LEADING ARTICLE

Biodrugs 2009; 23 (3): 137-153
1173-8804/09/0003-0137/$49.95/0

Progress on the Induction of Neutralizing Antibodies Against HIV Type 1 (HIV-1)

Michael Vaine, Shan Lu and *Shixia Wang*

Laboratory of Nucleic Acid Vaccines, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA

Abstract

Infection with HIV type 1 (HIV-1), the causative agent of AIDS, is one of the most catastrophic pandemics to affect human healthcare in the latter 20th century. The best hope of controlling this pandemic is the development of a successful prophylactic vaccine. However, to date, this goal has proven to be exceptionally elusive. The recent failure of an experimental vaccine in a phase IIb study, named the STEP trial, intended solely to elicit cell-mediated immune responses against HIV-1, has highlighted the need for a balanced immune response consisting of not only cellular immunity but also a broad and potent humoral antibody response that can prevent infection with HIV-1. This article reviews the efforts made up to this point to elicit such antibody responses, especially with regard to the use of a DNA prime-protein boost regimen, which has been proven to be a highly effective platform for the induction of neutralizing antibodies in both animal and early-phase human studies.

Since its discovery in the early 1980s, HIV type 1 (HIV-1) has been implicated in the deaths of >20 million individuals worldwide. According to the WHO estimates >33 million people are currently harboring the HIV-1 virus, many without knowledge of it until the development of AIDS at a later stage. With an estimated 2.5 million people infected globally in 2007 alone, the spread of HIV-1 shows little signs of slowing.[1] The best hope of controlling this pandemic is an effective prophylactic vaccine. While it is generally believed that both humoral and cellular immunity are required to provide protection against HIV-1 infection, there has never been a clear roadmap on how to achieve such a goal.

In the last two decades, a great deal of information and knowledge has accumulated regarding the various immune responses observed during infection with the HIV-1 virus and the development of prophylactic vaccines. Unfortunately, several late-phase clinical trials of HIV-1 vaccine candidates have failed to provide protection against infection or to reduce viral loads after infection in clinical efficacy studies.[2-4] At the same time, we have also witnessed enormous progress in the induction of humoral and cellular immunities against HIV-1 that has resulted from novel strategies of antigen design and vaccination approaches. These have allowed us to further investigate potential protective mechanisms and develop more effective vaccines against HIV-1 infection. The most recent phase IIb trial, the STEP trial, was a novel attempt to deliver an HIV-1 antigen using a non-replicating adenoviral vector, and was intended to prevent disease through the induction of a potent cellular immune response.[4] While scientists are still debating whether the inadequate levels of cellular immunity may be responsible for the failure of this candidate vaccine, this outcome has highlighted the need for the induction of a balanced immune response consisting of not only cellular immunity, but also a broad and potent neutralizing antibody (NAb) response. This article will review data from human and animal studies on efforts made up to now, to induce cellular and humoral antibody responses against HIV-1.

1. Limitations of T-Cell-Based HIV Type 1 (HIV-1) Vaccines

In recent years, the focus of the HIV vaccine field has largely been on the induction of strong cell-mediated immune responses against the virus. This is especially true for the large effort put forth in inducing strong cytotoxic T lymphocyte (CTL) responses directed against the virus. The focus on the induction of CTL responses was driven by a number of

discoveries implicating $CD8^+$ T cells as vitally important in the prevention and control of viral infection. Early work on the role of CTL responses in viral infection determined that the induction of CTLs is the primary correlate for the control of viremia in early HIV-1 infection.[5,6] These findings were corroborated by the discovery that $CD8^+$ T cells controlled viremia in simian immunodeficiency virus (SIV) infection in rhesus monkeys.[7] Additional evidence in human patients capable of controlling viral replication without therapy, so-called 'elite controllers', supported this notion further; strong and effective CTL responses correlated with viremic control in these individuals.[8,9]

The theory behind the design of a T-cell vaccine is that the presence of a strong and immediate CTL response at the time of viral exposure would, at a minimum, reduce viral loads in infected individuals by reducing acute viremia. This theory was supported by results from animal studies, which showed that strong CTL responses protected rhesus monkeys against challenge with a pathogenic HIV-SIV hybrid virus (SHIV).[10-12] Because of the success in raising strong T-cell immune responses and the protection seen in SHIV challenge models, the T-cell vaccine appeared to be an attractive platform for the development of an HIV-1 vaccine. However, despite the success in raising strong HIV-specific T-cell immune responses and good protective efficacy against SHIV challenges, the protective efficacy of these T-cell vaccines against more highly pathogenic viral challenges in rhesus monkeys was much less substantial.[13,14] Furthermore, the entire T-cell vaccine theory has been built on the 'post-infection' protection model because it considers the induction of 'sterilizing immunity' against HIV-1 as an impossible mission. It is therefore unfortunate, but not entirely surprising, that despite being well tolerated and immunogenic in humans, as shown in the STEP trial, T-cell-based vaccines alone ultimately proved ineffective in the best of cases and possibly detrimental in the worst.[15-18]

2. Challenges of Raising Antibody-Based Vaccines against HIV-1

While there will be continued effort to improve the magnitude and breadth of T-cell immune responses in the development of HIV-1 vaccines, there has been a renewed focus on the induction of functional antibody responses to HIV-1 as a means of providing an early and even possibly sterilizing immune response. The induction of a strong functional antibody response, in the form of broadly NAbs, is currently one of the most sought after goals in the field of HIV-1 vaccine development.

Unfortunately, HIV-1 contains an array of protective mechanisms, making the elicitation of a broad and potent NAb response an exceptionally difficult task. Much of the difficulty in raising functional antibody responses can be attributed to the high degree of diversity found in the envelope (Env) glycoprotein, the major target of NAbs to the virus.[19] In addition to the difficulty of overcoming high levels of sequence diversity, functional NAbs must also be able to overcome a series of intrinsic defenses present in the HIV-1 Env. These include high levels of glycosylation, epitope masking by variable loops, cryptic binding domains, the high degree of entropy present in the Env protein, and masking of functionally important domains by quaternary interactions resulting from trimerization of the Env complex.[20] Additionally, because HIV, as a retrovirus, integrates into the cell genome of the host, there exists only a very narrow window for NAbs to act before the establishment of an infection.

Despite all of the protective mechanisms that the virus utilizes, a number of monoclonal antibodies (mAbs) have been identified that are capable of neutralizing a relatively wide number of primary isolates.[21] To date, the only means of providing sterilizing immunity is by the passive transfusion of these mAbs before or shortly after viral challenge.[22-27] While relatively high levels of these antibodies were required to prevent HIV-1 or SHIV infection in animal models, the success of these studies demonstrates that sterilizing immunity based on an antibody-mediated mechanism is indeed feasible.

3. Raising Antibody Responses to HIV-1 Antigens

A significant amount of work has been done to raise high-quality, functional antibody responses against HIV-1. Much of this work has focused on two areas: (i) modulation of vaccine antigens; and (ii) the immunization regimens used to deliver these antigens. Early attempts to raise antibody responses to the virus primarily used HIV-1 Env glycoproteins from T-cell line-adapted (TCLA) viruses. This was before the realization that there was a significant difference in the antigenicity of Env glycoproteins derived from TCLA viruses and primary HIV-1 isolates.

In a study by Berman et al.,[28] chimpanzees received three immunizations of gp120 or transmembrane and cytoplasmic tail-truncated 'gp160' (gp140) formulated in aluminum hydroxide (alum). Based upon the presence of homologous NAb responses exceeding 1 : 160 in the immunized animals, a challenge with homologous HIV-1 isolate IIIB was carried out. While the two animals immunized with the gp160 variant became infected within 7 weeks of challenge, both of the gp120-immunized chimpanzees in this study remained free from viral infection 6 months after challenge.

The apparent success of this and other studies[29,30] led to the testing of TCLA-based recombinant proteins in clinical trials. Many of these trials, based on immunization with subunit Env glycoproteins derived from the TCLA isolates IIIB or MN, proved to be both safe and immunogenic.[31-34] These trials often succeeded in raising binding and NAb responses against TCLA viruses, similar to those seen in protected chimpanzees.[33,35,36] Based upon this information, two phase III efficacy trials were conducted, one in North America and the Netherlands[2] and a second in Thailand.[3] In the trial conducted in North America/Europe, >5400 individuals were enrolled to receive a bivalent vaccine consisting of two clade B recombinant (r)gp120 HIV-1 envelope antigens derived from isolates MN and GNE8 and adjuvanted in alum. All of the vaccinees who were studied generated positive binding antibodies to the homologous Env antigens, and many generated a homologous NAb response to the MN vaccine strain.[37] Despite this, the rates of infection did not differ between the placebo and vaccine groups, indicating that the vaccine was not efficacious.

Similar results were also observed in the trial conducted in Thailand.[3] This arm of the study enrolled >2500 intravenous drug users and differed from the North American/European arm of the trial only in the chosen antigens. In an attempt to better represent circulating virus at the Thailand location, a bivalent formulation, consisting of a clade B rgp120 HIV-1 Env antigen derived from strain MN, and a clade E rgp120 HIV-1 Env antigen derived from strain A244, was chosen. Again, gp120 binding antibodies and MN NAbs were generated as a result of immunization, but no difference in the rates of infection was observed between the placebo and vaccine arms of the trial.[3] The failure of these trials was actually predicted because *in vitro* neutralization assays performed before the start of these two trials had already demonstrated that antibodies elicited by TCLA Env antigens, such as MN, were not able to neutralize primary HIV-1 isolates. The failure of these trials may be due in part to the selection of antigens, specifically those derived from TCLA isolates, for the vaccine formulation. However, it still remains to be seen whether a selection of antigens more representative of those circulating amongst different populations may provide a protective response against viral challenge.

4. Raising the Quality of the Antibody Response Against HIV

Because of the proven ability of NAbs to provide sterilizing immunity in non-human primate-challenge models,[22-27] an enormous amount of effort has been put into designing immunogens that are capable of eliciting a functional NAb response (table I). These efforts have focused on a number of different techniques including, but not limited to, the manipulation of the Env sequence, making structural modifications to the Env antigens and increasing the immunogenicity of potentially important but poorly immunogenic epitopes.

