Journal of
AIDS & Clinical Research
ISSN: 2155-6113

Kong and Sattentau, J AIDS Clinic Res 2012, S8
DOI: 10.4172/2155-6113.S8-003

Review Article Open Access

Antigenicity and Immunogenicity in HIV-1 Antibody-Based Vaccine Design

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Abstract

Neutralizing antibodies can protect from infection by immunodeficiency viruses. However, the induction by active vaccination of antibodies that can potently neutralize a broad range of circulating virus strains is a goal not yet achieved, despite more than 2 decades of research. Here we review progress made in the field, from early empirical studies to today's rational structure-based vaccine antigen design. We discuss the existence of broadly neutralizing antibodies, their implications for epitope discovery and recent progress made in antigen design. Finally, we consider the relationship between antigenicity and immunogenicity for B cell recognition and antibody production, a major hurdle for rational vaccine design to overcome.

HIV-1 Vaccine Development and the Case for Inducing Neutralizing Antibodies

Vaccines have allowed the control of many infectious human diseases, and eradication of one [1]. However, despite having identified the causative agent of AIDS as HIV-1 and characterized in large part its biological characteristics, we are still far from developing a prophylactic vaccine against this virus. The fact that HIV-1 infected individuals cannot clear the virus means that there is a lack of evidence from natural infection regarding immune correlates of protection [2]. The extraordinary mutation rate of HIV-1 arising from an error-prone polymerase (reverse transcriptase, RT) and the high propensity for recombination are major obstacles. When pitted against the immune system, such an adaptable virus readily diversifies into numerous variants that can escape the cytotoxic T cell (CTL) and neutralizing antibody (NAb) responses, fuelling ongoing infection [3,4]. The high degree of variability in the global viral population leads to two broad conceptual approaches to vaccine design: 1) To target only regions of high conservation in the viral antigens; 2) To target multiple regions of relative variability in a 'cocktail' approach. In the current review we will focus on the first of these concepts.

A question that presents itself in vaccine design is whether the vaccine needs to induce sterilizing immunity (preventing infection) or may allow infection but protect from disease. It seems likely that most successful vaccines protect from disease rather than infection, since adaptive immunity relies heavily upon antigenic restimulation to mount a secondary response, and the required levels of antigen can usually only be obtained after limited replication of the pathogen in the host. In the case of HIV-1, it may not be sufficient to rely upon vaccine-mediated disease control post-infection, because once established in the host this virus is so extraordinarily able to evade the adaptive immune response. Although CTL are able to control HIV-1 replication in animal models of vaccination [5], their activation requires the infection of host cells, followed by recognition and killing. Thus unless CTL were able to clear all infected cells within a short period of time, fully aborting infection, it seems unlikely that true sterilizing immunity would be an outcome of this type of strategy. By contrast, Nab infused or actively elicited by immunization have provided complete sterilizing protection from experimental challenge in macaque models [2]. The same cannot be said of non-neutralizing Ab, since their infusion fails to protect from challenge in macaque models [6,7], or results in weak, non-significant effects [8], or may even increase viral transmission across mucosal surfaces [9]. A major caveat of protection from challenge by active vaccination however, is that to date it has only elicited Ab that neutralize, and protect from, unusually neutralization sensitive viral strains [2]. The logical conclusion from these considerations is that Ab-mediated sterilizing immunity is a desirable feature of an HIV-1 vaccine, but that achieving this by active vaccination requires considerable further work.

A number of conventional vaccine development strategies have been implemented to combat HIV-1. They include the use of live-attenuated and inactivated viruses, and isolated viral envelope glycoprotein (Env)-based subunit antigens. Whole inactivated vaccines have not shown much promise, and chemical inactivation processes may adversely modify Env, preventing or reducing NAb responses. An advantage of using a live-attenuated virus is that a broad range of immune responses is induced towards an entity that resembles the pathogen in almost every respect. However, the possibility of the attenuated virus reverting to pathogenicity is too significant a health issue [10]. In later studies more genes were deleted from SIV to further attenuate the virus, but this reduced vaccine efficacy and still failed to completely eliminate the risk of pathogenicity [11].

The HIV-1 Envelope Glycoproteins as Vaccine Antigens

HIV-1Envis the only target of NAb, and an Env-based subunit vaccine would, in principle, be safe and practical. Early experimental formulations in which the surface Env subunit gp120 was expressed in isolation showed that the antigen was safe and immunogenic in Phase I clinical trials [12]. Vaccinee sera, however failed to neutralize viruses other than the autologous, neutralization-sensitive isolate from which the gp120 sequence was derived [13]. This was explained by the observation that Ab in the sera from individuals vaccinated with gp120 mainly recognized linear epitopes within the hypervariable

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Received February 09, 2012; **Accepted** March 18, 2012; **Published** March 22, 2012

