinatorial peptide libraries obtained by chemical synthesis or phage display are tested for their ability to bind antiprotein Mabs that recognize either continuous or discontinuous epitopes (Irving et al. 2001). When the Mabs are directed to discontinuous epitopes, they may bind to peptides showing little or no sequence similarity with the antigen used to elicit the antiprotein antibodies. Antigenic cross-reactions occurring in the absence of sequence similarities may arise from the overriding attraction between hydrophilic and hydrophobic groups that occurs when a pattern of hydrophobic complementarity is conserved in dissimilar peptide sequences (Hanin et al. 1997; Van Regenmortel 1998). Such a phenomenon is responsible for the binding of complementary peptides specified by the sense and antisense strands of RNA molecules because their respective codons always code for amino acids of opposite hydrophobicity (Blalock 1990; Tropsha et al. 1992).

When Mabs used to detect mimotopes recognize a continuous epitope in a protein, there is sometimes extensive sequence identity between the mimotope and the continuous epitope. In the case of two neutralizing Mabs that recognized the same 7-residue sequence present in the surface glycoprotein of a murine coronavirus and in several mimotopes, it was found that the two Mabs presented little similarity in their heavy chain complementarity determining regions (CDRs), indicating that two paratopes that recognize similar sequences can be very different (Yu et al. 2000). It is also often found that Mabs harbouring unrelated paratopes are able to recognize very similar discontinuous epitopes (Malby et al. 1994; Lescar et al. 1995; Van Regenmortel 2009a), suggesting that it may be difficult to control the type of antibody that will be obtained when very similar epitopes are used as immunogens.

A limited number of mimotopes may also be able to induce antibodies that cross-react with the protein (Delmastro et al. 1997; Puntoriero et al. 1998; Larralde et al.

2007) and show a modest capacity to neutralize viral infectivity (Schellekens et al. 1994; Steward et al. 1995). The antigenic and immunogenic activities of mimotopes often depend on their conformation. For instance, when mimotopes are displayed on a phage, they may better mimic the conformation of a viral epitope than when they are used as free peptides or conjugated to a carrier protein (Irving et al. 2001; Zwick et al. 2001a; Dorgham et al. 2005). Mimotopes displayed on a phage protein may also be better elicitors of antibodies that cross-react with the virus and neutralize its infectivity (Yu et al. 2000). Although several mimotopes of HIV-1 epitopes have been isolated from combinatorial peptide libraries (Zwick et al. 2001a; Dorgham et al. 2005; Keller et al. 1993; Boots et al. 1997; Scala et al. 1999) none of them were able, when used as immunogens, to elicit adequate levels of neutralizing and protective antibodies.

What Do Mimotopes Mimic?

Many peptides that are given the label mimotope because they cross react with an antiprotein Mab do not necessarily bind only to residues of the known antiprotein paratope since they may interact with neighbouring residues that are part of a different paratope recognized by the mimotope. As a result, many so-called mimotopes do not really mimic the epitope recognized by the Mab but simply react with polyspecific immunoglobulin (Ig) molecules that always possess numerous potential binding sites for different epitopes.

The most commonly used procedure for assessing what a mimotope recognizes is to test whether it is able to inhibit the binding of the Mab to its homologous antigen. If the mimotope is found to compete with the antigen for binding to the Mab, this is usually taken as sufficient evidence that it mimics the epitope recognized by the Mab (Irving et al. 2001; Zwick et al. 2001a). This criterion for establishing mimicry is actually questionable since the binding inhibition could simply be due to the fact that steric hindrance prevents the mimotope from binding to a neighbouring paratope in the Ig binding pocket of the Mab. If an antibody recognizes two unrelated epitopes through separate, non-overlapping paratopes, it would of course make little sense to say that the two epitopes are antigenic mimics of each other. If the mimotope presents some sequence similarity with portions of the epitope, this is taken as evidence that the mimotope binds to the putative paratope of the Mab. However, it is equally plausible that the mimotope binds to its "own" specific site consisting of a neighbouring set of residues. Furthermore, if the mimotope is able to elicit antibodies that recognize the epitope believed to be mimicked, this is taken as confirming evidence that the mimotope exactly mimicks the epitope.

The antigenic mimicry displayed by a mimotope, however, does not imply that the peptide is a precise structural mimic making identical contacts with the paratope to those made by the epitope that elicited the antibody, since a modest similarity between two antigens may be sufficient to give rise to a cross-reaction involving overlapping but different paratopes. The concept of immunogenic mimicry is even

more difficult to define in structural terms than antigenic mimicry, since it involves undefined functional activities in the immunized host as well as unknown characteristics of the immunogen that elicit the appearance of cross-reactive antibodies (Irving et al. 2001).

The Specificity of Antibodies Is Limited Rather than Absolute

The concept of specificity in immunology has been difficult to define (Cohn 1997, 2005; Langman 2000; Cohen et al. 2004). The term specificity is derived from the word species and describes what is characteristic of a species (Van Regenmortel 1998). For centuries, biological species were considered to be separated by clear-cut discontinuities and in the nineteenth century bacteriologists believed that antisera raised against different species of bacteria were completely specific, allowing different species to be identified by serology with absolute certainty (Mazumdar 1995). This view was later questioned by Landsteiner (1962) who showed that serological cross-reactions between different cell types were caused by antibodies that reacted to different degrees with a wide range of cells. He demonstrated that there was no one-to-one relationship between an antigen and its antibody and that an antigen could elicit a wide spectrum of antibodies capable of cross-reacting with many antigens. This led to antigen-antibody interactions being regarded as much less specific than previously thought and is nowadays explained by the widespread occurrence of cross-reactions between different epitopes and paratopes. The work of Landsteiner led Talmage (1959) to suggest that immunological specificity was not the result of a unique antibody molecule recognizing each distinguishable antigen but arose from the combinatorial effect of several different antibodies recognizing separate epitopes on the same antigen. According to that interpretation, a specific antigen recognition or elimination process is always initiated by several binding events involving different antibodies.

Specificity has been defined as “the exact complementary relationship between an agent and something acted on” (Medawar and Medawar 1978), a definition which applies to the stereochemical complementarity observed between antigen and antibody, enzyme and substrate or receptor and ligand. In the case of antibodies, the recognition of a complementary antigen is mediated by the binding site of Ig molecules consisting of 50–70 hypervariable residues distributed over the six CDRs in the variable domains of the heavy or light Ig chains. Each immunoglobulin binding site can accommodate many overlapping or non-overlapping binding subsites of 10–20 residues, corresponding to paratopes able to bind different antigens. An Ig molecule may, for instance, possess two independent binding sites that allow it to bind simultaneously to phosphorylcholine and to *α*-D-galactopyranoside (Bhattacharjee and Glaudemans 1978). A more common situation is that different paratope subsites partly overlap, in which case binding to one epitope would prevent a second related or unrelated epitope from being accommodated at a nearby location in the Ig binding site. All antibody molecules are therefore polyspecific since they

always harbor numerous subsites corresponding to paratopes specific for different epitopes. The flexibility of CDR loops also contributes to the ability of Igs to adapt to a variety of epitopes (Ramsland et al. 1997; James et al. 2003; Jimenez et al. 2003). It is important to distinguish between an actual paratope and the virtual functional site of an Ig molecule consisting of all the residues of the six CDRs which, however, do not form a binding site for any particular epitope.