4.1 Centralized Antigens

One of the most highly criticized elements of the two failed phase III trials[2,3] was the antigen selection used in the vaccine – in particular, the inclusion of the TCLA-derived Env antigens from strain MN. The MN isolate is not representative of the majority of isolates circulating in the global population. Because of this, it should be of little surprise that this particular isolate, when used as an immunogen, did not elicit a broadly cross-reactive immune response. In an effort to design an immunogen more representative of the isolates more likely to be observed in an *in vivo* setting, the use of consensus or centralized envelope sequences has garnered some attention in recent years. One such study generated an artificial Env antigen based on the five constant regions of gp120 (C1 to C5) and the V3 loop of the group M consensus sequence.[38] Both gp120 and gp140 forms of this consensus M protein were investigated as immunogens in a guinea-pig model.[38] After five immunizations with either the gp120 or gp140 proteins administered in Ribi-CWS (cell wall skeleton) adjuvant, the resulting sera neutralized the sensitive isolates SF162 and Bx08 to a high titer. Additionally, positive NAbs against the more difficult-to-neutralize isolates, SS1196 and QH0692, were also generated. V3 peptide adsorption performed in an attempt to identify the specificity of the NAbs elicited with this construct demonstrated that while sensitive isolates were primarily neutralized by the antibodies to the V3 loop, other unknown antibody specificities were elicited that were largely responsible for neutralization of the more resistant primary isolates.

Based upon the ability of the first-generation consensus M immunogen to raise a functional, but limited, antibody response, a second-generation group M consensus *env* gene was also tested.[39] As opposed to the first-generation construct, this *env* gene encoded the consensus sequence for the entire Env protein, as opposed to primarily the constant regions. Some modifications to the variable loops of the Env were made as a result of the interpretation of the consensus sequences by the authors. These modifications resulted in the variable loops being slightly shorter than those of the average wild-type strains. Protein from different gp140 constructs of this consensus M *env* gene, when administered in Ribi-CWS adjuvant,

Table I. Envelope immunogen designs and their effect on neutralizing antibody (NAb) responses

Immunogen design	Concept	Immunogens tested	Result	References
Centralized sequences	To reduce the genetic distance between immunogens and primary isolates to elicit a more cross-reactive response	Group M consensus	Limited increase in the breadth of NAb response	38,39
		Subtype B consensus	Increased NAb potency over wild-type sequences	40
		Subtype C consensus	No appreciable increase in NAb response	41
		Ancestral B	No appreciable increase in NAb response	42
		Ancestral C	No appreciable increase in NAb response	41
Variable loop deletions	To make functionally important domains more accessible	V1/V2, V3, and V4 deletions	No appreciable increase in NAb response	43-45
		V2 deletions	Increased potency and small increases in the breadth of the NAb response	44,46-48
Glycosylation mutants	To shield irrelevant domains or expose important domains	Hyperglycosylation	Elimination of unwanted antibody specificities No improvement in overall NAb response	49,50
		Targeted deletion	Increased breadth and potency of NAb response in some isolates; no effect in others	51-54
Envelope trimers	To better mimic the natural state of functional trimer	Eliminate envelope cleavage site	NAb response study- and isolate-dependent	55,56
		Stabilized intermolecular interactions	Inconsistent increases in the potency of NAb titers to homologous isolates	57
		Envelope trimerized with heterologous motifs	Increased potency of NAb response	58-60
Epitope grafting	To make a neutralizable epitope more immunogenic when presented in a different context	MPER grafts	No increases in NAb response with specificity for graft	61,62

MPER = membrane-proximal external region.

successfully broadened the NAb response compared with immunization with wild-type sequences derived from the primary isolates JR-FL (clade B), 92RW020 (clade A), or 97ZA012 (clade C). However, the overall breadth of neutralizing activity was still very limited, with the sera still being unable to neutralize the majority of prototypic HIV-1 isolates. When this serum was tested for neutralizing specificity, a large proportion of neutralizing activity could be adsorbed with the V3 peptides. Because the V3 loop may not be accessible in a large fraction of primary isolates, the detection of a high percentage of V3-directed antibodies potentially explains the limited breadth of neutralization observed during immunization with this antigen.

In attempts to focus the antibody response more on a single subtype, subtype C ancestral and consensus genes have also been generated.[41] When these immunogens were administered to guinea pigs as three DNA immunizations, the resulting sera were capable of recognizing a greater breadth of contemporary clade C antigens than sera generated by immunization with a wild-type clade C immunogen. However, despite the increase breadth of cross reactivity, very little homologous or heterologous neutralization was observed. Only one of the immunized animals generated antibodies capable of neutralizing its homologous isolate, while none of the animals generated antibodies capable of neutralizing heterologous primary isolates. Similar studies have also been completed with subtype B ancestral[42] and consensus immunogens.[40] The immunogenic properties of the subtype B consensus immunogen was evaluated in guinea pigs after three DNA immunizations

encoding the different forms of the consensus B *env* gene.[40] The elicited humoral responses were then compared with those of guinea pigs immunized with the wild-type isolates CAAN5342.A2 and WITO4160.27. In this study,[40] the consensus B immunogens elicited NAbs to isolates with a range of sensitivities, including isolates SF162 and SS1196, and a subset of viruses representative of those found in acute infection, so-called tier 2 viruses,[63,64] phenomenon not observed with immunization with the wild-type immunogens. Based upon the successful neutralization of a number of isolates, Kothe et al.[40] continued their investigations to identify the mechanism of virus neutralizing activity observed in their study. An HIV-2 virus either pre-exposed to CD4 or containing a graft of the membrane-proximal external region (MPER) of gp41 was used as a means to identify the presence of co-receptor- or MPER-targeted NAbs. However, none of the immunized animals generated antibodies with these specificities.

4.2 Variable Loop Deletions

Generation of centralized immunogens among a large group of viral isolates is only one approach to enhancing the immunogenicity of HIV constructs. Others strategies have focused on structural modification of existing HIV-1 Env antigens. It has been well established that variable loops can protect functionally important domains.[65-68] Theoretically, deletion of variable loops should expose these functionally important domains, allowing antibodies to be elicited to the previously obscured regions. On the basis of this theory, a number of studies have modified the wild-type Env glycoproteins by deleting variable loops. One of the first such studies to investigate the role of variable loop deletion in altering the immunogenicity of parental Env glycoproteins evaluated the effects of V1/V2 and V3 deletions on the immunogenicities of several forms of the HXB2 Env glycoproteins.[43] This study evaluated the wild-type and variable loop-deleted forms of gp120, gp140, and gp160 immunogens delivered by DNA immunizations for their ability to raise binding antibodies and NAbs in rabbits. Results indicated that variable loop deletion of the gp140 and gp160 constructs increased the amount of binding antibodies elicited to the gp120 subunit of the Env glycoproteins. However, despite this increase in the binding antibody titers, immunization with the wild-type gp120 subunit was still the most effective way of eliciting these antibodies. This study also evaluated the ability to raise NAbs against the sensitive HIV-1 isolate IIIB. Immunization with the strictly wild-type gp120 elicited the highest NAb titers against this isolate. However, the elimination of the variable loops from this construct had a detrimental effect, eliminating all observed neutralizing activity. This is likely due to the extreme sensitivity of this isolate to variable loop-mediated neutralization. In addition, although the variable loop deletions increased binding antibody titers in the gp140- and gp160-immunized animals, none of these constructs managed to elicit NAbs against the IIIB isolate.

Another study looked at the effect of eliminating only the V2 loop of the SF162 isolate of HIV-1 on its ability to raise a humoral immune response.[46] In this study, rabbits were given DNA immunizations encoding a full-length SF162 gp140 immunogen, or one with a partial deletion of the V2 loop. The raised antibody responses were then tested for neutralization breadth and potency against a panel of homologous and heterologous isolates. When tested against the homologous SF162 isolate, more potently neutralizing sera were raised by immunization with the V2-deleted construct. In addition, neutralizing activity was observed more frequently and with high NAb titers against six other heterologous clade B isolates. This pattern of NAb response was also observed in rhesus macaques immunized with the same constructs followed by a protein boost with the homologous V2-deleted gp140. Again, more broad and potent NAb responses were raised when the V2-deleted construct was used as an immunogen compared with the wild-type SF162 immunogen.

Further study of V2 deletions characterized the changes in the antibody specificities elicited compared with immunization with the wild-type Env glycoproteins.[69] Interestingly, immunization of macaques with the V2-deleted construct resulted in the generation of high titers of antibodies to the V3 loop of SF162 as well as a modulated ratio of serum antibodies capable of being out-competed by soluble CD4 binding to gp120. The utility of V2 deletions in subtype C immunogens has also been evaluated.[47] Using the viral Env glycoproteins of the South African HIV-1 TV1 strain as a model subtype C immunogen, a comparison of the immunogenicities of unmodified and V2-deleted immunogens have been made. When the V2-deleted constructs were used, an increase in the potency of homologous neutralization was observed, similar to the results seen with the SF162 immunogen. Additionally, an increase, albeit a very limited one, in the breadth of neutralization against heterologous clade B and C viruses was also observed with the use of this V2-deleted gp140 contruct.

Derby et al.[44] characterized the humoral antibody responses raised by wild-type and variable loop-deleted gp140 constructs elicited in macaques and compared these with humoral responses elicited during chronic SHIV infections in macaques and heterologous HIV-1 infection in humans.[44] Interestingly, the quantity, quality, and specificity of humoral

antibody responses differed greatly between groups. In gp140-immunized animals, the gp120 subunit of the immunogen appeared to be more immunogenic than the gp41 subunit. This trend was not observed in SHIV-infected macaques or HIV-1 infected humans, where gp41 was equally if not more immunogenic than gp120. Additionally, the overall binding titers were also significantly lower in immunized animals than in infected ones. Another significant difference between immunized and infected animals involves the neutralizing specificity of the serum. While the variable loop modified constructs were very capable of neutralizing homologous SF162 virus, it was discovered that this is largely due to recognition of the V1 loop, a phenomenon not observed in infected animals. While this area is accessible on most HIV-1 viruses, it is highly polymorphic, and potentially explains the limited neutralization breadth that is observed in the gp140 immunized animals.

More drastic modifications to the HIV-1 Env glycoproteins have also been made. The removal of the V1/V2, V3, and V4 loops alone and in combination on an HXBc2 background have also been investigated.[45] While immunization of mice with all of these constructs resulted in high binding antibody titers to recombinant gp120, the highest NAb titers resulted from immunization with the wild-type Env glycoprotein. Immunization of mice with the V1/V2 and V3 deleted constructs elicited an antibody response with little to no neutralizing activity. Epitope mapping analysis revealed that, as expected, deletion of the variable loops can shift the targeting of elicited antibodies. However, oftentimes the shift in recognition is to areas that are not exposed on the surface of the glycoprotein. Because of results such as this, it is likely that simple deletion of variable loops will not provide the necessary increase in potency and breadth of neutralization to effectively combat an HIV-1 infection.