Citation: Kong L, Sattentau QJ (2012) Antigenicity and Immunogenicity in HIV-1 Antibody-Based Vaccine Design. J AIDS Clinic Res S8:003. doi:10.4172/2155-6113.S8-003

loops [14]. It was therefore no surprise that neither of the two HIV-1 vaccine candidates based upon gp120 that completed Phase III efficacy trials protected vaccines [15-18]. In an effort to more closely recapitulate the antigenic structure of the intact viral spike, many laboratories then turned towards expression of trimeric antigens based upon gp120 linked to the extraviral portion of the transmembrane glycoprotein gp41, termed gp140. These trimeric gp140-based antigens can be expressed directly without modification, with site-directed mutations to stabilize structure or unmask conserved receptor binding regions, or as fusion proteins with a trimerization motif [19]. Some incremental improvements in Nab activity have been noted, and sterilizing immunity against challenge has been elicited in the macaque model [20], but overall the Nab response to these modified antigens is only incrementally improved in breadth and potency compared to that elicited by gp120 [19,21,22]. Reasons for this may include: i) trimeric Env is conformationally unstable and fluctuates between 'open' and 'closed' states [23]; ii) when cleaved at the gp120-gp41 interface, unmodified gp140 dissociates into its component subunits in a virus strain-specific manner [24-26]; iii)soluble Env conformation is not antigenically equivalent to the functional membrane-embedded Env trimer as probed by monoclonal antibodies (mAb) with epitopes sensitive to quaternary protein folding [27]; iv) when compared to whole virus or vectored delivery of viral genes, soluble subunit vaccine preparations lack both the abundant T helper cell epitopes from other viral proteins important for T cell help of B cell activation, and lack presentation in an array to optimally trigger the B cell receptor (BCR), and may therefore lack immunogenicity compared to their virion-anchored counterparts [28].

This failure to elicit the appropriate quality and magnitude of Ab response has led to several questions. 1) Can HIV-1-infected individuals, and perhaps by extension Env vaccinated individuals, elicit Nab that bind highly conserved neutralization epitopes and thereby neutralize a broad range of viral variants (termed broadly neutralizing Ab or bNAb)? 2) If HIV-1 Env contains highly conserved neutralization epitopes such as the CD4 binding site (CD4bs), why are potent and broad-spectrum bNAb not elicited to those epitopes by active immunization? 3) What form of Env-based antigen might be designed to elicit bNAb?

Conservation of Antigenic Surfaces on Env and Isolation of bNAbs

The field has known for almost 2 decades that HIV-1-infected individuals can elaborate NAb responses to conserved neutralizing epitopes [2,29-31]. However, the frequency of these responses was unclear until a series of recent studies revealed that up to 25% of HIV-1-infected individuals may elicit bNAb responses [32-34]. Since overcoming the variation intrinsic to the circulating HIV-1 viral population will probably be the major challenge to eliciting NAb by vaccination, it is good news that such responses can be made relatively frequently in man. More recent optimism comes from the isolation of bNAbs from 'elite neutralizers' (HIV-1-infected individuals whose plasma neutralizes a broad range of viral isolates potently), that recognize, by definition, highly conserved structures on Env. Indeed, some bNAbs neutralize 70-90% of all circulating isolates [27,35-37], implying that their elicitation as individual or mixed specificities by vaccination might protect against the majority of circulating virus strains. The current 'crop' of bNAbs are summarized in [38] and those with structurally characterized epitopes in Figure 1. Existing bNAb epitopes fall into four broad regions: 1) the gp120 CD4bs; 2) a high mannose glycan patch on the outer domain of gp120 around the V3 loop area; 3) glycopeptide epitopes associated with the V1/V2 loops of gp120 that require quaternary Env interactions; 4) the Membrane Proximal External Region of gp41 (MPER) [38] (Figure 1). Others probably remain to be discovered, although the total number of conserved bNAb epitopes is predicted to be restricted [39,40], perhaps unsurprising given the very limited exposed protein surface area on Env. The isolation of bNAbs has direct relevance to vaccine design. Co-crystallization of the bNAb with its epitope yields information regarding which exposed conserved regions on the antigen can elicit an Ab response, and the epitope structure may help guide subsequent vaccine antigen design [41-44].

HIV-1 Neutralizing Antibody Evasion Mechanisms

Throughout the history of developing aNAb-based vaccine against HIV-1, a pressing question has emerged but remains largely unanswered: how can the biophysical properties of the antigen be translated to specific kinds of immune responses in a highly defined manner? What is clear from the work carried out previously using Env-based immunogens, is that the presence of an epitope on the antigen is no guarantee that that epitope will be recognised by B cells and responded to by Ab production. This invokes the concept of B cell immunodominance in antigen recognition. A definition of humoral immunogenicity is the capacity of an antigen to elicit Ab. Certain portions of the antigen may elicit more Ab than others and thus be more immunogenic, and this is termed immunodominance. B cell immunodominance may be defined experimentally by defining those surfaces of an antigen that elicit the greatest number and titre of antibody responses to those that elicit reduced or absent responses, or theoretically by modelling specific biophysical parameters of the antigen surface in question. B cell immunodominance is much less well understood than T cell immunodominance [45], but is substantially dictated in the case of HIV-1 Env by viral NAb evasion strategies which can be summarised as follows. 1) Use of extensive sequence variation [46,47]. 2) Presence of the glycan shield, which is largely immunogenically invisible to the host immune system, to mask protein surface from Ab binding [48-51]. 3) Use of conformational masking to avoid or reduce B cell recognition and Ab binding [52,53]. 4) Presence of non-functional, misfolded and precursor Env, gp41 'stumps' and shed monomeric gp120 as decoys for B cell recognition [54,55]. 5) Recessed or cryptic receptor and co-receptor binding sites [56-58]. If this evidence is taken together, it becomes clear that HIV-1 Env is an extraordinarily evolved machine that combines function (receptor binding and membrane fusion) with multiple layers of immune evasion. Probably the most difficult problems to overcome are the phenomenal level of sequence variability in Env combined with the very limited protein surface exposed on the intact trimeric spike. The most direct way to overcome this is to target precisely those conserved exposed regions as defined by bNMAb probes: the existence of such bNMAbs provides proof of principle for the existence of such conserved surfaces, and leads directly to structural analysis of these surfaces to guide subsequent antigen design.