Degeneracy of the Immune System and Fuzzy Recognition Sites

Many terms such as cross-reactivity, molecular mimicry, plasticity, promiscuity, degeneracy and polyspecificity have been used to describe the finding that a T cell receptor (TCR) and B cell receptor (BCR) can recognize many different ligands (Cohn 2005; Sperling et al. 1983; Parnes 2004). The term polyspecificity is particularly fitting because it captures two essential features of immune receptors, i.e. their ability to recognize multiple peptide/MHC (major histocompatibility complex) ligands or B cell epitopes as well as their capacity to distinguish between closely related ligands that present only very small structural differences (Wucherpfennig et al. 2007).

The term cross-reactivity does not imply that a particular mechanism is responsible for recognition but it suggests that there is a primary ligand for each TCR and BCR and that the binding of all other ligands are cases of cross-reaction. In reality, there is no single intrinsic or “real” epitope for any immune receptor but only a diverse group of potential ligands (Wucherpfennig et al. 2007). Cross-reactivity is also said to occur when an immune response and the resulting secreted antibodies directed at one antigen are able to interact with other antigens that did not stimulate the original reaction (Parnes 2004). Cross-reactivity then refers to a lack of specificity. Degeneracy is the ability of elements that are structurally different to perform the same activity or function. Degeneracy may yield the same or different functions depending on the context in which it is expressed (Edelman and Gally 2001). The genetic code, for instance, is degenerate because there are many more triplet codons than encoded amino acid residues. As a result, an enormous number of structurally distinct mRNA species could be translated to generate the amino acid sequence of any particular protein. Degeneracy is further increased when one includes variations in polynucleotides sequences that result in mutated but functionally equivalent gene products (Edelman and Gally 2001). In immunology, degeneracy refers not only to the ability of structurally different antibodies to perform the same recognition function but also to the ability of a single antibody to perform multiple functions like binding to different antigens. Degeneracy is a property of a system as a whole and not of its single components (Parnes 2004). The degeneracy of T cell recognition refers to the fact that peptide-binding motifs are polyspecific for MHC class molecules and that a single TCR can be activated by a large number of different peptides possessing limited sequence similarity. The degeneracy of the immunoglobulins

made by an animal ensures that the animal has the ability to make antibodies which recognize essentially any molecular motif which its immune system may encounter. If each immunological receptor could only recognize a single complementary ligand, the immune system would need one receptor for every possible epitope which would require orders of magnitude more receptors than there are cells in the immune system. It is only because of polyspecificity that a complete immune repertoire can be achieved by a much more restricted number of receptors (Wucherpfennig et al. 2007). Degeneracy is also a prerequisite to allow organisms to adapt over evolutionary time since natural selection can only operate among a population of dissimilar organisms (Edelman and Gally 2001).

The description of protein binding sites as fuzzy recognition sites was introduced by Moodie et al. (1996) to account for the finding that protein-ligand complementarity can be achieved by not just a single arrangement of amino acids but by a large number of alternative arrangements. Epitopes have also been called fuzzy recognition sites because there is no clear-cut minimum difference in binding affinity or in atomic positions at the antigen-antibody interface that can serve as an absolute yardstick for deciding that two epitopes are the same or not (Van Regenmortel 1998, 1999b). The use of fuzziness as a descriptor of reality is useful for handling concepts and entities that do not possess clear-cut boundaries (McNeill and Freiburger 1993). Whereas classical, Aristotelian logic analyses concepts and classes in terms of mutually exclusive dichotomies of the type: a glass is full or is not full and a statement is true or not true, fuzzy logic is a method to handle a continuum with innumerable steps (McNeill and Freiburger 1993; Van Regenmortel 2001b; Sadegh-Zadeh 2001). Linguistic descriptors are often assumed to refer to entities that possess sharp boundaries because they satisfy the propensity of the human mind to want to make clear-cut distinctions. The continuous nature of the spectrum of electromagnetic waves does not prevent us from distinguishing different colors and the continuity of geological rock formations does not stop us from giving different names to individual mountain peaks. In a similar way, the continuous nature of biological variation and of protein evolution does not prevent us from distinguishing and giving separate names to individual biological species, antigenic binding sites and antibodies (Van Regenmortel 1998).

Even the classical tenet of immunological theory which posits the need of the immune system to discriminate between self and nonself (Cohn 2005), has been interpreted in terms of fuzzy recognition sites (Leng and Bentwich 2002) and complex systems (Efroni and Cohen 2002). It used to be believed that T cells in the thymus were either clearly responsive or unresponsive to self and foreign antigens but there is now evidence that many self-reactive T cells which escape from clonal selection as a result of low affinity interactions with self-peptides, eventually become part of the general T cell repertoire (Sandberg et al. 2000). The occurrence of antibodies able to react with foreign antigens results from selective forces operating during immunization which depend on reactant concentration and on the probability of receptor binding and lymphocyte stimulation occurring above a certain triggering threshold (Foote and Eisen 1995; Batista and Neuberger 1998).

Epitopes and Paratopes Are Relational Entities

Once an Ig molecule has been found to bind to an antigen, it becomes possible to solve the structure of the antigen-antibody complex and to identify the paratope and epitope regions. Epitopes and paratopes are actually relational entities defined by their mutual complementarity and they depend on each other to acquire a recognizable identity. An epitope is thus not an intrinsic structural feature of a protein that could be identified in the absence of a particular interaction with a paratope (Van Regenmortel 1999a, 2009b). This relational dependence means that as soon as the epitope is slightly altered and binding to the antibody is affected, the paratope also is no longer the same. As a result, 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 identifiable in a protein is therefore equal to the number of different Mabs that can be raised against it. Using this criterion, the insulin molecule was shown to possess 115 epitopes (Schroer et al. 1983) and the BLysS molecule more than a thousand (Edwards et al. 2003). This analysis differs from the viewpoint advocated by Cohn (2005) which defines a “paratopic clan” as a family of paratopes, distinguishable one from the other, that are all functionally capable to recognize a given single epitope. Cohn further defines a “mimotopic array” as a set of epitopes, distinguishable one from the other, that are all able to interact functionally with a given single paratope.