4.3 Glycosylation Mutants

While immunizing with variable loop deletions have resulted in some increases in the quality of the antibody response, other less drastic alterations to the viral Env glycoproteins have also been evaluated for their effect on immunogenicity. One such modification is the alteration of the glycosylation pattern on the surface of the HIV-1 Env. The surface of the Env is very highly glycosylated, with carbohydrates encompassing up to 50% of the total molecular weight of the protein. It has been well documented that changes in the glycosylation pattern of the Env proteins can have significant effects on the antigenicity of the Env glycoproteins and neutralization sensitivity of the parental virus.[70-75] Because changes in the glycosylation pattern of these Env proteins can have a drastic effect on their phenotype, it may also be possible to modulate the immunogenicity of the protein by altering the glycosylation pattern. One potential use of changing the glycosylation patterns on the Env proteins is to dampen immune responses to undesirable epitopes. Because it is relatively difficult to raise antibodies to sugars that should rightfully be identified as 'self' by the immune system, the addition of glycans in unwanted areas should have the effect of focusing the humoral antibody response to desirable areas of the Env. Efforts have been made to this extent in attempts to focus antibodies to the CD4 binding site.[76] In this study, the addition of seven extra glycans eliminated binding of the undesirable non-NAbs, 15e, b6, b3, F91, and F105 while preserving the binding site of the broadly neutralizing CD4 binding site antibody IgG1 b12. Use of this immunogen in rabbits, however, produced mixed results. Rabbits immunized with this construct in Ribi adjuvant generated positive binding antibodies to wild-type Env glycoproteins but raised a highly limited NAb response.[49] Analysis of the immune sera also revealed very limited amounts of antibody with a specificity similar to that of mAb b12, an antibody specificity that this construct was intended to enhance. The neutralization results mirror this, with sera generated from this construct often being incapable of neutralizing even the highly sensitive isolates SF162 and HXBc2. As intended, immunization with the hyperglycosylated mutant did have the effect of dampening the elicitation of most of the weak NAbs similar to the mAbs b6 and F105. Unfortunately, it did not succeed in eliciting b12-like antibodies. This, in combination with lower levels of V3 crown-directed antibodies, may potentially explain the disappearance of the neutralizing activity in this type of serum.

Selvarajah et al.[50] further investigated the concept of dampening immune responses to unwanted areas through hyperglycosylation with the use of a hyperglycosylated trimeric gp140 construct. Trimerization of a hyperglycosylated gp140 construct through the use of a heterologous trimerization domain resulted in significantly reduced availability of the V2 and V3 loops, as measured by mAb binding. This was reflected in the immune sera resulting from protein-based immunizations with this construct. Neutralization of the sensitive isolate SF162 was dramatically reduced as a result of fewer antibodies being elicited to the variable loops of the virus. In the context of dampening the immune response to unwanted areas, this strategy succeeded; however, within the context of focusing the antibody response to more desirable areas, such as the CD4 binding site, this strategy still needs to undergo further development.

Hyperglycosylation of the Env to dampen immune responses to a particular region is only one strategy involving the manipulation of the glycosylation sites on the Env proteins. Another strategy involves the elimination of particular glycosylation sites in order to enhance the immunogenicity of the Env proteins. One such attempt at this eliminated N-linked glycosylation sites in the first and second variable loop of an infectious simian immunodeficiency virus isolate. This glycosylation mutant isolate was then used to infect rhesus monkeys. The resulting humoral antibody responses were then compared with those elicited by infection with the parental wild-type virus.[51] Relative to humoral antibody responses raised by the wild-type virus, the mutant glycosylated virus demonstrated a shift in specificity to the degycosylated region of the V1/V2 loop as well as an increase in the neutralizing activity of the sera. A second study showed that the elimination of a single N-linked glycan at the stem of the V2 loop in a 89.6 background could have dramatic effects on the phenotype, antigenicity, and immunogenicity of a model immunogen.[52] Immunization of macaques with a vaccinia vector encoding the glycan deleted construct, followed by boosting with recombinant protein, dramatically increased the potency of neutralization to homologous mutant and parental virus, as well as increasing the breadth of neutralization against a panel of heterologous clade B-derived primary isolates. However, whether these results will hold true in other Envs, or if they are specific to the 89.6 background, remains to be seen. A number of other studies have attempted to generate mutant forms of the virus by eliminating N-linked glycosylation sites; however, in each of them, it was shown that the mutants were no better immunogens than their parental virus.[53,54]

4.4 Envelope Trimerization

The native Env spike on an HIV-1 virus is a structure consisting of three subunits each of gp120 and gp41. It is possible that an effective immunogen may need to mimic this trimeric structure in order to elicit an effective NAb response. The creation of a trimeric mimic has proven to be a difficult task, mostly because gp120-gp41 and gp41-gp41 interactions on the surface of a virion are governed only by weak noncovalent interactions. Because this limitation makes the production and evaluation of trimeric immunogens a difficult task, a number of strategies have been employed in attempts to overcome this hurdle. One such strategy that has been employed is to eliminate the cleavage site that would normally result in the processing of the precursor gp160 into its mature gp120 and gp41 components. Further modification of this construct, by elimination of the transmembrane and intracellular tail of gp41, results in a relatively stable trimeric construct that can be used for immunogenicity studies. One such study that used this strategy immunized rabbits with a monomeric gp120 or trimeric gp140 construct, derived from the HIV-1 IIIB Env, in Ribi monophosphoryl lipid A plus squalene (MPL-SE) adjuvant.[55] The resulting antibody response was capable of binding gp120 and gp160 constructs from homologous and heterologous isolates. An increase in the potency of the NAb response in animals immunized with the trimeric gp140 construct was also observed against the TCLA viruses NL4-3 and MN. However, when this serum was tested against more prototypical primary isolates, no neutralizing activity was observed. Despite this, the trimeric gp140 constructs were also tested in macaques. The resulting sera were again capable of neutralizing TCLA strains of HIV-1. Epitope-mapping analysis of the sera revealed that usually >50%, and as high as 77%, of the neutralizing activity could be adsorbed using V3 peptides. The predominance of V3-directed antibodies and the limited exposure of this loop in primary isolates could potentially explain why so little neutralization of primary isolates was observed.

Other studies have also used the strategy of eliminating the cleavage site between gp120 and gp41 in an attempt to increase the yield of oligmers produced. One of these compared the immunogenicity of monomeric gp120 with oligomeric gp140 derived from the CD4-independent isolate R2.[56] After four protein-based immunizations in the very powerful AS02A adjuvant, antibody responses were tested for neutralizing activity against a large panel of primary isolates from clades B and C. In groups of three rabbits, at least two of three rabbits that received gp140-based immunizations were capable of neutralizing all but one of the viral isolates tested. In comparison, two of three rabbits that received monomeric gp120-based immunogens were only capable of neutralizing nine of the 46 isolates tested.

A second means used to stabilize the trimeric interaction and study its immunogenicity is through the addition of heterologous trimerization domains to the HIV-1 Env glycoproteins. One example of this introduced the GCN4 or foldon trimerization domain into the Yu2 gp140 background. Immunization with this trimerized construct was initially shown to be effective in raising a more potent NAb response in mice.[58] Vaccination of guinea pigs with these trimeric constructs using a wide variety of adjuvants generally increased the potency of neutralization against selected homologous and heterologous clade B isolates.[59] However, it did little to expand the breadth of neutralization of isolates when compared with immunization with monomeric gp120. Analysis of this neutralizing activity revealed that gp120-immunized animals had neutralizing activity directed primarily towards the V1 loop of the Yu2 virus,

with additional neutralizing activity directed towards the V3 loop. Interestingly, immunization with the gp140 constructs redirected neutralizing activity away from the V3 and V1 loops to other areas of the virus. Trimerized Yu2 gp120 constructs were also generated using the GCN4 motif and tested in rabbits.[60,77] The general pattern of an increase in potency of the neutralizing activity of the sera was again observed in this study. Interestingly, this study also looked at the effects of stabilizing the Yu2 gp120 in a CD4-bound state in its trimeric form through site-directed mutagenesis. The additional modifications of stabilizing the core in its trimeric form increased the potency of the elicited NAb response even further. However, the overall breadth of neutralization was not increased appreciably against the more difficult to neutralize isolates JR-FL and TRJO.58. Also interesting to note was that the specificity of this neutralizing activity differed between the two studies.[59,60] In guinea pigs immunized with the trimeric structures, most neutralizing activity could be attributed to V1 recognition, whereas in rabbits immunized with a similar construct, almost no neutralizing activity could be attributed to V1 reactivity.

Other attempts to induce trimerization of the Env glycoproteins have also been successful. Instead of the introduction of heterologous trimerization motifs, the stability of a normal Env trimer has been enhanced through the introduction of disulfide bonds to stabilize the gp120-gp41 interaction, and mutations in the gp41 region have been made to stabilize gp41-gp41 interactions.[78,79] These modifications allowed the trimer to be cleaved normally, while still maintaining a stable trimeric interaction. In a study evaluating the immunogenicity of this disulfide bond stabilized construct, high titers of binding antibody were elicited but only weak neutralizing activity was observed.[57] Specifically, the homologous JR-FL isolate, from which the immunogen was generated, was neutralized only sporadically upon immunization with this construct. Neutralization of the TCLA MN isolate of the HIV-1 virus was also evaluated in this study. Sera generated through immunization with this construct frequently neutralized this virus with a high titer; however, there was no trend for higher NAb titers being raised in the animals immunized with the trimer. A second study also evaluated the effects of trimerization by immunizing rabbits with a trimerized construct resulting from the deletion of the gp120-gp41 cleavage site, a trimerized construct based on an intermolecular disulfide bond, or immunization with monomeric gp120.[80] Again, in this study there was little improvement in the functional antibody response elicited by the trimeric proteins. Sporadic neutralization of the homologous isolate JR-FL was seen in trimer-immunized animals, as well as sporadic neutralization of the sensitive isolate BaL. However, almost no neutralization of resistant primary isolates was observed. Analysis of neutralizing sera in both of the above-mentioned studies indicated that neutralizing activity was not directed towards the V1/V2 loops of the virus or the MPER. In addition, only limited amounts of neutralizing activity could be assigned to the V3 loop, leaving the exact specificity of any neutralizing activity that was observed largely undefined.