Epitope Mimicry and Structure-Based Antigen Design

Structure-based vaccine design posits that if one has the structure of an Ab epitope, then that epitope can be engineered to be presented in a different context, and used to elicit the original Ab specificity by immunization [41,42] and Figure 2. This is an attractive concept, since our ability to generate a specific bNAb epitope could in theory lead to elicitation of large amounts of the corresponding bNAb specificity *in vivo*. The recent use of molecular 'scaffolds', small structurally-defined domains obtained from the protein data base that constrain and present conformationally-sensitive epitopes in a manner that precisely mimics

that on the intact antigen, has been a major advance in structure-based vaccine design [59,60]. However, whilst proof-of-principle evidence exists for the precise engineering of specific bNAb epitopes that are structurally almost identical to their native counterparts, their use as immunogens has generally been less fruitful. Early encouraging data were obtained using synthetic peptide epitope mimics of the gp120 V3 loop, in accord with its immunodominant nature [61-63] and coreceptor binding function [61]. The more successful variants of this target have been based upon selection of constrained epitopes using Ab binding [64] or epitope structure-based design [65]. However, it is well recognized that the V3 loop is usually only exposed to Ab binding on functional Env of unusually neutralization sensitive viruses [63], limiting the usefulness of this approach, although this view is challenged by some [66]. Nevertheless, the use of epitope mimics of the V3 loop provide direct proof-of-principle that such minimal engineered constructs can induce a NAb response against HIV-1.

The concept of generating a single specificity or a very limited set of related specificities of MAb from immunization with an epitope mimic differs from conventional vaccine approaches that rely upon polyvalent responses to complex antigens [67,68]. It has been suggested that structure-based vaccine design is fundamentally flawed, since the continuous and unpredictable nature of Ab epitopes on an antigen make reiteration of a particular Ab specificity by an isolated epitope mimic highly improbable[67-69].However, the outcome of the epitope mimic approach is likely to depend to great extent upon the intrinsic immunogenicity of the epitope in question, since as described above, isolated fragments of the gp120 V3 loop are able to induce robust oligoclonal Ab responses that are neutralizing for a subset of HIV-1 strains. This result contrasts with the failure, to date, of engineered epitope mimics of the MPER region of gp41 to elicit the desired specificity of HIV-1 Nab, although this failure has specific caveats relating to lipid recognition (see below and [59,70-72]). Thus a major question stemming from this debate is whether the immunogenicity of particular epitope or epitope mimic is entirely pre-determined by its intrinsic immunodominance, or whether the immunodominance of an epitope is relative and can be modulated experimentally. This question is discussed further in later sections.

MPER Epitope Mimics

The MPER peptide epitopes for bNMAbs 2F5 and 4E10 (Figure 1)

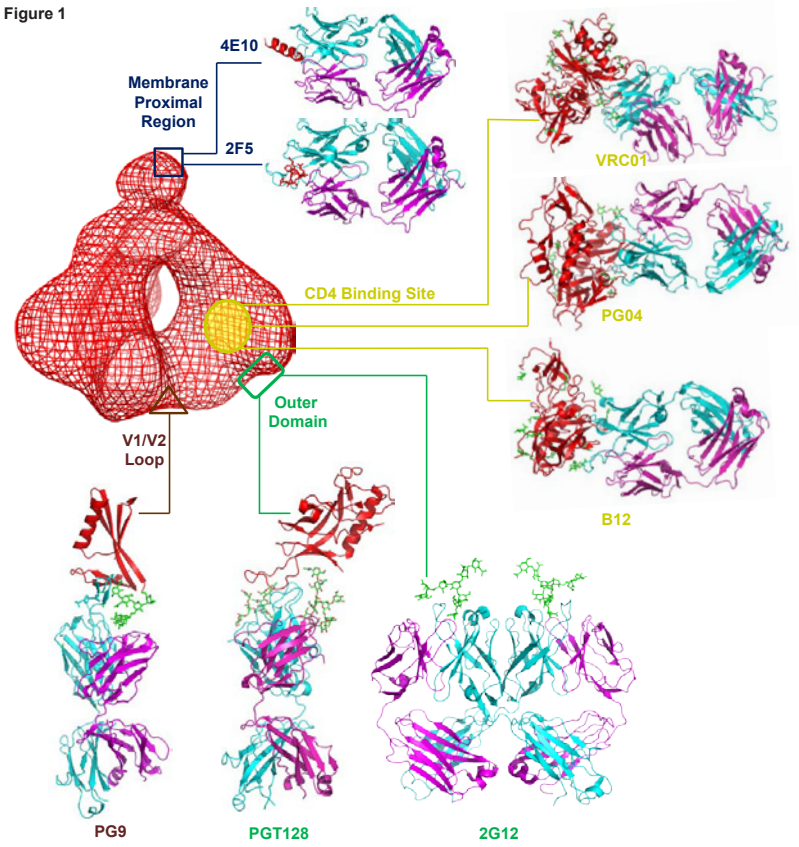

Figure 1: The epitopes of four classes of bNMAb.