Epitopes and Antigenic Sites

It is now accepted that the entire accessible surface of a protein harbours a large number of overlapping epitopes that can be detected only if Mabs that bind all these regions are available (Benjamin et al. 1984; Berzofsky 1985). This means that the same residues at the surface of a protein can always be part of different overlapping epitopes. Even if the majority of residues recognized by two different Mabs are the same, the chemical bonding pattern between epitope and paratope may be very different and involve totally unrelated CDR sequences (Van Regenmortel 2009a). When only a small number of Mabs are available for studying the antigenicity of a protein, investigators tend to give undue importance to spurious boundaries assumed to demarcate discrete antigenic regions and they disregard the fact that the protein surface is an antigenic continuum. This leads to an analytical bias that emphasizes immune responses elicited by single epitopes rather than fostering an examination of the beneficial neutralizing synergy that often result from the combined interactions of several independent epitopes and paratopes (Tilley et al. 1992; Laal et al. 1994; Zwick et al. 2001b). Restricting one’s attention to single epitopes also negates the insight of Talmage (1959) that specific interactions result from the combined effect of several independent recognition events.

Although it is impossible to draw distinct boundaries between overlapping epitopes, it is customary to refer to the existence of separate antigenic sites in

proteins, each one comprising many overlapping or non-overlapping epitopes. Each antigenic site is identified on the protein surface on the basis of a recognizable spatial location as well as by certain structural and functional properties. Several antigenic sites of HIV-1 Env spikes have been extensively studied, for instance the CD4 binding site (CD4bs) which is the region that allows the virus to attach to its primary CD4 receptor on the surface of target cells (Li et al. 2007; Berzofsky 1985; Zhou et al. 2007c), the V3 antigenic site (Zolla-Pazner 2004; Javaherian et al. 1989; Hioe et al. 2010) and the membrane proximate external region (MPER) of gp41 (Montero et al. 2008; Muster et al. 1993; Zwick 2005). It must be stressed that each HN antigenic site consists of several overlapping epitopes that may give rise to neutralizing synergy, a phenomena that may be overlooked if only one epitope recognized by a Mab is used in the study of an antigenic site.

Antibody Heterospecificity

Antibody heterospecificity is the ability of an antibody to bind more strongly to a different antigen than the one used for raising the antibody and is frequently observed when an antibody is tested against a series of analogs or mutants of the immunogen (Loor 1971; Al Moudallal et al. 1982; Underwood 1985; Harper et al. 1987). Heterospecificity is due to the fact that the clonal selection of B cells, which subsequently leads to antibody secretion in plasma cells, can be triggered by an immunogen endowed with only moderate affinity for a B cell receptor. Antibodies of low or moderate affinity will then be obtained which may react better with heterologous epitopes endowed with a superior degree of complementarity with the paratope (Van Regenmortel 2009b). In some cases, antibodies may have such a low affinity for the immunogen that they will not react with it at all and will only bind to a mutated antigen presenting a substitution that facilitates heterospecific binding. The technique of intragel cross-absorption of antisera is particularly suitable for detecting this phenomenon (Van Regenmortel 1966). It is well-known that following immunization with antigens, antisera are obtained that contain levels of total immunoglobulins that far exceed the levels of antibodies able to react in immunoassays with the administered antigen (Eisen 2001). A likely explanation of this difference is that B cell responses are degenerate and trigger the secretion not only of Igs that can function as detectable antibodies with the homologous antigen but also of heterospecific antibodies that have an affinity for the immunogen below the threshold required for binding to it although they may be able to bind to other, related antigens.

Specificity Versus Discrimination Potential

The specificity of antibodies is inversely related to their ability to cross-react with a variety of antigens (Berzofsky and Schechter 1981). Part of the confusion

surrounding the concept of specificity arises when no distinction is made between a single epitope and a multiepitopic antigen. If an investigator wishes to differentiate between two antigenically related proteins using a panel of Mabs, an antibody that recognizes the same epitope in both molecules would be called nonspecific whereas an antibody recognizing an epitope present in only one of them would be considered specific (Van Regenmortel 1998). Since an antibody would thus be called specific or non specific, depending on what the investigator is trying to achieve, it is preferable to refer to the discrimination potential of antibodies raised against a multiepitopic antigen. An antibody, of course, cannot be specific for a multiepitopic protein and it will at best only have a limited specificity for an individual epitope. That an antibody is always able to cross-react with many related epitopes is also demonstrated by the ability of large numbers of peptide analogs presenting amino acid replacements at each residue position to react with the same antibody (Getzoff et al. 1988; Geysen et al. 1988; Roberts et al. 1993).

Antibody Specificity and Affinity

It is often believed that antibody specificity is correlated with high affinity because it is expected that highly specific antibodies will possess a better stereochemical complementarity with their antigens than antibodies of lower affinity. There is, however, no necessary link between affinity and specificity since antibodies of lower affinity may discriminate better between two antigens than do antibodies of higher affinity. The reason for this is that low affinity antibodies tend to detect fewer cross-reactions than antibodies of high affinity since cross-reactions are usually weaker than homologous reactions and will more quickly drop below the level of detection (Van Regenmortel 1998). It must also be emphasized that in the same way that epitopes and paratopes are defined only through their mutual relationship, the affinity of an antibody can only be defined with respect to a binary relationship with a single antigen. In contrast, the specificity of an antibody is a ternary relational property that is meaningful only with respect to a more complex set of relations between one antibody and at least two antigens, the reason being that specificity arises from the ability of an antibody to react differently with two or more antigens and thus to discriminate between them.

Discontinuous Epitopes Cannot Be Extracted from Their Protein Environment in Order to Be Used as Vaccine Immunogens

The failure of reverse vaccinology in developing an HIV-1 vaccine is partly due to the unwarranted expectation that the structural delineation of discontinuous HIV-1

epitopes bound to neutralizing Mabs should allow these epitopes to be used as successful vaccine immunogens. As discussed above, a discontinuous epitope corresponds to a set of non-contiguous residues which cannot be isolated as a separate entity to show it possesses binding activity outside the protein context. Many unsuccessful attempts have been made to reconstitute the exact structure of discontinuous epitopes by chemical synthesis (Van Regenmortel 2009a and references therein, Timmerman et al. 2007) and it does not seem possible to reproduce their exact immunological activity in the absence of the scaffolding contribution of the peptide chain. As a result, the immunogenic capacity of a discontinuous epitope can only be evaluated by using the native protein in which it is embedded as an immunogen, a procedure that will always stimulate an heterogeneous immune response against the numerous epitopes of the protein. For instance, when antibodies are raised against the CD4bs surface of gp120, most of them do not possess the exceptional neutralizing capacity of Mab b12 (Pantophlet et al. 2003b).