4.5 gp41 Targeting (Epitope Grafts)

A series of neutralizable epitopes are found in the MPER of gp41. However, this region of gp41 has been shown to be poorly immunogenic. In efforts to increase the immunogenicity of this region, the linear epitopes in the MPER of gp41, recognized by broadly neutralizing antibodies, specifically 2F5 and 4E10, have been grafted into other areas of the Env. In one such study, transplantation of the 2F5 epitope into either the V1, V2, V3, or V4 loop was tested.[61] DNA-based immunizations of mice with these grafted constructs generated positive binding antibodies against this MPER epitope when it was placed in either the V1 or V3 loop. However, immunization of guinea pigs failed to raise positive antibody titers to the intact MPER when positioned in these same loops. Interestingly, repeated immunizations of the MPER graft in the V2 loop raised positive antibody responses targeted to the grafted epitope in guinea pigs. Despite the positive recognition of this epitope, however, no MPER-based neutralization was observed against the HIV-1 IIIB isolate.

The immunogenicity of the MPER epitope grafted into the V1/V2 loop was also assessed in mice and rabbits.[62] This grafted region was further manipulated by the addition or deletion of residues flanking the epitope in order to manipulate the exposure of the helix. These grafts were proven capable of binding the MPER-directed antibody 4E10, and their immunogenicity in rabbits was assessed. Again, while many gp120 binding antibodies were generated in this study, no NAbs targeted to the MPER region were detected.

5. Anti-Idiotypic Immunogens

Yet another interesting approach to generate an effective antibody response is to use anti-idiotypic antibodies as immunogens, to focus the antibody response on a desirable domain. Anti-idiotypic antibodies capable of binding a CD4 binding site-directed fraction of human sera from HIV-1 infected individuals have also been evaluated as immunogens.[81] This study enriched a fraction of CD4 binding site-directed

antibodies from four individuals with no disease progression in the long term, and used this fraction to generate anti-idiotypic mAb in mice. Two monoclonal antibodies were generated that were capable of binding b12 and were subsequently used in their Fab form adjuvanted in incomplete Freund's adjuvant as immunogens in rabbits. The best of the two Fab immunogens, P1, was capable of neutralizing the sensitive HIV-1 isolate HxB2 in three of the five rabbits. However, no data were reported on the neutralizing activity of this sera against the more representative primary isolates.

6. Vaccination Approaches for Delivery of HIV-1 Envelope Antigens

The ultimate goal of raising a strong antibody response against HIV-1 is to prevent infection with this virus. The design of an effective antigen to be used in a vaccine is only one part of the overall effort to generate a protective antibody response. A second, equally important task is to optimize the delivery method by which an optimal Env antigen can be administered to humans so that a successful antibody response can be raised. To pursue this goal, a number of different strategies have been implemented (table II). The use of traditional subunit-protein vaccines was attempted first, the details of which have been discussed (see section 4). However, the sheer difficulty of raising effective antibody responses against the virus has necessitated the use of novel immunization approaches.

6.1 Viral Vector-Based Vaccines

One of the novel immunization approaches is to use viral vectors to deliver HIV-1 antigens. One such vaccine candidate has been evaluated in a phase I clinical trial using an adenovirus vector to deliver HIV-1 Env antigens.[82] This study used an adenovirus delivery system that was made replication incompetent through the deletion of the E1 and E4 region as well as part of the E3 region of the viral genome. Inserted into the virus were genes encoding a Gag-Pol fusion protein, intended for elicitation of T-cell responses, as well as three gp140 Env genes, each derived from a single clade, A, clade B, and clade C HIV-1 isolate. Four weeks after immunization, 93% of healthy, uninfected adults were capable of recognizing the homologous clade B antigen by IP-Western blot. However, when antibody responses were measured by ELISA, only 50% of the individuals were capable of recognizing one of the three antigens used in the vaccine formulation. Despite the positive binding titers induced in some individuals, no neutralizing activity was detected against the highly sensitive isolate SF162 or the TCLA isolate HXB2. Therefore, while this vaccine proved to be relatively safe, it failed to generate a highly immunogenic humoral antibody response against even very sensitive strains of HIV-1.

Russell et al.[83] evaluated the use of a canarypox virus to deliver HIV-1 antigens. In this phase II human trial, uninfected individuals were immunized with the canarypox vector vCP1452 encoding the gp120 protein of the MN isolate fused to the gp41 region of the HIV-1 isolate LAI, plus the entire *gag* gene and CTL epitopes derived from the Nef and Pol proteins. The HIV-1 canarypox vaccine was either administered alone or boosted with a subunit protein boost of a bivalent formulation of gp120 derived from HIV-1 isolates MN and GNE8 for a total of four immunizations. Positive binding antibodies were raised against the Gag protein in 23–36% of individuals, depending on the immunization group. More relevantly, however, between 70% and 83% of individuals raised NAb responses against the homologous MN isolate. Notably, individuals who received only the canarypox vaccine candidate elicited lower titers of

Table II. Vaccination strategies and resulting neutralizing antibody (NAb) responses in clinical trials

Vaccination approach	Rationale	Result	References
Subunit protein	Subunit-based immunizations have been successfully used to raise antibody responses to a number of pathogens	Narrow NAb responses with no protective efficacy	2,3
Viral vectors	Raise a balanced T- and B-cell response	None or narrow, low-titer NAb responses elicited	82,83
DNA vaccines	Raise a balanced T- and B-cell response	Barely detectable NAb responses	84-86
Viral vector + protein	Viruses alone elicited only low-quality antibody responses that may be boosted with a recombinant protein	Increase in NAb titers with administration of protein boost	83
DNA + viral vectors	Adenoviruses may be capable of boosting low-titer immune responses resulting from DNA priming	Increased potency but still limited breadth of NAb response	87
DNA + protein	DNA immunizations can successfully prime a humoral antibody response, which can be augmented by boosting with a subunit protein	Low titer but broad NAb responses against a wide range of primary isolates	88

NAb against the MN isolate compared with recipients of rgp120. Neutralization of the heterologous IIIB isolate was also evaluated in a limited number of samples. When heterologous neutralization was taken into account, those individuals who received only the canarypox-based immunization fared significantly worse than those who received a subunit-protein booster. Specifically, individuals who received only the canarypox vaccine candiate never successfully neutralized the HIV-1 IIIB isolate, while 70% of individuals who received a subunit-protein boost were able to neutralize this isolate. The generation of antibody responses capable of neutralizing TCLA strains of virus using a similar canarypox prime-protein boost immunization regimen has also been mirrored in a number of other studies, demonstrating the utility of a heterologous prime-boost regimen.[89-92] Despite this ability, the overall quality of antibody responses in this trial does not appear to be better than that reported in trials conducted with strictly subunit protein-based immunizations. Notably, the ability of individuals to neutralize a significant number of neutralization resistant primary isolates has yet to be demonstrated.

6.2 DNA Vaccines

The sole use of DNA-based immunizations to raise HIV-1 specific antibody responses has also been tested in phase I human trials. In one trial, three DNA immunizations encoding three Env antigens, one each derived from clades A, B, and C, as well as the T-cell antigens, Gag, Pol, and Nef, were given to healthy human volunteers using a needle-free injection system.[84] Antibody responses from this trial were then evaluated by ELISA and NAb assay. Humoral antibody responses recognizing the clades A and C Env antigens were generated in 71% of individuals; clade B Env antigens were recognized in 64% of individuals in the trial. Despite the presence of binding antibodies, functional NAb were entirely lacking. None of the vaccinated individuals generated NAbs against the sensitive HIV-1 isolate MN, indicating the overall lack of immunogenicity of this approach in generating a strong antibody response.

These results are mirrored by a second human DNA-only vaccine trial.[85] This trial delivered DNA encoding a Gag-Pol-Nef fusion protein plus modified Env antigen constructs derived from clades A, B, and C via a needle-free injection system. Similar to other DNA-only trials, binding antibodies, as determined by ELISA, were raised in 60% of the individuals. However, once more there was a total lack of NAbs raised against the sensitive HIV-1 strain MN. A third trial utilizing only DNA-based immunizations encoding Gag, Pol, Env, Rev, Tat, and Vpu, delivered by traditional needle-based intramuscular injection, also failed to produce any detectable NAb titers against the HIV-1 isolates ADA or MN.[86] The sole use of DNA-based immunizations in humans has highlighted the fact that as a whole, the DNA vaccine is not very immunogenic by itself. However, it is puzzling that the immune sera cannot neutralize even highly sensitive HIV-1 isolates when positive binding antibody responses were clearly identified. One possible explanation is the use of a modified Env antigen (gp145)[93] in these DNA vaccine-alone studies.[85] It has multiple deletions in gp120 and gp41 domains,[93] resulting in most of the construct remaining cell associated. The combination of these mutations may have adversely affected the critical Env antigen conformation that is required for eliciting NAb responses.

6.3 DNA Vaccine Prime-Viral Vector Boost

Based upon the limited ability of vaccines utilizing a single modality to raise an effective antibody response against HIV-1, combinations of heterologous immunization approaches have also been attempted. One such study used a DNA prime and adenovirus boost to elicit cellular and humoral antibody responses in rhesus macaques.[94] This study used DNA and replication-defective recombinant serotype 5 adenoviruses (rAd5) expressing three HIV-1 Env antigens from clades A, B, and C, either alone or in combination, as well as a fused Gag-Pol-Nef construct intended to raise cell-mediated immunity. While this study generated strong cell-mediated immune responses to the virus, functional antibody responses were still somewhat lacking. Positive binding antibody titers were raised in immunized animals as well as positive NAb titers against the sensitive isolates HxB2 and SF162. Inhibition of three clade A isolates, UG29, UG031, and 44951 by 50% was observed in animals receiving a combination of all three Env immunogens. However, 50% inhibition of the majority of primary isolates from clades B and C was entirely lacking. Despite the low level of NAb responses, immunization did demonstrate positive effects after an 89.6P challenge. Immunized animals demonstrated better control of viral infection as well as better preservation of the CD4+ T-cell compartment.

