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Improving immunogenicity of HIV-1 envelope gp120 by glycan removal and immune complex formation

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Abstract

HIV-1 envelope (Env) gp120 is an important target for neutralizing antibody (Ab) responses against the virus; however, developing gp120 vaccines that elicit potent and broad neutralizing Abs has proven to be a formidable challenge. Previously, removal of an N-linked glycan at residue 448 by an N to Q mutation (N448Q) has been found to enhance the *in vitro* antigenicity of neutralizing epitopes in the V3 loop. In this study the mutated gp120 was first compared with wild type gp120 for immunogenicity in mice using a DNA prime and protein boost immunization regimen. The N448Q mutant did not elicit higher titers of anti-gp120 serum Abs and failed to generate anti-V3 Abs. The sera also had no virus-neutralizing activity, even though the mutant induced higher levels of lymphoproliferation and cytokine production. Subsequently, the N448Q mutant was used to construct an immune complex vaccine with the anti-CD4 binding site monoclonal antibody (mAb) 654. The N448Q/654 complex stimulated comparably high levels of serum Abs to gp120 and V3 as the wild type complex. However, Abs against the C1 and C2 regions in the gp120 core were more elevated. Importantly, the mutant complex also elicited higher titers of neutralizing Abs activity than the wild type counterpart. Similar results were achieved with a complex made with gp120 bearing an N448E mutation, confirming the importance of the N448-linked glycan in modulating gp120 immunogenicity. Neutralizing activity was directed to V3 and other undefined neutralizing epitopes. Improved immunogenicity of the immune complexes correlated with alterations in exposure of V3 and other Ab epitopes and their stability against proteases. These data demonstrate the advantage of combining site-specific N-glycan removal and immune complex formation as a novel vaccine strategy to improve immunogenicity of targeted Ab epitopes on critical regions of HIV-1 gp120.

Keywords

HIV-1; HIV-1 envelope; N-glycan; immune complex vaccine; antibody response

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Introduction

The envelope (Env) glycoprotein gp120, the viral antigen that mediates HIV-1 binding to CD4 and the chemokine receptor on target cells, is a critical target for virus-neutralizing antibodies (Abs). However, many efforts to make vaccines that elicit potent and broadly reactive neutralizing Abs against gp120 have not been successful. In addition to its renowned variability, HIV-1 gp120 is highly glycosylated with 20-30 N-linked glycans per molecule that accounts for half of this antigen's molecular weight. This heavy glycosylation contributes to neutralization-resistant phenotypes of HIV-1 isolates by masking critical neutralizing epitopes [1-6]. Glycans also influence immunogenicity of neutralizing epitopes on Env of HIV-1 and its simian counterpart SIV. Reitter and co-workers [7] reported that rhesus macaques infected with SIV lacking N-linked glycans in V1 had increased Ab titers to this region and higher neutralizing responses. Infection of rhesus macaques with SIVmac239 bearing up to five deglycosylating mutations also stimulated neutralizing Abs to higher, albeit variable, degrees than the wild type virus [8, 9]. However, prime-boost immunization with the quintuple deglycosylated Env vaccine did not result in improved neutralizing Ab responses [10]. Others have similarly observed no major difference between Ab responses elicited by wild type versus deglycosylated Env vaccines [11-13]. Nevertheless, in a more recent study Li and co-workers observed that removal of a specific N-glycan in the stem of V2 loop in HIV-1 89.6 Env increased virus neutralization sensitivity to broadly neutralizing Abs against the CD4-binding site, V3, and the CD4-induced epitope [14]. Significantly, immunization with this Env mutant induced higher and broader serum neutralizing Ab responses than that with the wild type Env, indicating that more effective Env vaccines may be generated by removal of select N-glycan(s).