Currently, there are four broad classes of bNMAb against the HIV-1 trimeric spike (red): Ab that bind the membrane proximal region (blue), the CD4bs (yellow), the oligomannose patch on the outer domain with or without implication of the V3 loop (green) and the V1/V2 domain (brown). Representatives of each class have been extensively structurally characterised. Here, these are shown alongside a low-resolution cryo-EM map of the unliganded gp160 spike (EMDID: 5019) (red mesh) oriented with the viral membrane on the top. In the gp41 portion, the two bNMAb that recognize the membrane proximal region are shown: 4E10 (PDBID: 2FX7) and 2F5 (PDBID:1TJG). Beneath that, in the gp120 portion, are bNMAb that recognize the CD4bs: VRC01 (PDBID: 3NGB), PG04 (PDBID: 3SE9) and b12 (PDBID: 2NY7). Also in the gp120 portion are bNMAb that bind the outer domain region around the V3 loop: 2G12 (PDBID: 1OP5) and PGT128 (PDBID: 3TYG). Finally at the bottom is a bNMAb that binds a quaternary-fold-dependent epitope in the V1/V2 domain: PG9 (PDBID 3U2S). Here, all structures are displayed in cartoon format with the heavy chains of the Ab coloured cyan, the light chains coloured magenta, the gp120 protein portions coloured red and the gp120 glycan portions coloured green.

are structurally defined [73,74], and have been mimicked in a number of laboratories by synthetic peptides that bound the bNM Abs with relatively high affinity. However, although capable of eliciting specific Ab responses to the peptide [30,70,71,75-81], MPER epitope mimetics have so far failed to elicit appropriately strong bNAb responses. This is despite recent results showing that scaffolded peptide epitope mimics induced an Ab response in mice that bound an epitope conformation almost identical to that bound by the starting bNAb, 2F5 [70]. The conclusions reached after a decade of intensive research are that conformation and appropriate lipid presentation of the epitope mimic are critical to eliciting Abs of the desired specificity [70,82] (Table 1). Thus the 2F5 epitope probably requires precise insertion into a lipid interface, thereby presenting the epitope in a manner allowing appropriate 'extraction' from the membrane [82]. Moreover, the steric effects of having the MPER peptide epitope 'sandwiched' between the glycans of the Env trimer and the lipid envelope may make direct Ab association with the lipid bilayer a requirement, in order to achieve the appropriate angle of approach to the peptide epitope [43,44,70]. These studies reveal that protein engineering has reached a level of sophistication whereby peptide mimics of conformational epitopes can be constructed with high fidelity, and can elicit Ab responses of appropriate peptide specificity. However they also demonstrate that we have yet to successfully combine recognition of peptide surfaces with concurrent recognition of other antigenic structures such as lipids and glycans (Table 1) to yield composite bNMAb epitopes such as those presented in the gp41 MPER. Finally, a further level of complexity is introduced by the fact that lipid association promotes an elevated level of autoreactivity with cell membranes and other host cell components, implying that self-tolerance mechanisms may need to be overcome to generate such Ab responses [83-85].

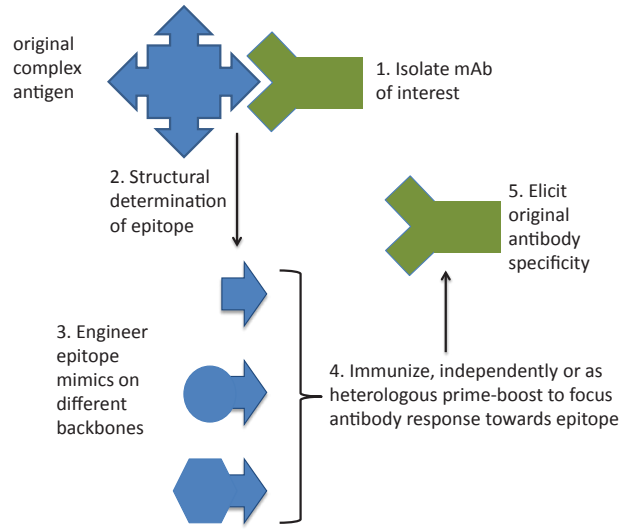

Figure 2: The structure-based vaccine antigen design strategy

The flow of steps in the process is shown here. The original complex antigen or a subcomponent of it is co-crystallized with a bNMAb to reveal atomic-level structural information about the epitope. The epitope is then recapitulated as a constrained mimic using scaffolds as backbones to present the epitope. If several different backbones are generated that present essentially the same epitopic structure, then these can be used in a series of heterologous immunizations to selectively amplify B cell responses to the epitope. If the strategy works, then the outcome would be the generation of a polyclonal B cell response containing bNMAb specificities that resemble the original template bNMAb.