Studies evaluating the elicitation of antibody responses using combinations of DNA plasmids and adenovirus vectors expressing HIV-1 proteins have also been performed.[87,95] In one study, immunization of rhesus macaques with a chimeric HxBc2/BaL gp145 construct delivered either by a DNA prime-adenovirus boost or by strictly repeated immunizations with rAd5 was evaluated.[87] Immunization with a single rAd5 vector generated higher binding antibody titers against the gp140

protein than immunization with only the DNA vaccine. However, repeated boosting with subsequent rAd5 immunizations did not enhance the antibody response in these animals. In contrast, the DNA-primed animals, when administered a rAd5 boost, demonstrated a rapid rise in Env binding antibody titers. Neutralizing activity raised by the two immunization approaches was also evaluated. Neutralization of the 89.6 isolate was found to be significantly greater in animals that first received a DNA prime, indicating the superiority of this combination immunization approach relative to immunization with only rAd5. However, the breadth of neutralization using the DNA prime rAd5 boost format was still somewhat limited; only about one-third of tested clade B isolates were neutralized by sera generated from immunization with either an 89.6 or chimeric HxBc2/BaL construct.

6.4 DNA Prime-Protein Boost

The use of DNA vaccines to raise humoral antibody responses against HIV-1 was first seen in the early 1990s, when it was shown that a DNA plasmid encoding HIV-1 Env glycoproteins derived from TCLA was capable of raising HIV-1-specific antibody responses in small animals.[96,97] The antibodies raised by this approach were capable of both binding recombinant Env glycoproteins and neutralizing the HIV-1 IIIB isolate. The utility of this approach was further demonstrated in an SHIV challenge model in cynomolgus macaques.[98] In this study, animals that received DNA immunizations generated a strong immune response that resulted in a lowered viral load compared with unimmunized animals. Additionally, in this study, one of four immunized animals was protected from viral challenge upon completion of the DNA immunization regimen.

Other than its obvious ability to generate an immune response, there are a number of positive aspects of DNA immunizations that make them an attractive option for use as a platform for an HIV-1 vaccine. The first of these is the endogenous production and processing of a chosen antigen. When a DNA immunization is given, antigen-encoding plasmids are taken up directly by cells at the injection site of the host, thereby making antigen production similar to that of a live attenuated vaccine. This allows the protein to undergo well regulated translation processes allowing for native folding, as well as normal post-translational modifications, such as glycosylation, of the antigen of interest. Additionally, because of the endogenous production of the antigen, the produced protein can be efficiently presented to the immune system through class I and class II major histocompatibility complexes, allowing for an efficient T-cell response to the antigen. Furthermore, the DNA vaccine has also proven to be a very safe alternative to subunit and live attenuated vaccines.[99-103] Because DNA vaccines are normally non-replicative and non-integrative, and can only encode the protein(s) of interest, they allow the researcher to elicit an antibody response with the specificity of a subunit vaccine and the native antigen processing of a live attenuated vaccine, all without the safety risk of reversion of an attenuated viral strain into a pathogenic one.

In addition to its relative safety, DNA-based immunizations provide an excellent platform for studying different properties of a particular antigen, screening of different immunogens,[104,105] identifying immunogenic and neutralizing domains of a target,[106] and identifying effective immunization regimens.[107]

Despite the ability of the DNA vaccine to generate an immune response against model antigens, a number of caveats still exist. One of these is a relatively low *in vivo* transfection efficiency, leading to low levels of antigen production. Because of this, a significant effort has been made to increase the potency of DNA vaccines. This involves studying different delivery mechanisms for the DNA itself, including electroporation,[108,109] needle-free jet systems,[110-112] gene gun,[109,112] and micro-needle injections,[113] all of which are intended to increase the efficiency of DNA delivery over a traditional intramuscular injection.[109]

Increasing the efficiency of DNA delivery is only one aspect of the effort to increase the potency of DNA immunizations. Work has also focused on the design of DNA constructs themselves in order to enhance antigen production on the level of the individual transfected cell. One of the primary improvements that has been made was the advent and implementation of codon optimization to maximize the efficiency of transfer (t)RNA usage in the cell.[114-116] Optimizing each codon to utilize the most prevalent tRNA present in the cell allows for more efficient protein translation, resulting in a higher quantity of antigen being produced. Other work to increase the amount of antigen produced has focused on manipulating the leader sequences and promoters of these constructs.[115] The simultaneous manipulation of all of these factors in a potential construct was shown to improve the immunogenicity of a gp120 protein in a mouse model.[115]

Despite improvements in the design of the DNA construct and increased efficiency in delivery, DNA immunization is still only capable of producing limited quantities of antigen at levels much lower than that delivered by inactivated or subunit vaccines. Because of this, as witnessed in DNA vaccine-alone human trials,[84,85] it is not immunogenic enough on its own to generate an effective antibody response against the virus. Despite this, DNA immunizations are highly effective in

priming the body's immune system and work best when used in combination with another immunization approach, usually with DNA administered as a priming immunization followed by a boost of another modality. One of the simplest and most effective of these combination approaches for the elicitation of humoral immunity is a DNA prime followed by a traditional subunit protein boost.[117]

Early studies using the DNA prime-protein boost approach utilized TCLA-derived Env glycoproteins in the vaccine formulation.[118] Rabbits in this study were immunized with DNA-based immunizations encoding gp120, gp140, or a replication incompetent form of HXB-2 and subsequently boosted with rgp160 derived from the HIV-1 isolate IIIB in incomplete Freund's adjuvant. While only limited binding antibody was generated after the DNA immunizations, boosting with recombinant protein greatly increased binding titers in immunized animals. Analysis of serum avidity elicited by each immunization regimen indicated that use of a combination approach elicited a higher avidity antibody response than use of DNA immunizations alone. The combination DNA prime-protein boost approach generated homologous NAb titers significantly greater than those observed with immunization of naive animals with recombinant protein only. A heterologous NAb response against MN and SF2 were also generated using this prime boost immunization regimen. Titers in immunized animals varied from 1 : 148 to >1 : 3000 against MN and from 1 : 37 to 1 : 269 against SF2. However, the use of a TCLA-derived immunogen, was shown to be incapable of generating a heterologous NAb response against more difficult-to-neutralize primary isolates.[118]

Extensive ground work has been carried out, demonstrating that a DNA prime-protein boost strategy is an effective means of raising antibody responses in both small animals and non-human primates.[119,120] However, many of these studies suffered from the inability to neutralize the more relevant primary isolates of HIV-1. One breakthrough, in an attempt to overcome the limitations of TCLA-derived immunogens, used the gp120 derived from the primary isolate JR-FL as a model immunogen.[121] In this study, rabbits were immunized in either a DNA prime-protein boost format or with only recombinant gp120 protein derived from the primary isolate JR-FL. Sera generated by both immunization approaches contained high levels of binding antibody to homologous Env glycoproteins, but the NAb response generated by each immunization regimen differed dramatically. One example of this was observed with the neutralization of the sensitive isolate SF162. Both immunization regimens were capable of generating a NAb response against this sensitive isolate; however, the DNA-primed animals did so with a much higher titer. Additionally, animals that received a DNA prime were capable of neutralizing the homologous strain of HIV-1, JR-FL, in a peripheral blood mononuclear cell-based neutralization assay. Inhibition of this neutralization-resistant primary isolate was not observed in animals that were immunized with protein only. Additionally, sera raised by the DNA prime-protein boost approach were also frequently capable of neutralizing other heterologous clade B isolates, including 1196 and 0692.

The superiority of the DNA prime-protein boost approach was proven again concurrently in a separate study looking at the effect of oligomerization on the elicitation of NAbs.[57] Here, the use of a DNA prime-protein boost approach elicited a 12-fold higher binding antibody response when compared with immunization with protein only, clearly demonstrating the potential of this platform for eliciting a functional antibody response to the virus. A third example of the effectiveness of the DNA prime-protein boost vaccination approach using monomeric gp120 immunogens generated a consistent NAb response against neutralization resistant primary isolates.[57] While the use of the DNA prime-protein boost approach was able to enhance the binding and NAb response elicited compared with immunization with subunit protein only, the overall breadth of neutralizing activity was still somewhat limited. The most likely explanation for this phenomenon was the use of only a single, subtype B Env glycoprotein in the vaccine formulation. One attempt that was made to increase the breadth of the neutralizing activity was to include multiple, genetically distinct Env glycoproteins into a single polyvalent formulation.[122] In this study, rabbits were immunized in a DNA prime-protein boost format consisting of either monovalent or polyvalent formulations of gp120 derived from clades A, B, C, D, E, F, and G. Sera generated by immunization with these constructs were then tested in a pseudovirus-based neutralization assay against a panel of 14 viruses from clades A to E. Data from this study revealed that immunization with a polyvalent formulation significantly increased the breadth of neutralization against this multiclade panel, nearly doubling the number of isolates neutralized compared with the monovalent immunization groups.[122]

The success of the DNA prime-protein boost immunization format further showed its promise in non-human primate studies. A modification of the polyvalent formulation above, consisting of two clade B gp120s, one clade C gp120, and one clade E gp120, plus Gag derived from NL4-3, was tested for its protective efficacy in rhesus macaques.[123] Animals received a combination of DNA and protein-based immunizations, and were subsequently rectally challenged with the R5 SHIV

BaL strain. At the time of challenge, animals had generated an antibody response capable of neutralizing the sensitive isolates of HIV-1, MN, and SF162, as well as the challenge strain of BaL. Immunization with the above regimen provided sterilizing immunity to four of six macaques from the SHIV challenge, based upon detection of viral RNA in the blood. Relative to the control group of seven naive animals who all became infected and demonstrated high viral loads, the remaining two immunized macaques that became infected demonstrated lower levels of viral RNA in the blood. Because the Gag antigen was from an HIV-1 isolate while the challenge virus was a SHIV, the protection of animals against infection was clearly mediated by Env-induced immunity, most likely due to anti-Env antibodies.[117]

Other studies have also confirmed the utility of the DNA prime-protein boost approach in non-human primates. One of these studies used this prime-boost approach in neonatal macaques.[124] Immunization of animals in this study with DNA encoding vpu and the IIIB Env glycoproteins, followed by boosting with recombinant IIIB derived gp160, protected 4 of 15 animals from a homologous IIIB intravenous SHIV challenge. Another study utilizing the DNA prime-protein boost approach immunized rhesus macaques with gp120 or gp160 forms of Env glycoproteins derived from HXBc2.[120] Homologous NAb titers >1 : 1000 were generated as a result of immunization with these constructs. Following an intravenous challenge of SHIV HXBc2, none of the immunized monkeys became infected, based upon negative re-isolation of HXBc2 at every bleed after challenge.