Our previous study identified a glycan at the C4 region (N448) that is important for the processing and presentation of nearby T helper epitopes [15, 16]. Interestingly, the removal of this glycan also enhanced antigenicity of neutralizing epitopes in the V3 loop, without affecting the other Ab epitopes or the CD4-binding capacity [15]. The current study was designed to evaluate whether the enhanced V3 antigenicity on gp120 lacking the N448-glycan could be exploited to improve elicitation of neutralizing Ab responses against V3. Two vaccination strategies were tested in mice. First, we utilized the DNA prime/protein boost protocol that has been shown to elicit cellular and humoral responses against HIV and other antigens [17-20]. Second, we constructed immune complex vaccines composed of gp120 and an anti-gp120 monoclonal antibody (mAb). Our previous studies demonstrated that besides N-glycan removal, immune complex formation of gp120 and anti-CD4-binding site mAbs also better exposes and enhances Ab reactivity against neutralizing epitopes on the V3 loop [21, 22]. Significantly, immune complex vaccines are capable of eliciting virus-neutralizing Abs in immunized mice, while uncomplexed gp120s with the same immunization protocol fail to stimulate any neutralizing Abs. Nevertheless, improvements in the immune complex vaccine platform are still required in order to achieve greater potency and breadth of neutralizing Ab responses. In this study we focused on improving the potency of anti-gp120 Ab responses by testing whether complexes made of gp120s lacking the N448-glycan and the anti-CD4 binding site mAb 654 were able to elicit higher titers of gp120-binding and virus-neutralizing Ab responses. To evaluate the critical parameters influencing elicitation of anti-gp120 Abs, the immunization data were correlated with the effects of N488-glycan removal and/or immune complex formation on Ab reactivity to specific gp120 epitopes *in vitro* and resistance of these epitopes to proteolytic digestion. The study demonstrated that a combination of glycan removal and immune complex formation enhanced the capacity of gp120 to elicit Ab responses in part as a result of improved antigenicity and stability of specific epitopes on the gp120 antigen.

Material and Methods

Antigens, mAbs, and DNA

Recombinant gp120 proteins of HIV-1 BH10 (a molecular clone of LAI) with the wild-type sequence or with N448Q or N448E mutations were generated in transfected CHO-L761h cells as described previously [15, 16]. For comparison, these same proteins were also produced in the human embryonic kidney cells 293T, and elicited comparable levels of anti-gp120 serum IgG as the CHO-derived gp120 proteins in the DNA prime-protein boost vaccination study (data not shown). The gp120_{core} and gp120_{core}+V3 proteins of HIV-1_{YU2} were kindly provided by Dr. Richard Wyatt (IAVI Center for Neutralizing Antibodies, Scripps Institute). The outer domain gp120 (OD1) protein of HIV-1_{YU2} was kindly provided by Dr. Joseph Sodroski (Dana-Farber Cancer Institute, Harvard Medical School of Public health Boston MA).

Forty seven peptides (20-mers overlapping by 10 amino acids) spanning gp120 of HIV-1 HXB-2 (a molecular clone of LAI), including peptide 40 (KQIINMWQKVGKAMYAPPIS), peptide 18 (TQACPKVSFEPIPIHYCAPA) and peptide 41 (KAMYAPPISGQIRCSSNITG), were obtained from National Institute for Biological Standards and Control Centre for AIDS Reagents (EU Programme EVA/AVIP). The V3_{HXB-2} peptide (NTRKRIRIQRGPGRAFVTIG) was purchased from Sigma.

Human gp120-specific mAbs used in this study were generous gifts from Dr. Susan Zolla-Pazner and Dr. Mirosław Gorny (New York University School of Medicine). The sheep polyclonal Ab, D7324, directed to C-terminus of gp120 was purchased from Aalto Bio Reagents Ltd, Dublin, Ireland.

All gp120-encoding plasmids used for DNA immunizations were prepared with the pEE14 expression vector [15, 16]; these same plasmids were also used for the production of soluble gp120 proteins described above. The plasmids were produced in JM109 E. coli, isolated and purified using an Endo-Free plasmid purification column (Qiagen, Valencia, CA), and quantified based on optical density at 260 nm. The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pHXB2-env from Dr. Kathleen Page and Dr. Dan Littman.