Mimics of Epitopes Dependent Upon Tertiary and Quaternary Env structure

Much recent effort has gone into designing polypeptide mimics of discontinuous epitopes, with the gp120 CD4bs being most relevant to current HIV-1 vaccine design. This surface is, by definition as a contact surface for a non-polymorphic receptor, relatively highly conserved, although it is flanked by, and contains, islands of variability and conformational flexibility [86]. A sub-component of the CD4bs, however, is highly conserved and conformationally stable, and has been termed a 'site of vulnerability' [87]. This region contains epitopes for very potent and broad NMAb such as VRC01 [36,88]. Early attempts to isolate epitope mimics used phage expression libraries linked with binding to the only available bNMAb, IgG1b12 (b12), for selection. Although peptide mimics were isolated, the affinity of b12 for the resulting mimics, where tested, was still substantially below that of the affinity for the native gp120 [89-92]. It was therefore not surprising when immunization studies with these polypeptides failed to elicit robust b12-like responses or detectable neutralization. A more sophisticated approach has recently been adopted that relies upon reiterating the b12 epitope molecular surface by constraining a polypeptide into the correct conformation using a designed molecular scaffold [93]. The scaffold was created by selecting molecules from the protein structural database that had the appropriate 'fit' for constraining the b12 mimic into the correct fold, then the mimic was reiteratively modeled and mutated within the epitope and backbone until binding affinity for b12 attained that of the native antigen (~10nM). Structural analysis revealed a strong similarity between binding of b12 to the

Antibody	Epitope region	Epitope type	Structure known?	Hurdles to overcome	Key references
2F5, 4E10	gp41MPER	Continuous, conformational and composite (protein-lipid)	Yes, but only in context of antibody-peptide complex	Instructing B cells to recognize peptide-lipid antigens. Overcoming immune tolerance mechanisms without inducing autoimmune pathology	[70,71,73,74,82]
b12 VRC01	gp120CD4bs	Discontinuous protein	Yes, in context of core gp120 and also at lower resolution with cryo-EM in context of trimer	Focusing B cell responses towards 'site of vulnerability', overcoming low immunogenicity, instructing antibody 'angle of approach' to trimer	[37,38,86,87,88,93,112, 114,127]
PG9, PG16	gp120 quaternary V1/V2	Continuous, conformational, composite (protein-glycan)	Yes in context of constrained mimetic	Instructing B cells to recognize glycopeptide epitopes	[95]
PGT 128	Gp120 glycopeptideV3	Continuous, conformational, composite (protein-glycan)	Yes in context of outer domain construct of gp120	Instructing B cells to recognize glycopeptide epitopes	[97]
2G12	gp120 glycan	Discontinuous glycan	Yes in context of synthetic glycans	Instructing B cells to recognize glycans. Presenting glycan arrays that appear foreign to host and may not require breaking of immune self tolerance	[101]

Table 1: A summary of bNMAb families with their epitope type, structure and major hurdles to overcome to develop an antigen capable of eliciting bNMAb.

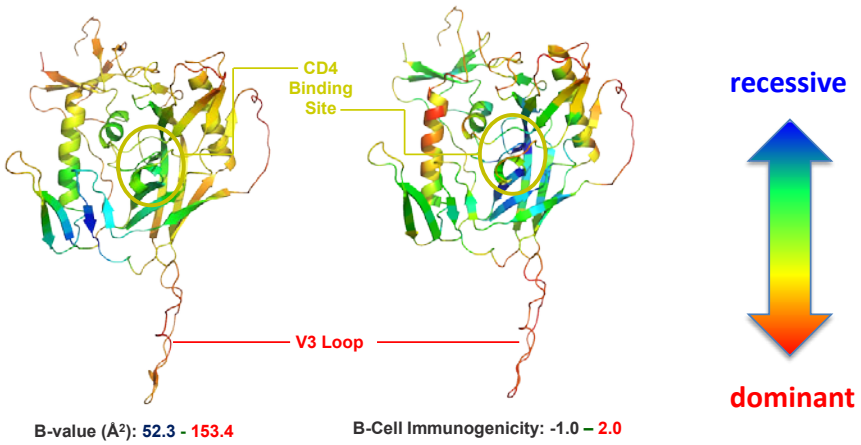

Figure 3: HIV-1 gp120 flexibility and predicted B-cell immunogenicity.

Here the structure of gp120 containing an intact V3 loop (PDBID: 2B4C) is shown in cartoon representations coloured by heat gradient (blue = least, red = most), according to either its crystallographic b-value -a measure of flexibility (left), or to its predicted b-cell immunogenicity by the BEprot server (right). The two representations are oriented to fully display the CD4bs, which has been circled in yellow.

mimic and to native gp120 [93]. Moreover, this scaffolded epitope was selectively unable to bind another weakly neutralizing CD4bs MAb b13, implying strong specificity for b12. This is an impressive engineering achievement that sets the scene for development of further mimics of complex discontinuous epitopes.

Other recent studies have generated interest in bNMAbs that bind epitopes requiring conservation of the quaternary fold of Env. The prototype for this family is the 2909 mAb that binds an epitope dependent upon residues within the gp120 V2 and V3 regions [94]. Whilst this MAb is exceptionally potent, it is highly virus strain-specific. By contrast, the clonally-related bNMAbs PG9 and PG16 neutralize 70-80% of viral strains tested with high potency [27]. Their epitopes are dependent upon maintenance of Env folding, to the extent that no soluble glycoprotein, even when trimerized, maintains the epitope in its native, high affinity conformation [27]. The epitope of PG9 is contained within a region of the V1/V2 domain, and has been recapitulated using a scaffolded glycopeptide mimetic [95]. Within the epitope the PG9 paratope contacts a single β-strand and two flanking glycans that form a mini-canyon structure [95,96]. The independent solution of the structure of another bNMAb of different specificity, PGT 128 that binds an epitope in the gp120 V3 loop, reveals fascinating insight into convergence of molecular recognition. Both PG9 and PGT 128 bind a similar glycopeptide-canyon structure using related structural solutions [95-97]. This convergence suggests that the limited area of conserved protein surface on HIV-1 Env has selected B cell clones that can penetrate the glycan shield and bind conserved peptide sequences that would otherwise be masked by the glycans. Such recognition appears to require an unusually long CDR H3 loop and a high degree of affinity maturation, potentially making active elicitation of this type of Ab difficult (Table 1). However this is now experimentally testable using the epitope mimics made for co-crystallisation purposes, or structurally related antigens, as immunogens.