Based on the successful protection of non-human primates in an SHIV protection model, the DNA prime-protein boost approach was tested in a phase I clinical trial.[88] Again, a polyvalent Env glycoprotein formulation was used, this time consisting of five Envs from clades A, B, C, and E. After three DNA immunizations and two protein boosts, humoral antibody responses were evaluated by solid-phase antibody binding and NAb assays. Immunization with this polyvalent Env glycoprotein vaccine formulation and regimen elicited broad and high titer binding antibody responses in all 27 individuals evaluated in the trial against gp120 antigens from clades A to H of HIV-1, as evaluated by ELISA and Western blots. Additionally, NAb responses were detected in 100% of individuals against the sensitive HIV-1 isolates MN, NL4-3, and SF162 at titers as high as 1 : 2000. Neutralization of the homologous primary isolates included in the vaccine was also frequently observed. Specifically, neutralizing activity was detected in >60% of individuals against the subtype C vaccine strain 96ZM652. In addition to eliciting NAbs against sensitive and homologous isolates, which has not been demonstrated in some other trials,[125] the new polyvalent DNA prime-protein boost regimen also generated neutralizing activity against difficult-to-neutralize heterologous primary isolates from clades A, B, C, D, and E in a high-throughput, pseudotyped, virus-based neutralization system.[88] Positive NAb titers were identified in each of the 22 vaccinees included in the analysis, with about 60% of the vaccinees having positive NAbs against 80–100% of pseudotyped viruses included in the assay. This represents a significant improvement over the NAb responses reported with immunization via protein, DNA, or viral vectors alone, as well as combinations of viral vector prime-protein boost and DNA prime-viral vector boost studies. Additionally, while this vaccine was successful in generating a cross-reactive antibody response, it also proved to be safe and well tolerated.[126]

The underlying mechanism as to why the DNA prime-protein boost immunization regimen is more effective at raising a functional antibody response is still not fully understood. One potential explanation for this is that the avidity of the antibody response generated against the HIV-1 Env glycoproteins by a prime-boost immunization is higher than that generated by immunization with either modality alone.[118,121] These studies indicated that boosting with recombinant protein vastly increased the avidity of the antibody response elicited by DNA priming. The generation of a higher avidity antibody response may be one of the defining features necessary to elicit effective neutralization of HIV-1.

A second possibility as to why the DNA prime-protein boost approach elicits a higher quality antibody response has also been recently investigated. Comparison of antibody specificities generated by immunization with only recombinant protein or with the DNA prime-protein boost approach has revealed that the incorporation of a DNA prime alters the specificity of elicited antibodies.[127] Based upon an enhanced breadth of NAb response observed in the DNA-primed animals, the specificities of the antibodies elicited by each immunization approach was studied. Recognition of linear peptides derived from the group M consensus sequence revealed that immunization with either approach generated binding antibodies to the C1 and V3 regions of the Env. However, immunization with the DNA prime-protein boost approach also elicited antibodies with specificities for the flanking regions of the V1/V2 loop, the C2 region, and the junction of the V5 and C5 regions. Mapping of these regions onto the crystal structure of liganded gp120 revealed that these uniquely recognized regions all mapped very closely to known contact residues for CD4. Through the use of a mAb

competition assay, it was confirmed that increased titers of CD4 binding site-directed antibodies were present in animals that received a DNA prime. Interestingly, however, V3-directed antibodies were also observed to be elicited in higher titers in the DNA-primed animals. Further investigation into this phenomenon revealed that while the V3-directed antibodies were more prevalent in the DNA-primed animals, they did not play a significant role in the neutralization of primary isolates. This left the increased levels of CD4 binding site-directed antibodies as the most likely mechanism for the observed enhanced NAb response. With recent studies implicating the CD4 binding site as a primary target for individuals with broadly neutralizing activity,[128] the ability of a DNA prime-protein boost approach to elicit antibodies with this specificity is a remarkably important attribute of this platform.

7. Conclusion

The development of an effective HIV-1 vaccine still faces numerous challenges before it becomes a reality. These challenges include the enhancement of the antibody response to neutralize a wide array of viral isolates and to do so with a high degree of potency. However, with current work underway in using a polyvalent Env formulation delivered by the DNA prime-protein boost approach to elicit broadly cross-reactive antibody responses, this challenge may not prove to be insurmountable.

Acknowledgments

This work was supported in part by US National Institutes of Health (NIH) research grants R29AI40337 and R01AI65250 to Shan Lu. Michael Vaine was supported by NIH training grant 5 T32 AIO7349-18. The authors have no other affiliations or financial involvement with any organization or entity with a financial interest or conflict with the subject matter or materials discussed in this manuscript, apart from those disclosed.

References

1. UNAIDS/WHO. Global AIDS epidemic continues to grow 2006 [online]. Available from URL: http://www.who.int/hiv/mediacentre/news62/en/index.html [Accessed 2009 Apr 29]
2. Flynn NM, Forthal DN, Harro CD, et al. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis 2005 Mar 1; 191 (5): 654-65
3. Pitisuttithum P, Gilbert P, Gurwith M, et al. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J Infect Dis 2006 Dec 15; 194 (12): 1661-71
4. Buchbinder SP, Mehrotra DV, Duerr A, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 2008; 372: 1881-93
5. Borrow P, Lewicki H, Hahn BH, et al. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 1994 Sep; 68 (9): 6103-10
6. Koup RA, Safrit JT, Cao Y, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994 Jul; 68 (7): 4650-5
7. Schmitz JE, Kuroda MJ, Santra S, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 1999 Feb 5; 283 (5403): 857-60
8. Bernard NF, Pederson K, Chung F, et al. HIV-specific cytotoxic T-lymphocyte activity in immunologically normal HIV-infected persons. AIDS 1998 Nov 12; 12 (16): 2125-39
9. Pontesilli O, Klein MR, Kerkhof-Garde SR, et al. Longitudinal analysis of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte responses: a predominant gag-specific response is associated with non-progressive infection. J Infect Dis 1998 Oct; 178 (4): 1008-18
10. Amara RR, Villinger F, Altman JD, et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 2001 Apr 6; 292 (5514): 69-74
11. Shiver JW, Fu TM, Chen L, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 2002 Jan 17; 415 (6869): 331-5
12. Barouch DH, Santra S, Schmitz JE, et al. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augumented DNA vaccination. Science 2000 Oct 20; 290: 486-92
13. Casimiro D, Wang F, Schleif W, et al. Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with dna and recombinant adenoviral vaccine vectors expressing Gag. J Virol 2005; 79 (24): 15547-55
14. Liang X, Casimiro DR, Schleif WA, et al. Vectored Gag and Env but not Tat show efficacy against simian-human immunodeficiency virus 89.6P challenge in Mamu-A*01-negative rhesus monkeys. J Virol 2005 Oct; 79 (19): 12321-31
15. HIV vaccine failure prompts Merck to halt trial. Nature 2007 Sep 27; 449 (7161): 390
16. Steinbrook R. One step forward, two steps back: will there ever be an AIDS vaccine? N Engl J Med 2007 Dec 27; 357 (26): 2653-5
17. Pantaleo G. HIV-1 T-cell vaccines: evaluating the next step. Lancet Infect Dis 2008 Feb; 8 (2): 82-3
18. Ledford H. HIV vaccine may raise risk. Nature 2007 Nov 15; 450 (7168): 325
19. Korber B, Gaschen B, Yusim K, et al. Evolutionary and immunological implications of contemporary HIV-1 variation. Br Med Bull 2001; 58: 19-42
20. Pantophlet R, Burton DR. GP120: target for neutralizing HIV-1 antibodies. Annu Rev Immunol 2006; 24: 739-69
21. Burton DR, Stanfield RL, Wilson IA. Antibody vs HIV in a clash of evolutionary titans. Proc Natl Acad Sci U S A 2005 Oct 18; 102 (42): 14943-8
22. Conley AJ, Kessler II JA, Boots LJ, et al. The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. J Virol 1996 Oct; 70 (10): 6751-8
23. Emini EA, Schleif WA, Nunberg JH, et al. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature 1992 Feb 20; 355 (6362): 728-30
24. Hofmann-Lehmann R, Vlasak J, Rasmussen RA, et al. Postnatal pre- and postexposure passive immunization strategies: protection of neonatal macaques against oral simian-human immunodeficiency virus challenge. J Med Primatol 2002 Jun; 31 (3): 109-19
25. Hofmann-Lehmann R, Vlasak J, Rasmussen RA, et al. Postnatal passive immunization of neonatal macaques with a triple combination of human monoclonal antibodies against oral simian-human immunodeficiency virus challenge. J Virol 2001 Aug; 75 (16): 7470-80