Immunization

For the DNA priming–protein boost vaccination study, C57BL/6 and BALB/c mice (female, > 6 weeks old from the Jackson lab, 5 mice per group) were first injected intramuscularly with plasmids encoding wild type or mutant gp120s (50 µg in 100 µl PBS per animal). At weeks 3 and 6, mice were boosted subcutaneously with wt gp120 or N448Q gp120 (5 µg per animal) along with adjuvant QS21 (20 µg per animal), provided by Aenus Inc., Lexington, MA in a total volume of 100 µl at two separate sites in the back of each mouse. A control group of animals were immunized with PBS and QS21 adjuvant. Blood was collected two weeks after the last immunization, sera from each group were pooled, and then stored at -80 °C until use.

For immunization with immune complexes, BALB/c mice (female, > 6 weeks old from the Jackson lab, 5 animals per group) were injected intraperitoneally with immune complexes containing wild type or mutant gp120s and mAb 654-D (designated herein as 654). The immune complexes were prepared (3 µg gp120 and 9 µg mAb in 50 µl per animal) and mixed with adjuvant (25 µg MPL and 250 µg DDA in 50 µl per animal per dose; Sigma, St. Louis, MO) immediately prior to injection as described previously [21, 22]. The immune complex vaccines were administered 3 times, at 2-week intervals. Blood was collected two weeks after the last immunization, and the sera from each group were pooled. The animal

studies were carried out according to the protocols approved by the VA New York Harbor Healthcare System and New York University Institutional Animal Care and Use Committees.

ELISA to assess serum Ab response

Serum Ab responses were determined using ELISA as described previously [21]. Briefly, different gp120 proteins or peptides were coated on the ELISA plates and reacted with serially diluted mice sera. Serum Ab binding was detected using alkaline phosphate-conjugated secondary Ab to mouse total IgG or IgA or specific IgG isotypes. Peptide epitope mapping was performed using pools of peptides spanning gp120_{HXB-2}. Except for one pool that contained only two peptides (C5: 46-47), pools of five peptides were prepared by combining equal amounts of individual peptides and tested in ELISA at a final concentration of 2 µg/ml for each peptide.

HIV-1 neutralization assay with TZM/bl target cells

Virus neutralization was measured using HIV-1 pseudoviruses or infectious viruses with TZM-bl target cells as described previously [21, 22]. HIV-1_{LAI} was grown in mitogen-activated PBMCs. Pseudoviruses with HIV-1 Env SF162 or HXB-2 were produced by transfecting 293T cells using the ProFection mammalian transfection system (Promega, Madison, WI). Prior to neutralization assay, sera were heat-inactivated (56°C for 30 min). Virus was incubated for 1 h at 37 °C with diluted sera and then added to TZM-bl cells in the presence of diethylaminoethyl (Sigma-Aldrich, St Louis, MO) and Indinavir (1 µM). Virus infection was determined after 48 h using the Bright-Glo Luciferase Assay System (Promega, Madison, WI). The anti-V3 mAb 447/52D was included in the assay as a positive control. For peptide absorption assay, test peptides were first added to serum for 1 h prior to addition of virus. The following peptides were used: V3 peptide, a peptide pool covering C1 (aa 80-140) and C2 (aa 232-191), and a scrambled control peptide (IGPGRATRPNNNFYTTGTRKSIH). The final peptide concentration used was 40 µg/ml. Comparable data were obtained with peptide concentrations of 5, 80, and 180 µg/ml (data not shown).

Lymphoproliferation and cytokine secretion

Lymphoproliferation was assessed using the standard ³H-thymidine incorporation assay as described previously [23]. Briefly, freshly isolated splenocytes from immunized mice were resuspended in complete RPMI medium supplemented with ultra low IgG FBS (Gibco) and then incubated at 2 × 10⁵ cells/well with antigens or with medium alone for 4 days. The cells were pulsed with ³H-thymidine for 16–22 hr prior to harvest. To measure cytokine production, culture supernatants of spleen cells stimulated with or without antigens were collected at 72 h and measured by using a custom configured Bio-Plex multiplex assay (BioRad, Hercules, CA) detected on a Luminex 200.