Glycan-based Mimics

HIV-1 Env is heavily glycosylated, which helps HIV-1 evade Ab recognition but also reduces the accessibility of its receptor binding sites [48,50]. These opposing selective pressures restrict the number of glycans to 15-32 [48,49]. When further packed into a trimer, gp120 glycans are so crowded that their enzymatic processing may be inhibited, affecting the type of glycans produced [98]. Such tight clustering of glycans allows for Ab recognition, since high density packing of this sort is absent in the host even though the individual glycans are host-derived [35,95,97,99]. The 2G12 bNMAb (Figure 1) binds to such a tight cluster of oligomannose glycans on the outer domain of gp120 [99-101], giving an example of the immune system taking offensive action against this viral defence. Speculation [102] that synthetic glycan epitopes might elicit Ab responses specific for those antigens has been rewarded [103,104], although the resulting Abs did not significantly cross-react with gp120. Modification of yeast polysaccharide biosynthesis created an immunogen that did induce gp120-reactive Abs, but these were only very modestly neutralizing [105]. Future approaches may need to recreate more precisely the orientation and presentation of the glycans to achieve higher affinity binding leading to increased neutralization activity. In this regard the recent isolation of several new glycan binding bNMAbs is of particular interest [35].

Understanding B Cell Immunodominance

Structure-based rational vaccine design proposes that direct and intentional modifications to the biophysical profile of an antigen can result in the controlled focusing of the humoral immune response to any selected antigenic surface [41,59,106]. However, this hypothesis relies entirely on the assumption that one or more B cell clones cells will recognise any antigenic fragment presented to them and respond by producing Ab identical to, or similar to, those used as a template to design the antigen. This concept makes assumptions about B cell immunodominance that may be naive. Whereas the biophysical profile of antigen-Ab interaction is strictly chemical and highly quantitative, the immune response consists of several interdependent biological processes that can only be qualitatively described. These processes involve both intrinsic and extrinsic factors including, but not limited to, the fate of antigen *in vivo*, the use of adjuvants, VDJ region recombination, T cell help, affinity maturation and self-tolerance [68,107]. Despite this complexity, the effects of certain biophysical properties of the antigen on the Ab response are partially understood and will be discussed here.

Immunodominance may be experimentally determined by

mapping serum activity through binding competition assays using a panel of mAb with well defined epitopes, and by measurement of B cell clonal frequency to specific epitopes by production of mAbs or Ab sequences. Additionally, since there are clear biochemical determinants of protein binding such as surface accessibility and hydrophilicity, it may be possible for Ab binding propensity to be modelled and subjected to computational prediction. Here we used used COBEpro [108], a discontinuous B-cell epitope prediction software that takes into account accessibility and side chain orientation, to predict the Ab binding propensity of a gp120 construct with intact V3 loop (Figure 3). The computational prediction shows that it is most likely for Ab to bind the V3 loop along with some other well-exposed portions such as the V4 loop and the inner domain. Interestingly, V3 loop immunodominance is what one would predict from previously published empirical results [14,61]. The algorithm gives high scores to those regions of the protein that appear to be more accessible, an expected result as there would be more ways for different BCR to bind a region when it is more exposed. The CD4bs is strikingly immunorecessive, no doubt as a partial consequence of being a shallow recessed canyon [86]. Another parameter used for predicting Ab binding propensity is flexibility [109,110]. If applied to gp120, it would also select the flexible hyper variable loops as being the most immunogenic. Indeed when a measure of flexibility, the b-value of gp120 is directly compared to the BEprot prediction, the resulting pictures are remarkably similar with high b-value regions matching high BEprot predicted B-cell immunodominance (Figure 3). Some supporting experimental evidence comes from a recent effort to elicit Ab against a transplanted epitope of gp41. The immunogenicity of the epitope graft correlated significantly with its flexibility [70]. In this case, greater flexibility may mean greater number of adopted shapes for BCR recognition, allowing a greater variety of B cell clones to bind and become activated.

A third parameter is BCR affinity for antigen since that is an important factor for B-cell survival within the germinal centre during affinity maturation. A detectable B-cell response requires at least micromolar affinity of BCR for antigen [111]. Optimizing this affinity would mean being able to measure binding of the antigen to the highly variable germ line receptors in every patient, which may be possible through personalized deep sequencing followed by variable region expression [112]. However, modification of an antigenic surface to promote BCR binding may well introduce novel ways of binding it that may not be present in the original antigen. This type of directed BCR evolution would result in the maturation of B cells whose receptors ultimately may not recognize the original antigen.

Epitope Focussing and Other Strategies to Modify B Cell Immunodominance

Focussing B cell responses away from immunodominant regions of an antigen towards subdominant (immunorecessive) regions is an attractive concept that deserves further investigation. However, its success is based upon the concept that immunodominance is relative, so that removal of an immunodominant segment will increase the immunogenicity of a previously more immunorecessive segment. This would suggest some form of competition in immunogenicity, as has been described for T cell epitopes [113], but for which there is not yet compelling supportive evidence for B cells. Conversely, if immunodominance is absolute, based solely on the constraints imposed by biophysical recognition of a surface by a given BCR, then there is no reason to suppose that such a strategy will work. However, as discussed above, Ab responses are not governed by biophysical parameters alone. The immunodominance of a surface may be influenced by biological factors such as the local frequency of B cells carrying appropriate BCRs, availability of T cell help and masking of epitopes by pre-existing Ab and/or receptor binding (as in the case of CD4-gp120 binding).