26. Mascola JR, Lewis MG, Stiegler G, et al. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol 1999 May; 73 (5): 4009-18
27. Mascola JR, Stiegler G, VanCott TC, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 2000 Feb; 6 (2): 207-10
28. Berman PW, Gregory TJ, Riddle L, et al. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature 1990 Jun 14; 345 (6276): 622-5
29. el-Amad Z, Murthy KK, Higgins K, et al. Resistance of chimpanzees immunized with recombinant gp120SF2 to challenge by HIV-1SF2. AIDS 1995 Dec; 9 (12): 1313-22
30. Berman PW, Murthy KK, Wrin T, et al. Protection of MN-rgp120-immunized chimpanzees from heterologous infection with a primary isolate of human immunodeficiency virus type 1. J Infect Dis 1996 Jan; 173 (1): 52-9
31. Schwartz DH, Gorse G, Clements ML, et al. Induction of HIV-1-neutralising and syncytium-inhibiting antibodies in uninfected recipients of HIV-1IIIB rgp120 subunit vaccine. Lancet 1993 Jul 10; 342 (8863): 69-73
32. Belshe RB, Clements ML, Dolin R, et al. Safety and immunogenicity of a fully glycosylated recombinant gp160 human immunodeficiency virus type 1 vaccine in subjects at low risk of infection. National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group Network. J Infect Dis 1993 Dec; 168 (6): 1387-95
33. Belshe RB, Graham BS, Keefer MC, et al. Neutralizing antibodies to HIV-1 in seronegative volunteers immunized with recombinant gp120 from the MN strain of HIV-1. NIAID AIDS Vaccine Clinical Trials Network. JAMA 1994 Aug 10; 272 (6): 475-80
34. Graham BS, Keefer MC, McElrath MJ, et al. Safety and immunogenicity of a candidate HIV-1 vaccine in healthy adults: recombinant glycoprotein (rgp) 120: a randomized, double-blind trial. NIAID AIDS Vaccine Evaluation Group. Ann Intern Med 1996 Aug 15; 125 (4): 270-9
35. Pitisuttithum P, Berman PW, Phonrat B, et al. Phase I/II study of a candidate vaccine designed against the B and E subtypes of HIV-1. J Acquir Immune Defic Syndr 2004 Sep 1; 37 (1): 1160-5
36. Francis DP, Gregory T, McElrath MJ, et al. Advancing AIDSVAX to phase 3: safety, immunogenicity, and plans for phase 3. AIDS Res Hum Retroviruses 1998 Oct; 14 Suppl. 3: S325-31
37. Gilbert PB, Peterson ML, Follmann D, et al. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. J Infect Dis 2005 Mar 1; 191 (5): 666-77
38. Gao F, Weaver EA, Lu Z, et al. Antigenicity and immunogenicity of a synthetic human immunodeficiency virus type 1 group M consensus envelope glycoprotein. J Virol 2005 Jan 2005; 79 (2): 1154-63
39. Liao HX, Sutherland LL, Xia SM, et al. A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses. Virology 2006 Sep 30; 353 (2): 268-82
40. Kothe DL, Decker JM, Li Y, et al. Antigenicity and immunogenicity of HIV-1 consensus subtype B envelope glycoproteins. Virology 2007 Mar 30; 360 (1): 218-34
41. Kothe DL, Li Y, Decker JM, et al. Ancestral and consensus envelope immunogens for HIV-1 subtype C. Virology 2006 Sep 1; 352 (2): 438-49
42. Doria-Rose NA, Learn GH, Rodrigo AG, et al. Human immunodeficiency virus type 1 subtype B ancestral envelope protein is functional and elicits neutralizing antibodies in rabbits similar to those elicited by a circulating subtype B envelope. J Virol 2005 Sep; 79 (17): 11214-24
43. Lu S, Wyatt R, Richmond JF, et al. Immunogenicity of DNA vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with and without deletions in the V1/2 and V3 regions. AIDS Res Hum Retroviruses 1998; 14 (2): 151-5
44. Derby NR, Kraft Z, Kan E, et al. Antibody responses elicited in macaques immunized with human immunodeficiency virus type 1 (HIV-1) SF162-derived gp140 envelope immunogens: comparison with those elicited during homologous simian/human immunodeficiency virus SHIVSF162P4 and heterologous HIV-1 infection. J Virol 2006 Sep; 80 (17): 8745-62
45. Kim YB, Han DP, Cao C, et al. Immunogenicity and ability of variable loop-deleted human immunodeficiency virus type 1 envelope glycoproteins to elicit neutralizing antibodies. Virology 2003 Jan 5; 305 (1): 124-37
46. Barnett SW, Lu S, Srivastava I, et al. The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. J Virol 2001 Jun; 75 (12): 5526-40
47. Lian Y, Srivastava I, Gomez-Roman VR, et al. Evaluation of envelope vaccines derived from the South African subtype C human immunodeficiency virus type 1 TV1 strain. J Virol 2005 Nov; 79 (21): 13338-49
48. Srivastava IK, Stamatatos L, Kan E, et al. Purification, characterization, and immunogenicity of a soluble trimeric envelope protein containing a partial deletion of the V2 loop derived from SF162, an R5-tropic human immunodeficiency virus type 1 isolate. J Virol 2003 Oct; 77 (20): 11244-59
49. Selvarajah S, Puffer B, Pantophlet R, et al. Comparing antigenicity and immunogenicity of engineered gp120. J Virol 2005 Oct; 79 (19): 12148-63
50. Selvarajah S, Puffer BA, Lee FH, et al. Focused dampening of antibody response to the immunodominant variable loops by engineered soluble gp140. AIDS Res Hum Retroviruses 2008 Feb; 24 (2): 301-14
51. Reitter JN, Means RE, Desrosiers RC. A role for carbohydrates in immune evasion in AIDS. Nat Med 1998 Jun; 4 (6): 679-84
52. Li Y, Cleveland B, Klots I, et al. Removal of a single N-linked glycan in human immunodeficiency virus type 1 gp120 results in an enhanced ability to induce neutralizing antibody responses. J Virol 2008 Jan; 82 (2): 638-51
53. Bolmstedt A, Sjolander S, Hansen JE, et al. Influence of N-linked glycans in V4-V5 region of human immunodeficiency virus type 1 glycoprotein gp160 on induction of a virus-neutralizing humoral response. J Acquir Immune Defic Syndr Hum Retrovirol 1996 Jul; 12 (3): 213-20
54. Quinones-Kochs MI, Buonocore L, Rose JK. Role of N-linked glycans in a human immunodeficiency virus envelope glycoprotein: effects on protein function and the neutralizing antibody response. J Virol 2002 May; 76 (9): 4199-211
55. Earl PL, Sugiura W, Montefiori DC, et al. Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. J Virol 2001 Jan; 75 (2): 645-53
56. Zhang PF, Cham F, Dong M, et al. Extensively cross-reactive anti-HIV-1 neutralizing antibodies induced by gp140 immunization. Proc Natl Acad Sci U S A 2007 Jun 12; 104 (24): 10193-8
57. Beddows S, Schulke N, Kirschner M, et al. Evaluating the immunogenicity of a disulfide-stabilized, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. J Virol 2005 Jul; 79 (14): 8812-27
58. Yang X, Wyatt R, Sodroski J. Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. J Virol 2001 Feb; 75 (3): 1165-71
59. Li Y, Svehla K, Mathy NL, et al. Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolate trimeric and monomeric envelope glycoproteins in selected adjuvants. J Virol 2006 Feb; 80 (3): 1414-26
60. Dey B, Pancera M, Svehla K, et al. Characterization of human immunodeficiency virus type 1 monomeric and trimeric gp120 glycoproteins stabilized in the CD4-bound state: antigenicity, biophysics, and immunogenicity. J Virol 2007 Jun; 81 (11): 5579-93
61. Liang X, Munshi S, Shendure J, et al. Epitope insertion into variable loops of HIV-1 gp120 as a potential means to improve immunogenicity of viral envelope protein. Vaccine 1999; 17: 2862-72

62. Law M, Cardoso RM, Wilson IA, et al. Antigenic and immunogenic study of membrane-proximal external region-grafted gp120 antigens by a DNA prime-protein boost immunization strategy. J Virol 2007 Apr; 81 (8): 4272-85

63. Mascola JR, D'Souza P, Gilbert P, et al. Recommendations for the design and use of standard virus panels to assess neutralizing antibody responses elicited by candidate human immunodeficiency virus type 1 vaccines. J Virol 2005 Aug; 79 (16): 10103-7

64. Li M, Gao F, Mascola JR, et al. Human immunodeficiency virus type 1 Env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J Virol 2005 Aug; 79 (16): 10108-25

65. Stamatatos L, Cheng-Mayer C. An envelope modification that renders a primary, neutralization-resistant clade B human immunodeficiency virus type 1 isolate highly susceptible to neutralization by sera from other clades. J Virol 1998 Oct; 72 (10): 7840-5

66. Pinter A, Honnen WJ, He Y, et al. The V1/V2 domain of gp120 is a global regulator of the sensitivity of primary human immunodeficiency virus type 1 isolates to neutralization by antibodies commonly induced upon infection. J Virol 2004 May; 78 (10): 5205-15

67. Johnson WE, Sanford H, Schwall L, et al. Assorted mutations in the envelope gene of simian immunodeficiency virus lead to loss of neutralization resistance against antibodies representing a broad spectrum of specificities. J Virol 2003 Sep; 77 (18): 9993-10003

68. Cao J, Sullivan N, Desjardin E, et al. Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope glycoprotein. J Virol 1997 Dec; 71 (12): 9808-12

69. Srivastava IK, VanDorsten K, Vojtech L, et al. Changes in the immunogenic properties of soluble gp140 human immunodeficiency virus envelope constructs upon partial deletion of the second hypervariable region. J Virol 2003 Feb; 77 (4): 2310-20

70. Back NK, Smit L, De Jong JJ, et al. An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization. Virology 1994 Mar; 199 (2): 431-8

71. Kang SM, Quan FS, Huang C, et al. Modified HIV envelope proteins with enhanced binding to neutralizing monoclonal antibodies. Virology 2005 Jan 5; 331 (1): 20-32

72. McCaffrey RA, Saunders C, Hensel M, et al. N-linked glycosylation of the V3 loop and the immunologically silent face of gp120 protects human immunodeficiency virus type 1 SF162 from neutralization by anti-gp120 and anti-gp41 antibodies. J Virol 2004 Apr; 78 (7): 3279-95

73. Reynard F, Fatmi A, Verrier B, et al. HIV-1 acute infection Env glycomutants designed from 3D model: effects on processing, antigenicity, and neutralization sensitivity. Virology 2004 Jun 20; 324 (1): 90-102

74. Koch M, Pancera M, Kwong PD, et al. Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. Virology 2003 Sep 1; 313 (2): 387-400

75. Huang Z, Chou A, Tanguay J, et al. Levels of N-linked glycosylation on the V1 loop of HIV-1 Env proteins and their relationship to the antigenicity of Env from primary viral isolates. Curr HIV Res 2008 Jun; 6 (4): 296-305

76. Pantophlet R, Wilson IA, Burton DR. Hyperglycosylated mutants of human immunodeficiency virus (HIV) type 1 monomeric gp120 as novel antigens for HIV vaccine design. J Virol 2003 May; 77 (10): 5889-901

77. Pancera M, Lebowitz J, Schon A, et al. Soluble mimetics of human immunodeficiency virus type 1 viral spikes produced by replacement of the native trimerization domain with a heterologous trimerization motif: characterization and ligand binding analysis. J Virol 2005 Aug; 79 (15): 9954-69

78. Binley JM, Sanders RW, Clas B, et al. A Recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. J Virol 2000; 74 (2): 627-43

79. Sanders RW, Vesanen M, Schuelke N, et al. Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. J Virol 2002 Sep; 76 (17): 8875-89