When a multiepitopic protein is used as immunogen, various epitopes will selectively bind to the B cell receptors present in the immunized host for which they possess sufficient affinity. However, the epitopes will not preferentially bind to those rare receptors, which in addition to recognizing the epitope, are responsible for the subsequent secretion by plasmocytes of antibodies that neutralize viral infectivity. The selection of B cell receptors during immunization is blind to the presence of neutralizing anti-viral activity in the antibodies that will be secreted since this biological function becomes effective only during the subsequent infection of a host. Furthermore, even if a single discontinuous epitope could somehow be used as an immunogen, the probability is still extremely low that a selectable B cell receptor would be present in the immunized host that exactly matches the variable immunoglobulin gene and CDR sequences of the Mab used for identifying the discontinuous epitope. Unfortunately, we do not know how to elicit neutralizing rather than non-neutralizing antibodies since it is not clear which features of an immunogen and immunized host are responsible for the cross-protective immunogenic activity that is required from any vaccine candidate (Zwick and Burton 2007; Parren and Burton 2001; Van Regenmortel 2006; Reading and Dimmock 2007).

The Unsuccessful Search for Immunogens Was Able to Elicit B12-like Antibodies

The difficulties encountered when one attempts to design a vaccine immunogen on the basis of the known structure of a neutralizing antibody recognizing a discontinuous HIV-1 epitope are clearly demonstrated in the case of the b12 antibody. This Mab which was obtained from a phage-displayed Fab library derived from an HIV-1 patient (Burton et al. 1994) recognizes a conserved discontinuous epitope overlapping the CD4bs of gp120. It neutralizes a wide range of primary and TCLA HIV-1 isolates by inhibiting the binding of gp120 to the CD4 receptor. Antibody b12

has an 18-residue long CDR H3 region, a feature present in many anti-CD4bs human Mabs, allowing them to penetrate into a hydrophobic cavity on the gp120 surface. Using a docking model of b12 onto gp120, several contact residues of the epitope were identified and their contribution to binding activity was evaluated by alanine mutagenesis (Saphire et al. 2001). The gp120 structure that was used for docking b12 was the only one available at the time and corresponds to that of a monomeric core complexed with CD4 and with the Fab 17b (Kwong et al. 1998), and it may therefore not correspond to the conformation of the trimeric CD4bs seen by antibodies and by the immune system. It is known that when gp120 binds to CD4, it undergoes a large conformational change which allows the virus to bind to one of its chemokine coreceptors (Labrijn et al. 2003; Decker et al. 2005).

In an attempt to identify an HIV-1 vaccine immunogen capable of eliciting b12-like neutralizing antibodies, peptide libraries displayed on filamentous phage have been used to select peptides that bind b12 (Zwick et al. 2001a). A 15-residue peptide labeled B2.1 with the sequence H₁ERSYMFSDLENRCI₁₅ was found to bind b12 with considerable affinity when it was present as a disulfide-bridged homodimer involving two C₁₄ residues. The residues S₈, D₉, C₁₄ and I₁₅ of peptide B2.1 were present in several of the selected mimotope peptides and were considered to be responsible for most of their binding activity (Zwick et al. 2001a). The peptide B2.1 shows significant homology to the D-loop region (residues: R₂₇₃SVNFTDNAKTII₂₈₅) of gp120 and appeared to be an exact conformational mimic of the D-loop region. When the immunogenicity of B2.1 was tested in mice and rabbits no antibodies were obtained that cross-reacted with gp120, indicating that B2.1 was not an effective candidate vaccine immunogen.

In another study, additional mimotopes reacting with b12 were obtained which possessed the same consensus sequence M/VhSD (where *h* represents an aromatic amino acid) found in the D2.1 peptide (Dorgham et al. 2005). Second and third generation libraries were constructed which gave rise to the improved consensus sequence G/SLL/MVWSDEL/H that reacted even more strongly with b12. Phage-displayed peptides containing this sequence competed with the binding of gp120 to b12 and were therefore considered to mimic the epitope recognized by b12. In contrast to what had been found with the B2.1 peptide, immunization with these more active phage-displayed mimotopes were able to elicit Env-specific antibodies, although immunization with the mimotopes conjugated to a carrier protein did not. Cross-reactive immune sera from immunized mice, however, did not neutralize laboratory-adapted strains of HIV-1 *in vitro*, showing that these improved mimotopes were also ineffective vaccine immunogens (Dorgham et al. 2005). The conclusion that immunization with peptide mimics of discontinuous epitopes is not a feasible approach for developing epitope-targeted vaccines was corroborated in a recent study of mimotopes that mimicked discontinuous epitopes of three well-characterized proteins (Irving et al. 2010).

The inability of mimotopes reacting with the b12 antibody to elicit b12-like antibodies is due to the fact that these linear peptides do not reproduce the complex structural features of the discontinuous epitope recognized by Mab b12 and which presumably induced its formation. This was clearly demonstrated when the crystal

structure of a complex between Fab of b12 and the B2.1 peptide was determined at 1.8 Å (Saphire et al. 2007). Only three of the critical binding residues of the B2.1 peptide (residues F₇, S₈ and D₉) were found to form side-chain contacts with the b12 paratope while other residues important for the binding activity (Y₅, C₁₄, I₁₅) only helped to stabilize the peptide structure. The B2.1 peptide is able to bind b12 not because it mimics the D-loop of gp120 but because it reacts with a different paratope subsite in b12 from the one that recognizes the complex discontinuous epitope determined by docking and mutagenesis studies (Zwick et al. 2001a, 2003a; Pantophlet et al. 2003b). Since the B2.1 peptide mimicks at best a very small portion of the discontinuous epitope recognized by b12, it is not surprising that this peptide is unable to elicit antibodies that cross-react with gp120 (Saphire et al. 2007).

In an attempt to understand why Mab b12 possesses potent neutralizing properties whereas the majority of anti CDRbs Mabs do not, more than 60 residues of the CD4bs were mutated to alanine to determine whether these substitutions affected the ability of gp120 to bind to CD4 and to neutralizing and non-neutralizing Mabs (Pantophlet et al. 2003b). Many substitutions were found to affect the binding of neutralizing and non-neutralizing Mabs in the same way, indicating a high degree of overlap in the epitopes recognized by these anti-CD4bs antibodies. Some of the mutations enhanced Mab binding but such an effect was never observed for CD4 binding, suggesting that the virus has evolved toward an optimal gp120-CD4 interaction. Although there were some individual differences between the effects of substitutions on the binding of different Mabs, no clear picture emerged that explained why b12 possessed a unique neutralizing capacity. Since certain residues in the V1/V2 and V3 loop of gp120 were able to affect the binding of b12, it was suggested that non-neutralizing anti-CD4bs Mabs might interact with the gp120 surface using a different orientation from the one used by b12 (Pantophlet et al. 2003b). It is known that all anti-CD4bs Mabs bind monomeric gp120 similarly, independently of their neutralizing capacity, whereas only b12 is able to bind trimeric, functional gp120 presumably through neotopes present only on gp120 oligomers. This may be the reason why only native gp120 trimers are able to elicit an effective neutralizing antibody response (Parren and Burton 2001; Sattentau and Moore 1995). All attempts to find immunogens able to elicit b12-like antibodies were based on the premise that such immunogens must reproduce exactly the structure of the epitope recognized by b12 because it was assumed that the antigenic activity of an epitope is always accompanied by a related immunogenic activity. This assumption implies that antigenicity which is a chemical property arising from a molecular recognition process involving complementary antibodies is equivalent to immunogenicity which is a biological property leading to antibody secretion that occurs only in the context of an immunized host (Van Regenmortel 2010). Immunogenicity depends on intrinsic properties of the immunogen but also on extrinsic factors controlled by constituents and properties of the host immune system such as the host Ig gene repertoire, appropriate BCRs, cytokines, etc. (Berek 1992; Harwood and Batista 2010). All these extrinsic factors did contribute to the occurrence of the neutralizing antibody initially used to identify the putative vaccine epitope but they cannot be expected to be present in identical form in the host that will be vaccinated