Env trimers and modified trimers

Considering these parameters, it is possible to formulate and test various hypotheses regarding the directed modification of immunogenicity. A common hypothesis is that maximizing the accessibility to Env surfaces recognized by bNAbs while minimizing accessibility to surfaces recognized by non-NAbs would result in a focused immune response to those surfaces recognized by bNabs [87,114]. One way to modify accessibility is to use Env in its native trimeric fold as found on the virion surface. It has long been observed that Ab neutralization correlated with binding to trimer instead of gp120 monomer [115-117]. Moreover, trimer-stabilizing motifs such as GCN4 fused to gp140 elicited sera that better neutralized homologous and heterologous primary HIV-1 isolates than monomeric gp120-elicited sera [118]. Superior immunogenicity was also observed for gp140 trimers stabilized by intramolecular disulphide bonds relative to monomeric gp120 [22], possibly as a result of increased BRC cross-linking and B cell activation. The recent discovery of Nabs with broad activity that are trimer specific also encourages its use [27,95,119,120]. However, the trimer in situ on the virion surface is relatively labile, shedding gp120 in a virus strain-dependent manner [53,121-123]. This problem is exacerbated when the trimer is solubilized by expression in a truncated soluble form without transmembrane anchor. Use of trimerization motifs may help stabilize the trimer, but the resulting oligomer may not represent the functional spike on the virus [124,125], as for example determined by binding of quaternary epitope-specific bNMAb [27]. This leads to the more general problem of defining the structure of a proper trimer mimic of the functional spike. Indeed, most spikes on a virion are defective [48,54] and so the only defining indication for proper oligomerization is functionality in viral entry. Also, like the flexible gp120 monomer [126], the trimer may sample many quaternary conformations, making the idea of a single proper trimer untenable. Cross-linking of the trimer to stabilize it into its different conformers followed by selection of appropriate trimer forms using bNABs such as PG9 is a potential strategy. Low resolution (~20Å) cryo-electron tomograms of the trimer offer useful information on how it is organized [127]. However, these densities will average data from all spikes, both functional and defective; parameters for selection of only functional spikes would be difficult to define. If testing the hypothesis requires a pure preparation of the functional Env, either in membrane anchored or soluble form, then it appears much further work on the trimer structure will be necessary.

Eliminating or modifying unwanted antigenic regions

Another way to modify epitope accessibility is to excise portions of the protein that influence the immunodominance and surface exposure of the overall protein. For example, macaque sera against V2 loop (which partially masks the CD4bs) -truncated gp140 trimers showed increased breadth of neutralization activity [128]. A more extreme excision removed the entire inner domain of gp120, resulting in stable outer domain only constructs [129-131]. Mouse immunization with a clade C outer domain containing an intact V3 loop resulted in predominantly V3 specific Ab responses [131], testifying again to the immunodominance of this loop. Moreover, although not assayed for in this study, excision introduces new surfaces at the cutting zones as well as altered surfaces around the regions that were cut, creating neo-epitopes. Distal structural effects as a result of conformational changes

are also possible. Such neo-epitopes may be more immunodominant than the regions of interest. Empirical studies with further modified outer domain constructs will be required to test this approach.

Another approach was based upon creating a chimeric antigen that contained elements of the CD4bs within a 'resurfaced', or antigenically modified, molecular context [36]. To achieve this, the gp120 'core' antigen from HIV-1, lacking the V1/V2 and V3 loops, was substituted in regions surrounding the CD4bs with amino acids from the homologous simian immunodeficiency virus (SIV) molecule, which is structurally related to HIV-1 gp120 but differs substantially at the amino acid level. In this way the HIV-1 CD4bs was conserved within an antigenically distinct SIV context. Additional glycan sequons were added to neutralization-irrelevant surfaces to exclude irrelevant B cell recognition. Use of this construct allowed the isolation of the highly potent and broadly reactive CD4bsmAb VRC01 and its bNMAb relatives from an HIV-1-infected patient, highlighting its focused antigenicity for the HIV-1 CD4bs [36]. Immunogenicity studies with this type of construct are underway.

Glycan masking and unmasking

A related strategy for focusing Ab responses towards a specific epitope or epitope region relates to maintaining the surface of interest within the native composite antigen whilst physically masking regions irrelevant to induction of bNAb. Approaches to this involve incorporation of additional N-linked glycosylation sequons on surfaces and loops of Env [132-134]. Application of this masking strategy was by engineering multiple N-linked glycan sequons into the gp120 coding sequence that were modeled to occlude protein surfaces apart from theCD4bs [135]. The optimization and expression of these constructs resulted in a higher level of gp120 glycosylation, an antigenic profile that reflected selective masking of neutralization-irrelevant gp120 surfaces, and optimal exposure of the b12 epitope [133,135,136]. However immunogenicity of the hyperglycosylated antigens was globally reduced *in vivo*, and did not reflect their antigenicity in that no detectable b12-like responses were elicited [133,137]. Mutations required to introduce the new glycans decreased protein expression levels and therefore must have had a negative impact on folding efficiency. While some of the protein stabilizing effects of glycans are known, especially on the peptide backbone, the overall conformational effect of excising or adding glycans is unpredictable [138-140]. In order to avoid destabilizing effects, statistical analysis of the protein environment around glycans can be used as a guide to select the best sites for modification [141]. Only those environments in which glycosylation is most likely to be observed should be targeted. For example, turns and random coils are preferred over helices, and certain shapes such as small pockets or convex hills appear to be better for glycosylation. Ultimately, common biochemical rules such as not perturbing protein core packing or disrupting salt bridges would also apply.