80. Beddows S, Franti M, Dey AK,et al. A comparative immunogenicity study in rabbits of disulfide-stabilized, proteolytically cleaved, soluble trimeric human immunodeficiency virus type 1 gp140, trimeric cleavage-defective gp140 and monomeric gp120. Virology 2007; 360: 329-40

81. Burioni R, Mancini N, De Marco D, et al. Anti-HIV-1 response elicited in rabbits by anti-idiotype monoclonal antibodies mimicking the CD4-binding site. PLoS ONE 2008; 3 (10): e3423

82. Catanzaro AT, Koup RA, Roederer M, et al. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. J Infect Dis 2006 Dec 15; 194 (12): 1638-49

83. Russell ND, Graham BS, Keefer MC, et al. Phase 2 study of an HIV-1 canarypox vaccine (vCP1452) alone and in combination with rgp120: negative results fail to trigger a phase 3 correlates trial. J Acquir Immune Defic Syndr 2007 Feb 1; 44 (2): 203-12

84. Catanzaro AT, Roederer M, Koup RA, et al. Phase I clinical evaluation of a six-plasmid multiclade HIV-1 DNA candidate vaccine. Vaccine 2007 May 16; 25 (20): 4085-92

85. Graham BS, Koup RA, Roederer M, et al. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 DNA candidate vaccine. J Infect Dis 2006 Dec 15; 194 (12): 1650-60

86. Mulligan MJ, Russell ND, Celum C, et al. Excellent safety and tolerability of the human immunodeficiency virus type 1 pGA2/JS2 plasmid DNA priming vector vaccine in HIV type 1 uninfected adults. AIDS Res Hum Retroviruses 2006 Jul; 22 (7): 678-83

87. Mascola JR, Sambor A, Beaudry K, et al. Neutralizing antibodies elicited by immunization of monkeys with DNA plasmids and recombinant adenoviral vectors expressing human immunodeficiency virus type 1 proteins. J Virol 2005 Jan; 79 (2): 771-9

88. Wang S, Kennedy JS, West K, et al. Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers. Vaccine 2008; 26: 3947-57

89. Gupta K, Hudgens M, Corey L, et al. Safety and immunogenicity of a high-titered canarypox vaccine in combination with rgp120 in a diverse population of HIV-1-uninfected adults: AIDS Vaccine Evaluation Group Protocol 022A. J Acquir Immune Defic Syndr 2002 Mar 1; 29 (3): 254-61

90. Thongcharoen P, Suriyanon V, Paris RM, et al. A phase 1/2 comparative vaccine trial of the safety and immunogenicity of a CRF01_AE (subtype E) candidate vaccine: ALVAC-HIV (vCP1521) prime with oligomeric gp160 (92TH023/LAI-DID) or bivalent gp120 (CM235/SF2) boost. J Acquir Immune Defic Syndr 2007 Sep 1; 46 (1): 48-55

91. Evans TG, Keefer MC, Weinhold KJ, et al. A canarypox vaccine expressing multiple human immunodeficiency virus type 1 genes given alone or with rgp120 elicits broad and durable CD8+ cytotoxic T lymphocyte responses in seronegative volunteers. J Infect Dis 1999 Aug; 180 (2): 290-8

92. Belshe RB, Stevens C, Gorse GJ, et al. Safety and immunogenicity of a canarypox-vectored human immunodeficiency virus type 1 vaccine with or without gp120: a phase 2 study in higher- and lower-risk volunteers. J Infect Dis 2001 May 1; 183 (9): 1343-52

93. Chakrabarti BK, Kong WP, Wu BY, et al. Modifications of the human immunodeficiency virus envelope glycoprotein enhance immunogenicity for genetic immunization. J Virol 2002 Jun; 76 (11): 5357-68

94. Seaman MS, Xu L, Beaudry K, et al. Multiclade human immunodeficiency virus type 1 envelope immunogens elicit broad cellular and humoral immunity in rhesus monkeys. J Virol 2005 Mar; 79 (5): 2956-63

95. Seaman MS, Leblanc DF, Grandpre LE, et al. Standardized assessment of NAb responses elicited in rhesus monkeys immunized with single- or multiclade HIV-1 envelope immunogens. Virology 2007; 367: 175-86

96. Wang B, Ugen KE, Srikantan V, et al. Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc Natl Acad Sci U S A 1993 May 1; 90 (9): 4156-60

97. Lu S, Santoro JC, Fuller DH, et al. Use of DNAs expressing HIV-1 Env and noninfectious HIV-1 particles to raise antibody responses in mice. Virology 1995; 209 (1):147-54

98. Boyer JD, Wang B, Ugen KE, et al. In vivo protective anti-HIV immune responses in non-human primates through DNA immunization. J Med Primatol 1996 Jun; 25 (3): 242-50

99. Roy MJ, Wu MS, Barr LJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 2000 Nov 22; 19 (7-8): 764-78

100. Wang R, Doolan DL, Le TP, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science 1998; 282 (5388): 476-80

101. Calarota S, Bratt G, Nordlund S, et al. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. Lancet 1998 May 2; 351 (9112): 1320-5

102. MacGregor RR, Boyer JD, Ugen KE, et al. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. J Infect Dis 1998 Jul; 178 (1): 92-100

103. Ugen KE, Nyland SB, Boyer JD, et al. DNA vaccination with HIV-1 expressing constructs elicits immune responses in humans. Vaccine 1998; 16 (19): 1818-21

104. Richmond JF, Mustafa F, Lu S, et al. Screening of HIV-1 Env glycoproteins for the ability to raise neutralizing antibody using DNA immunization and recombinant vaccinia virus boosting. Virology 1997 Apr 14; 230 (2): 265-74

105. Mustafa F, Richmond JF, Fernandez-Larsson R, et al. HIV-1 Env glycoproteins from two series of primary isolates: replication phenotype and immunogenicity. Virology 1997 Mar 3; 229 (1): 269-78

106. Wang S, Chou TH, Sakhatskyy PV, et al. Identification of two neutralizing regions on the severe acute respiratory syndrome coronavirus spike glycoprotein produced from the mammalian expression system. J Virol 2005 Feb; 79 (3): 1906-10

107. Robinson HL, Montefiori DC, Johnson RP, et al. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. Nat Med 1999; 5 (5): 526-34

108. Heller LC, Ugen K, Heller R. Electroporation for targeted gene transfer. Expert Opin Drug Deliv 2005 Mar; 2 (2): 255-68

109. Wang S, Zhang C, Zhang L, et al. The relative immunogenicity of DNA vaccines delivered by the intramuscular needle injection, electroporation and gene gun methods. Vaccine 2008 Apr 16; 26 (17): 2100-10

110. Aguiar JC, Hedstrom RC, Rogers WO, et al. Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device. Vaccine 2001 Oct 12; 20 (1-2): 275-80

111. Haensler J, Verdelet C, Sanchez V, et al. Intradermal DNA immunization by using jet-injectors in mice and monkeys. Vaccine 1999 Feb 26; 17 (7-8): 628-38

112. Trimble C, Lin CT, Hung CF, et al. Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. Vaccine 2003 Sep 8; 21 (25-26): 4036-42

113. Prausnitz MR. Microneedles for transdermal drug delivery. Adv Drug Deliv Rev 2004 Mar 27; 56 (5): 581-7

114. Deml L, Bojak A, Steck S, et al. Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the human immunodeficiency virus type 1 Gag protein. J Virol 2001 Nov; 75 (22): 10991-1001

115. Wang S, Farfan-Arribas DJ, Shen S, et al. Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. Vaccine 2006 May 22; 24 (21): 4531-40

116. zur Megede JZ, Chen MC, Doe B, et al. Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 *gag* gene. J Virol 2000; 74 (6): 2628-35

117. Lu S. Combination DNA plus protein HIV vaccines. Springer Semin Immunopathol 2006 Nov; 28 (3): 255-65

118. Richmond JF, Lu S, Santoro JC, et al. Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. J Virol 1998; 72 (11): 9092-100

119. Barnett SW, Rajasekar S, Legg H, et al. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. Vaccine 1997 Jun; 15 (8): 869-73

120. Letvin NL, Montefiori DC, Yasutomi Y, et al. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. Proc Natl Acad Sci U S A 1997 Aug 19; 94 (17): 9378-83

121. Wang S, Arthos J, Lawrence JM, et al. Enhanced immunogenicity of gp120 protein when combined with recombinant DNA priming to generate antibodies that neutralize the JR-FL primary isolate of human immunodeficiency virus type 1. J Virol 2005 Jun; 79 (12): 7933-7

122. Wang S, Pal R, Mascola JR, et al. Polyvalent HIV-1 Env vaccine formulations delivered by the DNA priming plus protein boosting approach are effective in generating neutralizing antibodies against primary human immunodeficiency virus type 1 isolates from subtypes A, B, C, D and E. Virology 2006 Jun 20; 350 (1): 34-47

123. Pal R, Wang S, Kalyanaraman VS, et al. Immunization of rhesus macaques with a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 vaccine elicits protective antibody response against simian human immunodeficiency virus of R5 phenotype. Virology 2006 May 10; 348 (2): 341-53

124. Rasmussen RA, Hofmann-Lehman R, Montefiori DC, et al. DNA prime/protein boost vaccine strategy in neonatal macaques against simian human immunodeficiency virus. J Med Primatol 2002 Feb; 31 (1): 40-60

125. Goepfert PA, Tomaras GD, Horton H, et al. Durable HIV-1 antibody and T-cell responses elicited by an adjuvanted multi-protein recombinant vaccine in uninfected human volunteers. Vaccine 2007; 25: 510-8

126. Kennedy JS, Co M, Green S, et al. The safety and tolerability of an HIV-1 DNA prime-protein boost vaccine (DP6-001) in healthy adult volunteers. Vaccine 2008 Aug 18; 26 (35): 4420-4

127. Vaine M, Wang S, Crooks ET, et al. Improved induction of antibodies against key neutralizing epitopes by human immunodeficiency virus type 1 gp120 DNA prime-protein boost vaccination compared to gp120 protein-only vaccination. J Virol 2008 Aug; 82 (15): 7369-78

128. Li Y, Migueles SA, Welcher B, et al. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. Nat Med 2007 Sep; 13 (9): 1032-4

Correspondence: Dr *Shixia Wang*, Room 304, Laboratory of Nucleic Acid Vaccines, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Lazare Research Building, Worcester, MA 01605, USA.
E-mail: shixia.wang@umassmed.edu