ELISA to compare Ab reactivity of uncomplexed versus complexed gp120s

Assessment of Ab reactivity against V3 and C2 epitopes on uncomplexed gp120s versus gp120/654 complexes was performed as described previously [21, 22]. Briefly, wild type and mutant gp120s, either alone or in complex with mAb 654, were captured with sheep anti-C5 Ab onto ELISA wells, and then reacted with biotinylated anti-V3 694/98D (designated herein as 694) or anti-C2 1006-30D (designated herein as 1006) mAbs. The mAb binding was detected by alkaline phosphate-conjugated streptavidin.

Proteolysis assay

Proteolysis assays with cathepsins were performed as previously described by Yu and co-workers [24] with minor modifications. Briefly, gp120s either alone or in complex with mAb 654 were incubated with cathepsins L, S or D individually (cathepsin/gp120 ratio of 1:25) in a 37°C water bath for 1 h in a digestion buffer prepared according to manufacturer's directions (Sigma-Aldrich, St Louis, MO). The digestion products were then captured with sheep anti-C5 Ab onto ELISA plates and probed with biotinylated human mAbs to V3, C2, or V2. The mAb binding was detected by alkaline phosphate-conjugated streptavidin.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.

Results

Immunization with wild type gp120 or gp120 lacking the N448-linked glycan by DNA prime and protein boost

To evaluate immunogenicity of gp120 that lacks the N448-linked glycan, we initially used a DNA prime and protein boost vaccination regimen. C57BL/6 and BALB/c mice were primed intramuscularly with DNA encoding gp120 with the N448Q mutation or the wild type sequence and then boosted twice, three weeks apart, via a subcutaneous injection with the corresponding mutated or wild type gp120 proteins in the presence of adjuvant QS21. Animals immunized with no gp120 (adjuvant alone) was tested in parallel as a negative control. Serum Ab responses were studied two weeks after the last injection. First, the levels of gp120-specific Abs were measured in ELISA. Antibody (Ab) response to the wild type gp120 was stimulated to comparable levels in C57BL/6 mice immunized with wild type or mutant gp120s (Figure 1A). Control mice had no detectable gp120-specific IgG responses. The Ig isotype analyses further showed that the anti-gp120 Abs were made of mostly IgG1, with much lower levels of IgG2a, IgG3 and IgA, indicating the elicitation of Th2 response in these immunized mice. However, anti-V3 Abs were not detected in the sera of mice immunized with either wild type or mutant gp120s (Figure 1B), indicating that the enhanced V3 antigenicity demonstrated by N448Q gp120 *in-vitro* was not sufficient to augment its immunogenicity upon vaccination. Correspondingly, low levels of neutralization were detected against pseudovirus bearing homologous HIV-1 HXB-2 Env (Figure 1C). When BALB/c mice were immunized with wild type or N448Q gp120s using the same DNA prime/protein boost protocol, the N448Q gp120 also elicited similar levels of anti-gp120 serum IgG as the wild type, although the mutant stimulated lower levels of anti-gp120 IgG1 (Supplementary Figure 1). Anti-gp120 IgG2a and IgA were not generated by wild type or mutant gp120s. In addition, anti-V3 Abs also were not induced, and neutralizing activity was undetectable in either group.

Next, we evaluated whether the N448Q mutation had any effect on T cell responses against gp120. Splenocytes of the immunized C57BL/6 mice were tested for lymphoproliferation and cytokine secretion in response to wild type gp120 (Figure 2A). Interestingly, higher lymphoproliferation was achieved following immunization with the N448Q gp120 than that with the wild type gp120. The increased proliferation was accompanied by higher production of cytokines IL-10 and IL-6. IFN- γ secretion, on the other hand, was comparable for both groups of immunized mice. No difference was also seen with MIP-1 β production, while IL-4 was not detectable in either group (data not shown). Animals receiving only adjuvant had no detectable response to gp120.