An opposite approach has been to remove or modify glycans on gp120 to better expose neutralizing epitopes [142,143]. Deletion of glycans can sensitize viruses to neutralization by both CD4bs and CD4i Ab. Ultimately; however, it is unknown whether elimination of glycans adds neo-epitopes that skew the Ab response away from nAb induction. Moreover, Ab affinity to gp120 epitopes can be drastically altered by the presence of glycans that are very distant from them, suggesting a largely unpredictable reshuffling of the entire glycan shield whenever any glycan is modified [48,133]. This shifting of the glycans might be better understood given increased understanding of biophysical parameters gleaned from structures. However, with the exception of the unliganded SIV gp120 [144], structures of gp120 with full glycans remain elusive. Furthermore, the glycan shield can be properly understood only in the context of the trimer, in which the majority of solvent exposed protein surface would be occluded. As with previously described methods of altering accessibility, progress in testing the glycan approach would require much empirical experimentation with different glycan modifications as well as use of structural information as a guide for designing the modifications. A more subtle approach adopted recently is to reduce glycan complexity to maintain the total number of glycans present, but decrease their steric encumbrance. Production of gp120 in insect cells or mammalian cells treated with Kifunensine [145], or grown in mammalian cells lacking N-acetylglucosaminetransferase I (GnTI) [146], resulted in glycoproteins carrying oligomannose 'stumps' lacking sialic acid termination. This led to increased exposure of the CD4bs in both studies, which encouragingly translated to increased CD4bs immunogenicity [145].

Molecular Mimicry and The Case of Anti-Idiotypes

In the early days of HIV vaccine design, the anti-idiotype hypothesis was invoked as a possible method of generating NAb responses by using Ab as antigens. The concept was based around the idea that an Ab recognising an antigen could itself be used as an antigen for immunisation, and secondary Ab raised against the first Ab's paratope would then recognise the antigen. Looking back this seems naive, as making a 'carbon copy' of a complex three-dimensional surface that could then be used to generate an exact mirror image *in vivo* seems extremely unlikely to take place at any detectable frequency. Indeed, this was the outcome of the empirical analysis, since several laboratories carried out multiple immunisations in mice but failed to obtain convincing proof-of-principle for the concept [147]. A later analysis of molecular mimicry undermined the entire concept of using anti-idiotype networks as a strategy with any practical utility [148], after which it was abandoned for HIV vaccine purposes. The anti-idiotype approach is not directly comparable to current rational vaccine design, not least because of the wealth of detailed structural information available to guide current attempts. Nevertheless, the idiotype story provides a cautionary backdrop to the idea that antigenicity can be predictably converted to immunogenicity.

Conclusion

Phenomenal progress has been made in the past few years regarding our understanding of the structure of bNMAb epitopes on HIV-1 Env. Sophisticated epitope mimics have been engineered that are excellent structural look a likes of the starting epitope within the native antigen. The major challenge now is to determine whether an Ab response to such mimics can be elicited that recapitulates the starting bNMAb in specificity and affinity (Table 1). We already know from previous studies with complex antigens such as AbV regions (anti-idiotype strategy) that eliciting Ab to specific protein epitopes can be very difficult. The biological hurdles implicit in 'training' B cells to recognize composite epitopes comprised of protein-glycan or protein-lipid are unknown, but the apparent rarity of such specificities probably testifies to the complex B cell evolution required to elicit them. One strategy that has yet to be tested is a form of instructive evolution of B cells in which one 'teaches' the B cell to see each component of a complex protein or composite epitope one step at a time, from germ line BCR to plasma cell secreting high affinity Ab. Having access to phylogenetically dissected bNAb lineages may help illuminate a path along which B cells could be matured towards a particular antigenic endpoint [112].

It has been proposed that rational vaccine antigen design may represent an elevation of vaccine research from an empirical exercise to

a scientific discipline [58]. Others have argued that the structure-based vaccine design concept is set in opposition to empirical studies [67,68]. However, as the current literature clearly attests, rational vaccine design it is not a purely theoretical exercise devoid of experimental trial-and-error investigation. Optimization of antigens for Ab binding, with possible optimization of a series of antigens for iterative BCR activation, heterologous prime and boost regimens to focus responses towards conserved regions and use of diverse immunization schedules and adjuvants can all be exhaustively tested by experiment. Rational vaccine design was born from the frustration arising from the failure of conventional strategies and, as described above, challenges the basic quantitative/qualitative divide that separates the chemical description of an antigen and its biological effect on immunity. It is a hypothesis that invites much empirical testing, and while its ultimate practical goal may be an effective vaccine for HIV-1, its study will undoubtedly deepen our understanding of immunology in a reductionist manner that goes along with the general spirit of synthetic biology that has taken hold of contemporary research.

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This article was originally published in a special issue, **Vaccine research: HIV** handled by Editor(s). Dr. Marc Van Regenmortel, University of Strasbourg, France