with the same antigen. Another reason that makes it impossible to extrapolate from antigenic to immunogenic structures is that the structure of an epitope determined in a crystallographic complex is also influenced by processes of induced fit, conformational selection and mutual adaptation of the two binding partners (Rini et al. 1992; Wilson and Stanfield 1994; Berger et al. 1999; Bosshard 2001; Goh et al. 2004). As a result, the particular antigenic structure revealed in an antigen-antibody complex does not necessarily correspond to the immunogenic structure that was recognized by B cell receptors during the immunization process and should be present in the vaccine immunogen (Van Regenmortel 2011b). When tested in an immunoassay, a peptide may bind to an anti-protein neutralizing Mab by an induced fit mechanism driven by the pre-existing structure of the Mab. However, the same induced fit will not occur when the peptide is used as immunogen and is recognized by B cell receptors that have no special affinity for the cognate intact protein.

The assumption that immunogenic potential can be deduced from antigenic structures has led several groups to modify gp120 molecules in order to improve their vaccine potential. For instance, recombinant gp120s were generated that no longer were able to bind non-neutralizing anti CD4bs Mabs while retaining their capacity to bind b12, an approach called immunofocusing (Pantophlet and Burton 2003). It was hoped that such a re-engineered gp120 would acquire the immunogenic capacity of eliciting only neutralizing antibodies, somehow mirroring the altered antigenic reactivity (Pantophlet et al. 2003b). As was to be expected, there have been no subsequent reports indicating that the attempt to induce an immunogenic functionality in gp120 that mirrored the antigenic properties of the engineered constructs was successful. Recently a stabilized gp120 antigen, better able to bind neutralizing antibodies, has been developed by rational design (Wu et al. 2010). The CD4bs of Env was re-engineered by deleting the V1 and V3 loops of the full gp120 molecule and introducing cross-links between different regions of the core protein. One of the re-engineered stabilized surfaces which reacted strongly with Mab b12 but not at all with four non-neutralizing CD4bs Mabs was used to select CD4bs-directed Mabs from memory B cells derived from a clade B-infected donor. A small number of CD4bs-specific memory B cells was obtained from about 25 millions peripheral blood mononuclear cells of the donor. One selected Mab (VRC01) neutralized about 90% of nearly 200 HIV isolates from multiple clades and had a much broader neutralizing capacity than b12 (Wu et al. 2010). The authors suggested that their results do provide an important insight for future HIV-1 vaccine design, although they presented no evidence that their rationally designed antigen is also a superior immunogen able to induce VRC01-like polyclonal antibodies in humans.

Continuous Epitopes of Viral Proteins Are Not Effective Vaccine Immunogens

Although many linear peptide sequences in proteins cross-react with antibodies raised against the protein, they mostly are unable to elicit antibodies that cross-react with the native protein. The lack of cross-reactive immunogenicity of peptides

(Van Regenmortel 2006) can be explained as follows. When an antiprotein antibody is tested for its ability to react with a peptide in an immunoassay, it may select one of the many available peptide conformations or it may bring it about by induced fit, the result in both cases being a detectable cross-reaction. In contrast, when the same peptide is confronted with a variety of B cell receptors during immunization, different conformations of the peptide may be recognized by separate receptors, leading to the subsequent appearance of a variety of anti-peptide antibodies. However, there is no reason why the peptide would bind preferentially to those rare receptors which in addition also cross-react with a more complex epitope present only in the native protein. Since a peptide corresponding to a continuous epitope is nearly always a poor mimic of a protein discontinuous epitope, it is unlikely that a significant proportion of the elicited anti-peptide antibodies would react with the cognate protein. Antibodies which do not recognize viral proteins will, of course, possess no neutralizing activity. It is sometimes possible to improve the cross-reactive immunogenicity of a peptide by increasing the conformational similarity between peptide and intact protein, for instance by cyclization of the peptide, but this approach rarely succeeds in producing immunogens able to elicit neutralizing antibodies (Van Regenmortel 2009a, and references therein; Joyce et al. 2002; Ho et al. 2005). As mentioned earlier in the case of discontinuous epitopes, linear peptides corresponding to continuous epitopes, when used as immunogens, will not preferentially select and bind to the rare B cell receptors which in addition to cross-reacting with the native protein also trigger a B cell differentiation process giving rise to antibodies endowed with a functional protective activity. Although a few mimotopes of several continuous epitopes of different viruses, including HIV-1, have been found to be able to elicit antibodies that cross-react with the virus and possess some neutralizing activity (Yu et al. 2000; Steward et al. 1995; Scala et al. 1999), no successful protective peptide-based antiviral vaccine has been developed and marketed for human use (Hans et al. 2006). Two 6-residue long continuous epitopes located in the conserved region of MPER of gp41 have been regarded as promising HIV-1 vaccine candidates (Zwick 2005) and many groups have attempted to turn them into effective vaccine immunogens (Montero et al. 2008). It seems highly unlikely, however, that these short linear peptides which are part of larger complex discontinuous epitopes eliciting potent neutralizing Mabs, will be able to induce an effective protective immune response (Van Regenmortel 2011b).