To investigate whether the higher anti-gp120 T cell response observed in mice immunized with the N448Q mutant was due to alterations in T cell recognition of epitopes around

position 448 [25], T cell response to two overlapping peptides representing the wild type gp120 sequence from positions 421 to 440 (peptide 40) and from positions 432 to 450 (peptide 41) were analyzed (Figure 2B). Lymphoproliferative responses to both peptides were significantly higher in mice immunized with N448Q than in mice with wild type gp120. Peptides 40 and 41 also elicited higher production of Th2-associated cytokines IL-10 and IL-6, respectively ($p < 0.001$), while secretion of Th1 cytokine IFN- γ in response to these peptides was significantly reduced when the mice were immunized with N448Q as compared to wild type gp120. In contrast, the responses to peptide 18 from the distant C2 region were not significantly altered (Figure 2B). These data indicate that deletion of the N448 glycosylation site increases Th2 response to gp120 and in particular to epitopes near residue 448. However, this enhanced Th2 response did not further improve anti-gp120 serum Ab titers above those achieved by immunization with wild type gp120 and did not result in induction of any virus-neutralizing Abs.

Immunization with immune-complex vaccines made with wild type gp120 or gp120 mutants lacking the N448-linked glycan

Our previous studies have shown that the immunogenicity of gp120 can be significantly enhanced when this antigen is administered as an immune-complex vaccine constructed with an anti-CD4-binding site mAb. Immunization with such a complex elicits virus-neutralizing Abs that are directed to epitopes in the V3 loop and other regions yet undefined. Hence, we sought to examine whether a more potent Ab response could be elicited by the immune complexes made with gp120s lacking the N448-glycan. BALB/c mice were immunized three times at two-week intervals with wild type or mutant gp120s complexed with the anti-CD4 binding site mAb 654 along with the MPL/DDA adjuvant. Two different mutations that remove the N448-linked glycan, N448Q and N448E, were tested for comparison. Control groups of mice immunized with uncomplexed gp120 or only adjuvant were also tested in parallel. The data show that mutant or wild type gp120s complexed with mAb 654 elicited high levels of gp120-specific serum IgG (Figure 3A). In contrast, animals immunized with uncomplexed wild type gp120 had a much lower titer of gp120-specific serum IgG (Supplementary Figure 2), in agreement with our earlier observations [21, 22]. Notably, the titers generated by the gp120/654 complexes were about five-fold higher than those achievable with DNA prime/protein boost vaccination regimen (Figures 1A and 3A). Half maximal anti-gp120 Ab titers of 1:12,000 and 1:2,500 were elicited by N448Q gp120/654 complex and N448Q gp120 DNA prime/protein boost, respectively. A similar pattern was observed with wild type gp120. Unlike the DNA prime/protein boost immunization, immunization with each of the three gp120/654 complexes also successfully elicited anti-V3 Abs (Figure 3A). There was a trend of higher anti-gp120 and anti-V3 Ab titers elicited by the N448Q complex and the N448E complex as compared to the wild type gp120 complex, but the differences did not reach statistical significance. However, when the sera were tested for binding to gp120 core lacking V1, V2, V3, the N and C termini, significantly higher titers of Abs were detected following immunization with the mutant complexes. Higher titers were also seen with Abs against gp120 core that retains V3, but not with Abs against the protein containing only the outer domain of gp120 (OD1) (Figure 3A), suggesting that the mutant complexes augment Ab response against the inner domain of gp120.

To probe this issue further, immune sera were tested at a dilution of 1:200 for ELISA reactivity against pools of overlapping peptides spanning the entire gp120 sequence. Due to the limited availability of the specimen, sera from the N448E complex-immunized mice were not used in this experiment. The data in Figure 3B show that sera from the N448Q complex-immunized mice displayed significantly higher Ab reactivity against four of the peptides pools as compared to sera from mice receiving the wild type gp120 complex, with

the greatest differences observed with peptide pools representing the C1 and C2 regions in the inner domain of gp120 (C1:6-10 and C2:21-25; $p < 0.001$). Peptide pools from the C2-V3 and V4-C4 regions were also recognized better, but the differences were much smaller ($p < 0.01$).

Next we sought to determine whether the anti-gp120 Abs elicited by the mutant complexes mediated neutralizing activity against HIV-1 with wild type Env gp120. Neutralization was examined initially with HIV-1 pseudovirus expressing the homologous Env HXB-2. Neutralizing activity with IC_{50} of 1:80 was detected in sera of mice immunized with the wild type gp120 complex (Figure 4A). Sera from mice immunized with adjuvant alone had no neutralizing activity. Sera of mice immunized with uncomplexed gp120 had low neutralizing activity ($IC_{50} < 1:50$; Supplementary Figure 2), consistent with previously reported findings [21, 22]. Significantly, the complexes with N448Q or N448E elicited even more potent neutralizing activity, with IC_{50} titers reaching up to 1:170. A similar result was observed with neutralization of infectious HIV-1 LAI (Figure 4C). However, no neutralizing activity against a heterologous virus SF162 was detected (Figure 4B).

To determine the Ab specificities contributing to the neutralization, sera were pre-treated with the V3 peptide known to encompass the principal neutralizing epitope for HIV-1 LAI. As similar results were obtained with the two mutant gp120 complexes, only sera from mice immunized with the N448Q complex were tested and compared with sera from animals immunized with the wild type complex. The V3 peptide treatment of sera from the N448Q complex-immunized animals reduced the neutralizing activity by approximately 25%, with IC_{50} remaining at 1:70 (Figure 4C). By contrast, the addition of V3 peptide to sera from mice immunized with the wild type gp120 complex decreased neutralization such that the IC_{50} titer went below 1:50. To assess whether the neutralizing activity observed in sera from the N448Q complex-immunized mice was also mediated in part by Abs to C1 and C2 that were induced to higher levels by the N448Q complex (Figure 3B), the sera was pre-treated with peptides encompassing the C1 and C2 regions (C1:6-10 and C2:21-25, respectively) and then tested in the neutralization assay. Unlike that with V3 peptide, pre-treatment with the C1 and C2 peptides had no effect on neutralizing activity (Figure 4D), indicating that the enhanced Ab response induced against the C1 and C2 peptides by the N448Q complex has no virus-neutralizing activity. All together these data demonstrate that the mutated gp120/654 complexes were more immunogenic than the wild type gp120 complex in eliciting non-neutralizing and neutralizing Abs against the homologous virus HIV-1 LAI. The neutralization was directed in part against V3 epitopes, but a significant fraction also targeted neutralizing epitopes that remained undefined.

Alterations in antigenicity of wild type and mutant gp120 when complexed with mAb 654

We surmise that enhanced immunogenicity of the gp120/654 immune complexes is associated with increased accessibility of specific epitopes in wild type and mutant gp120s as a result of allosteric changes in the gp120 structure upon binding by the anti-CD4 binding site mAb 654. To test this idea, we compared the reactivity of uncomplexed N448Q versus the N448Q/mAb 654 complex with mAbs against V3 (694), C2 (1006), and V2 (697) in ELISA. Wild type gp120 complexed or not complexed with mAb 654 were also tested for comparison. Higher reactivity to V3 was observed with N448Q as compared to wild type gp120 in their uncomplexed forms, but V3 reactivity was further enhanced in the N448Q/654 complex ($p < 0.05$ at the lowest dilution vs. uncomplexed N448Q) (Figure 5A). The N448Q complex also had a slightly higher level of V3 reactivity than the wild type complex but the difference was not significant. Moreover, the immune complexes displayed enhanced anti-C2 reactivity as compared to the respective uncomplexed gp120s, and the increase was greater with the N448Q complex ($p < 0.001$ vs. N448Q) than the wild type complex ($p < 0.01$ vs. wild type gp120) (Figure 5B). In contrast, V2 reactivity was lower against the wild type