The Special Case of Terminal Regions in Proteins

The terminal regions of proteins frequently harbour continuous epitopes because they tend to be surface-oriented and are more hydrophilic and mobile than internal regions (Thornton and Sibanda 1983; Westhof et al. 1984; Tainer et al. 1985; Pellequer et al. 1994). A major purpose of epitope prediction is to replace intact antigen molecules by linear synthetic peptides for use as diagnostic reagents for detecting viral antibodies (Leinikki et al. 1993) or as immunogens for raising

antipeptide antibodies able to cross-react with a parent protein (Walter 1986; Muller 1999b). The most effective strategy in this case is to select 10-residue long synthetic peptides corresponding to the N- and C-terminal regions of the protein since these have the highest probability of cross-reacting antigenically with the protein. Methods for predicting which continuous epitopes in proteins are likely to be effective vaccine immunogens have been notoriously unsuccessful (Ponomarenko and Van Regenmortel 2009; Greenbaum et al. 2007; Blythe and Flower 2005; Caioli 2010) and only very limited success has been obtained when certain terminal peptides were used as vaccine immunogens. Vaccination with the N-terminal 15-residue peptide of the VP2 protein of canine parvovirus, for instance, was able to protect dogs against virus infection (Langeveld et al. 1994). Another example is the C-terminal region of the VP1 protein (residues 200–213) of foot-and-mouth disease virus, which is able to elicit high levels of neutralizing antibodies (Bittle et al. 1982). Residues 200–213 of one VP1 subunit are located next to the immunodominant disordered loop (residues 141–160) of an adjacent subunit (Rowlands et al. 1983), and both regions contribute to a single discontinuous neotope (Parry et al. 1989). The ability of the terminal regions of proteins to induce antibodies that cross-react with the native protein is partly due to the fact that these regions possess an intrinsic disorder which makes them very similar to the unstructured peptides (Uversky et al. 2005). Although some disordered regions in proteins may be recognized preferentially by antipeptide antibodies, the majority of viral epitopes capable of inducing a neutralizing antibody response are actually complex, discontinuous topographic sites rather than terminal linear peptides.

The HIV-1 V3 Antigenic Site

Loops and turns in proteins are often immunodominant antigenic regions and the V3 loop in HIV-1 has for many years been considered the principal neutralizing domain of the virus (Javaherian et al. 1989; Spear et al. 1994). Although it is designated as the third variable domain of gp120, the V3 loop has many conserved features such as a constant size of about 35 residues, a conserved type II turn at its tip and a disulfide bond at its base (Jiang et al. 2010). The semiconserved nature of the V3 loop allows it to play an important role in the recognition of viral co-receptors, but its sequence variability has led investigators to question its value as a target for vaccine-induced antibodies. Another reason that led investigators to doubt the value of the V3 loop as a vaccine candidate is that they looked for the presence of anti V3 antibodies in human anti-HIV-1 antisera by absorbing the sera with linear V3 peptides (Van Cott et al. 1995a). Since this absorption step did not remove the antibodies that neutralized primary HIV-1 isolates, it was concluded that the neutralizing antibodies were not directed against the V3 loop. In fact, human sera that neutralize both TCLA isolates and primary isolates from several HIV-1 clades contain antibodies that recognize a cyclic V3 conformation present in the virus and in disulfide-bonded V3 loops but absent in linear V3 peptides (Zolla-Pazner 2004; Gorny et al. 2002;

Hioe et al. 2010; Kayman et al. 1994; Gorny et al. 2006). The V3 loop is very flexible and is able to adopt different conformations when it binds to different Mabs (Stanfield et al. 1999). It was found that alternative β -hairpin conformations can be induced in the same V3 peptide, depending on whether it binds to Mab 447-52D that neutralizes both R5 and X4 viruses or to Mab 5b which neutralizes only X4 viruses (Rosen et al. 2005). This shows that the epitope conformation observed in a Mab-epitope complex can result from an induced fit process and is therefore not a reliable guide for deciding which immunogen conformation should be used for eliciting a certain type of neutralizing antibody. It is not yet clear whether intrinsically flexible V3 loops should be constrained in a particular conformation to increase their potential value as vaccine candidates (Mester et al. 2009). In the case of the immunodominant GH loop (residues 141–160) of the VP1 protein of foot-and-mouth disease virus, a stabilized all-D retro loop synthesized with D-amino acids was found to induce higher levels of neutralizing antibodies than the corresponding L-peptide (Briand et al. 1997; Carver et al. 1997; Nargi et al. 1999).

One of the Many Hurdles to Be Overcome by an HIV-1 Vaccine Is the Antigenic Variability of the Virus

There are several reasons why the strategies that were used successfully in the past to develop effective vaccines against many viral diseases are not applicable to HIV-1 (Hilleman 1992; Kusters and Almond 2010; Virgin and Walker 2010): (1) Since the natural immune response in HIV-1 infected individuals does not clear the infection, there is no natural immunological mechanism that the vaccine could imitate. An HIV-1 vaccine would thus have to surpass the normal immune response induced by infection and would for instance have to generate a far stronger response against conserved epitopes than what occurs during infection; (2) HIV-1 integrates into the host genome and establishes a latent pool of infected cells that conceal the virus from immune recognition; (3) The virus progressively destroys the immune system; (4) HIV-1 isolates exhibit an enormous antigenic variability arising from the error-prone viral reverse transcription which can produce as much as 35% sequence diversity in gp120 between viral subtypes and 10% diversity in gp120 in a single infected individual.

Although an increasing number of broadly neutralizing Mabs have recently been isolated from infected human donors (Stamatatos et al. 2009; Scheid et al. 2009; Walker et al. 2009; Corti et al. 2010; Mascola and Montefiori 2010), such antibodies are rarely induced by experimental immunization. It seems that during HIV-1 infection, the immune response is mostly directed against highly variable and accessible Env regions such as the V1, V2 and V3 loops rather than against functionally important but less accessible conserved domains such as the receptor and co-receptor binding sites. The propensity of the immune system to respond to immunodominant, strain-specific highly variable antigenic sites instead of to

subdominant conserved epitopes that are able in principle to elicit a more broadly protective immunity, has been called deceptive imprinting because it allows the virus to escape immune pressure (Nara and Garrity 1998). Attempts to overcome deceptive imprinting by redirecting the immune response to less strain-specific epitopes have been made using a strategy called immune refocusing. By introducing several new glycosylation sites into the V1 and V3 loops, it was possible for instance to obtain immunogens that induced a broader neutralization response that was effective against heterologous strains (Tobin et al. 2008). Some of the reported immunization results, however, are difficult to interpret because linear instead of cyclized loop peptides were used in inhibition experiments aimed at determining the specificity of the induced antibodies. Attempts have also been made to redirect the immune response to conserved neutralization epitopes by deleting some or most of the variable loops from Env, but the results were not satisfactory (Lu et al. 1998; Kim et al. 2003). Other approaches to overcome the problem of extensive antigenic variability include using as immunogen either artificial consensus sequences based on the most common amino acid in each position in an alignment (Gaschen et al. 2002; McBurney and Ross 2009), or alternatively combinatorial convergent peptide libraries consisting of thousands of V3 peptides known as mixotopes that are recognized as a single entity by immune receptors (Gras-Masse et al. 1999). Other strategies to deal with the existence of many antigenic variants by means of polyvalent vaccine cocktails have also been proposed (Korber and Gnanakaran 2009). Recent results have shown that the V3 loop may, in fact, be able to induce a neutralizing response against many different HIV-1 clades, indicating that a polyclonal response to V3 may have a much greater neutralizing breadth than that displayed by any single Mab or even a cocktail of Mabs (Hioe et al. 2010; Jiang et al. 2010; Pantophlet et al. 2007).