and N448Q complexes as compared to the respective gp120s (Figure 5C). In view of the fact that the V3, C2, and V2 regions of gp120 are not part of or immediately adjacent to mAb 654's epitope in the CD4-binding site of gp120 core, these results indicate that the binding of mAb 654 induces significant conformational changes in the overall gp120 structure that modulate accessibility of the distinct gp120 epitopes for Abs. The data also suggest that higher Ab reactivity of V3 and C2 on the immune complexes is one factor that contributes to the enhanced immunogenicity of these complexes *in vivo*.

Alterations in susceptibility of gp120 epitopes to proteolysis upon immune complex formation

In addition to modulating gp120 antigenicity, the binding of mAb 654 to gp120 also has been shown to render gp120 less susceptible to proteolytic digestion [26, 27]. Increased stability of gp120 epitopes on the immune complexes may then improve their immunogenicity *in vivo*. Nevertheless, the effects on specific gp120 epitopes have not been evaluated. To address this question, we examined the ability of cathepsins L, S, or D to disrupt V3 and C2 epitopes as recognized by mAbs 694 and 1006, respectively. These cathepsins are expressed by antigen-presenting cells and play a role in proteolytic processing of various antigens [28-30]. Wild type gp120 and the N448Q mutant were tested as uncomplexed antigens and as immune complexes with mAb 654. After treatment with each of the cathepsins, the digested products were probed for reactivity with the anti-V3 mAb 694 or the anti-C2 mAb 1006 in ELISA and compared to undigested controls. The data demonstrate that digestion of wild type and N448Q gp120 with cathepsin L reduced V3 reactivity by 40-60% (Figure 6A, left panel). For wild type gp120, complexing with mAb 654 did not protect V3 from this cathepsin as V3 reactivity was further reduced to 35%. Nevertheless, V3 reactivity was preserved better in context of the N448Q gp120/654 complex than in the wild type complex ($p < 0.001$). Cathepsins S and D had no effects on V3 reactivity of wild type or mutant gp120s, regardless of whether the gp120 antigens were complexed or not complexed with mAb 654.

In contrast to V3, the C2 epitope in uncomplexed wild type or mutant gp120 antigens was highly susceptible to cathepsins L, S, and D, such that only 10-20% of C2 reactivity was retained after digestion (Figure 6A, right panel). The loss of C2 reactivity was specific and was not accompanied by parallel destruction of epitopes in the V2 or V3 loops which flank the C2 region at its amino and carboxyl ends, respectively (Supplementary Figure 3). Interestingly, complexing with mAb 654 rendered the C2 epitope more resistant to each of the three cathepsins. Moreover, the C2 epitope was more resistant to cathepsin L in context of the N448Q complex than in the wild type gp120 complex. Of note, this C2 epitope was also most accessible for Ab binding on the N448Q/654 complex (Figure 5B). These data indicate that the binding of mAb 654 to wild type or mutant gp120s significantly increases not only the antigenicity but also the stability of the C2 epitope against proteolysis. Increased antigenicity and stability of the C2 epitope were displayed most in the N448Q/654 complex, and corresponded with the higher level of anti-C2 Abs induced by immunization with the N448Q/654 complex (Figure 3B). Similar results were observed with the V3 epitope in context of the N448Q/654 complex, albeit at lower extents. Hence, immunogenicity of gp120 epitopes is influenced by the accessibility of these epitopes for Ab binding and their stability against enzymatic degradation.