Rational Design Versus Empirical Immunogenicity Trials in HIV-1 Vaccine Development

A pervading theme in current vaccine research is the belief that rational design offers the best prospects for the much needed development of effective vaccines against HIV-AIDS, tuberculosis and malaria (Bramwell and Perrie 2005; D'Argenio and Wilson 2010; McElrath and Haynes 2010; Sette and Rappuoli 2010). In the case of HIV-1, this viewpoint has led to the claim that “rational design represents the only approach that can elevate vaccine research from an empirical exercise to a scientific discipline” (Karlsson-Hedestam et al. 2008). It is argued here that such a view which denigrates the time-honoured empirical, trial-and-error approach that gave us all our present day effective vaccines is highly misleading and could even possibly jeopardize future efforts at developing an HIV-1 vaccine (Van Regenmortel 1999b, 2001b). It is not always clear what scientists mean when they refer to rational design and it may be helpful to briefly analyze these terms. The label rational tends to be

used to describe any approach that relies on the probable outcome of an intervention as predicted from available scientific knowledge. The term rational is also used to describe common sense decisions such as focusing on known antigenic regions of a viral protein as primary targets for a vaccine (Obeid et al. 1995). Since all scientific work is based on reason, logic and currently accepted scientific theories, it makes little sense to oppose rational and empirical approaches since all scientific knowledge is derived from empirical observations made in the course of experiments that are necessarily planned and analyzed in a rational manner. It is therefore misleading to imply that trial-and-error experimentation is not rational since all novel findings and discoveries arise from the unpredictable outcome of controlled experiments rather than from purely rational, deductive thinking (Van Regenmortel 1999b). The term design can be defined as the deliberate and intelligent conceiving of an artificial object or process. The designer's task is to pose and solve an inverse problem, namely to imagine on the basis of existing knowledge what would bring about a desired outcome (Bunge 2003). Plausible solutions can only be tested by trial and error until the preset goal is attained (Van Regenmortel 2007). Since scientists need to secure funding for their research, it is tempting for them to suggest that their current understanding of the complexity of the immune system is sufficient to allow them to design immunogens and to predict the type of antibodies they will elicit.

Rational design terminology implies that there is a causal relationship between the structure of an antibody and its function, giving rise to the assertion: "structure determines function". Although it is true that a biological activity always depends on an underlying structure, the structure does not possess causal efficacy on its own in bringing about a certain activity. Causal relations are relations between successive events and not between two material objects nor between a structure and an event (Van Regenmortel 2002a). There is no unique causal relation between the structure of an antibody and its neutralizing capacity since the occurrence of neutralization is influenced by a large number of immunological and pathophysiological factors arising from complex interactions between antibody, pathogen, and host. The isolation of the potent neutralizing Mab VRC01 (Wu et al. 2010) and the elucidation of its structure (Zhou et al. 2010), as well as the isolation of many additional broadly neutralizing antibodies from the serum of infected donors (Walker et al. 2009; Corti et al. 2010; Mascola and Montefiori 2010; Binley et al. 2008; Gray et al. 2009), has been interpreted as providing valuable molecular information that should facilitate rational vaccine design (Burton and Weiss 2010). The search for additional broadly neutralizing Mabs has been stimulated by the finding that it is possible to provide sterilizing protection in non-human primates by administering neutralizing Mabs prior to viral challenge. However, this type of successful immunotherapy using neutralizing antibodies derived from infected individuals does not in any way tell us how such antibodies can be induced by vaccination.

It is precisely our lack of knowledge regarding which immunogens are able to induce such antibodies in certain infected individuals which prevents us from developing an HIV-1 vaccine by design. Furthermore, the polyspecificity of antibody molecules and the degeneracy of the immune system imply that it is futile to search for the single "intrinsic" epitope of a neutralizing Mab and for the putative

unique immunogenic structure capable of inducing protective antibodies. It has been claimed (Virgin and Walker 2010) that our failure to develop an HIV-1 vaccine is due to the fact that we do not understand what type of immune response is needed for protection. We can of course surmise that effective CD4, CD8 and B-cell responses are needed but this is of little help since we do not know how to elicit the required responses by immunization. One could also argue that studying the mechanism of a preventive or therapeutic HIV-1 vaccine will become possible only once an effective vaccination protocol has been discovered empirically. Figure 2 lists many of the questions that we need to answer if we want to be able to rationally design an HIV-1 vaccine (Van Regenmortel 2001b; Virgin and Walker 2010; Ebensen and Guzman 2008). It has been suggested that rational design may not be the best strategy to follow (Tobin et al. 2000) since it is not possible, for instance, to predict which combination of several substitutions in a protein may lead to new desired properties that are not manifested when the protein presents only one substitution. This has led to the conclusion that an improved protein function is more likely to be obtained by successive rounds of random mutation, recombination and selection than by rational design (Van Regenmortel 2002a and references therein). What is of course possible is to rationally design an antigen to fit a single Mab (Wu et al. 2010; Villen et al. 2006) or to improve by molecular design the paratope binding efficacy of a Mab intended for passive immunotherapy. What is not possible is to rationally design an

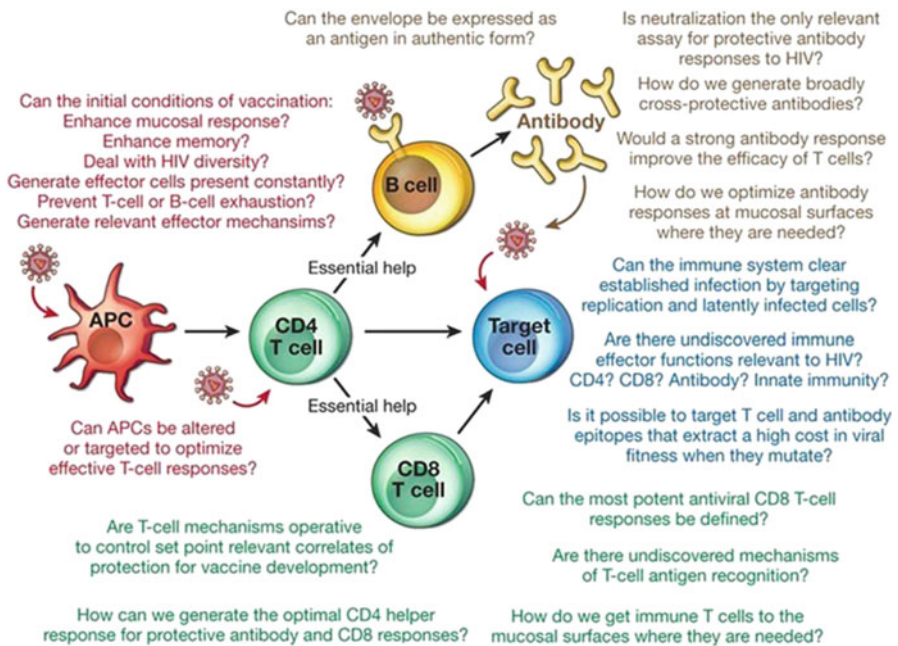


Fig. 2 Unanswered questions regarding the factors and mechanisms believed to be important for inducing an effective HIV-1 vaccine immune response (Virgin and Walker 2010)

immunogenic molecule that will elicit a protective polyclonal antibody response of predetermined efficacy.

The existence among HIV-1 infected individuals of long-term non-progressors (LTNPs) and elite controllers (ECs) has given rise to the hope that the elucidation of the mechanisms responsible for their occurrence may help HIV-1 vaccine design (Poropatch and Sullivan 2011). LTNPs which make up between 2 and 5% of all HIV-1 seropositive individuals (Okulicz et al. 2009) maintain low plasma levels of HIV-RNA and elevated CD4+ T cell levels whereas the even rarer EC individuals have undetectable levels of viral RNA. A combination of viral, genetic and immunological factors has been implicated in the ability of these slow progressors to control HIV-1 infection. The principal viral factor believed to slow disease progression is HIV-1 strain attenuation arising from mutations in viral accessory genes that cause a reduction in virus infectivity and replication (Poropatch and Sullivan 2011). If control of disease progression in these individuals results from an infection with attenuated, poorly replicating viruses, it is not clear how this could be made use of in vaccine development. The same applies to host genetic and immunological factors that promote control of infection such as chemokine receptor polymorphisms or the presence of certain protective HLA class I alleles in LTNP and EC individuals. Although some slow progressors have been shown to be able to induce broadly neutralizing antibodies against multiple HIV-1 strains (Villen et al. 2006; Simek et al. 2009), the relevance of this anti-Env immune response to vaccine design is also not obvious since we do not know which immunogens were able to induce such a response. It is, indeed, generally recognized that the major bottleneck in designing an HIV-1 vaccine lies in our ignorance of which immunogens are capable of inducing a broadly neutralizing immune response (Stamatatos et al. 2009; Mascola and Montefiori 2010; McElrath and Haynes 2010). As discussed in this review, a successful design strategy requires that we should know which immunogens and immunological mechanisms are likely to lead to the appearance of protective immunity following vaccination. Unfortunately even a vaccine that induces high levels of neutralizing antibodies will not necessarily protect against virus infection (Mascola 1999). Many investigators have recently stressed the need for embarking on large scale research programs in basic and preclinical immunology which they believe will give us the understanding required for designing vaccines (Virgin and Walker 2010; McElrath and Haynes 2010). Although nobody would quarrel with the need for more basic research in immunology, the track record in vaccinology shows that effective vaccines in the past were always discovered by trial-and-error immunization trials rather than by rational design based on available structural and immunological knowledge. The widespread current expectation that discoveries in biomedicine and vaccinology are likely to result from structure-based molecular studies, “omics” type investigations (Sette and Rappuoli 2010) or systems biology approaches (Pulendran et al. 2010) rather than from empirical trials may well turn out to be counterproductive if it inhibits investigators from undertaking exploratory trial-and-error experiments. Empirical immunogenicity trials based on a less than perfect understanding of the complexities of the immune response and of the nature of dynamic and transitory HIV-1 epitopes may still be the key to discovering a

promising HIV-1 vaccine candidate. Although epitope mapping with neutralizing Mabs and structural analyses may help investigators to identify which regions of the viral surface should be targeted by a vaccine, it must be emphasized that antigen mapping on its own will not lead to the discovery of vaccine immunogens that can only be revealed following experimental immunogenicity trials. Identifying the many cross-reactive epitopes that a neutralizing Mab can recognize is an entirely different task from discovering empirically which vaccine immunogens are able to induce a protective immune response.

Once an effective immunogen has been identified empirically, it may still take many years of basic research to establish its mechanism of action. As is the case with most present day successful vaccines, it is even conceivable that we may never fully understand why an empirically discovered vaccine is actually effective. This is a salutary reminder that discovering an effective vaccine empirically should have a higher priority than elucidating the mode of action of an ideal HIV-1 vaccine. Basic immunological research, therefore, should not stand in the way of rational immunogenicity trials nor should it restrict the financial resources that must be made available to implement them.

Conclusions

Creative imaginative thinking is required to choose credible candidate vaccines and to invent plausible hypotheses to help select judicious vaccine regimens, dosages, adjuvants, delivery routes and effective T cell help. Instead of misrepresenting experimental hypothesis testing as a case of rational design, it would seem preferable to openly acknowledge that the empirical testing of immunogens, which initially should take the form of small human trials, still offers the best hope of discovering an effective HIV-1 vaccine. There is increasing doubt that developing a preventive vaccine able to provide prophylactic immunity to stop HIV-1 infection from occurring altogether is a realistic enterprise. There is indeed no evidence that it is possible to induce sufficiently high levels of broadly neutralizing antibodies at mucosal surfaces to achieve that aim. In recent years, much research has been directed instead to the development of therapeutic HIV-1 vaccines for treating people who are already infected with HIV-1. It has been shown, for instance, that immunization with the conserved HIV regulatory proteins, Tat, Rev. and Nef, which are the first proteins expressed after HIV infection, is able to stimulate the immune system of patients receiving anti-retroviral therapy, enabling them to partially control virus replication (Caputo et al. 2009). Other therapeutic vaccines are currently being investigated (García et al. 2011; Li et al. 2011) and it may become possible to assess their value more rapidly in the future by using so-called «adaptive trial designs» aimed at screening out poor vaccines while extending the evaluation of efficacious ones (Corey et al. 2011). In addition, since many methods are now available to chemically inactivate HIV-1 while retaining the conformational and functional integrity of the gp proteins (Van Regenmortel 2011b and references therein), it

may be justified to re-examine the possible value of a killed HIV-1 vaccine, provided the total innocuity of inactivated and genetically modified virus can be established.

The unexpected promising results of the Thailand “RV144” trial, initially condemned as ill-conceived and unjustified (Burton et al. 2004b; McNeil et al. 2004; Belshe et al. 2004; Jefferys and Harrington 2004) but which turned out to be the first human vaccine trial showing modest protection against HIV infection (Rerks-Ngarm et al. 2009; Letvin 2009), demonstrate that empirical trials will always be needed since their outcomes are inherently unpredictable.

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