Discussion

This study investigated the use of mutated HIV-1 gp120 as a vaccine immunogen to elicit higher titers of anti-gp120 Ab response effective against the virus. We focused on enhancing titer or potency, but not breadth, of the Ab response by testing recombinant gp120 derived from Env of HIV-1 LAI, a subtype B laboratory-adapted strain in which the principal

neutralizing epitopes, located in the V3 loop, are already defined. Two approaches to improve V3 immunogenicity were studied: 1) removal of a single glycan at position 448 by substituting the N448 residue with Q or E, and 2) immune complex formation with the anti-CD4-binding site mAb 654. Each of these strategies has been shown previously to enhance antigenicity (i.e. *in vitro* Ab reactivity) of neutralizing V3 epitopes [15, 21, 22]. The human anti-CD4-binding site mAb 654 was selected for preparing the gp120/mAb complexes because it has a relatively high binding affinity for gp120 and forms a stable immune complex with enhanced V3 reactivity [26]. No murine anti-CD4-binding site mAbs displaying similar properties are available. Data from this study demonstrate that using the DNA prime/protein boost protocol, immunization with the mutant gp120 did not elicit anti-V3 Abs and failed to stimulate higher titers of anti-gp20 serum Abs than the wild type gp120. The sera also had very weak neutralizing activities comparable to that achieved with the wild type gp120. By contrast, immune complexes made with N448Q or N448E were potent in eliciting higher titers of both neutralizing and non-neutralizing Abs than the wild type complex. Neutralizing activity was directed against V3 and other undefined epitopes, while non-neutralizing Abs were stimulated to higher levels against the inner domain of gp120 including the C2 region. The contribution of these non-neutralizing Abs in protection against HIV-1 remains unclear but these Abs might be important for antiviral activities such as antibody-dependent cell-mediated cytotoxicity and antibody-dependent cell-mediated virus inhibition [31, 32]. The uncomplexed gp120 was not able to induce neutralizing Abs (Supplementary Figure 2C and [21, 22]), similar to the DNA prime/protein boost data (Figure 1C). These data demonstrate the advantage of combining a select gp120 mutation and immune complex formation for designing a more potent vaccine immunogen that is capable of eliciting higher titers of Ab responses against HIV-1.

Although the DNA prime/protein boost immunization with the N448Q mutant elicited comparable levels of gp120-binding and virus-neutralizing Ab titers as that with the wild type gp120, the mutant elicited higher levels of gp120-specific lymphoproliferation and secretion of Th2-associated cytokines. Significantly, the responses against peptides in the vicinity of residue 448 were significantly enhanced, suggesting that the removal of N448-linked glycan may promote the processing and/or presentation of nearby Th epitopes that result in better induction of Th responses to these epitopes. This finding could not be predicted from our previous *in vitro* data which showed that the same glycan removal reduced recognition of epitopes immediately upstream of residue N448 by both human and mouse CD4 T cell lines [15]. The reasons for this discrepancy are not known, but these data reaffirm the difficulty of extrapolating *in vitro* antigenicity of a vaccine to its immunogenicity *in vivo*. Enhanced immunogenicity of the gp120 mutants was further demonstrated by the ability of the immune complexes made with N448Q or N448E gp120s to induce higher titers of binding and neutralizing Abs than the wild type complex. It should also be noted that the DNA priming/protein boost protocol induced much higher titers of anti-gp120 Abs than immunization with gp120 protein alone (Figures 1A vs. Supplementary Figure 2A), confirming the significant contribution of DNA priming as reported previously [17, 20, 33, 34]. In these two protocols, animals were immunized three times with either one DNA prime plus two protein boosts or with three injections of proteins only, but the protein only immunization resulted in a very low level of anti-gp120 Abs that was almost comparable to the sham control. Nevertheless, DNA prime/protein boost vaccination, even with the N448Q mutant displaying enhanced V3 reactivity, was not adequate for eliciting Abs against neutralizing V3 epitopes.

In contrast to gp120 alone, immune complexes of gp120 and mAb 654 were highly potent in inducing virus-neutralizing Ab responses. Importantly, using the N448 mutant complexes we were able to further enhance the titers of neutralizing and non-neutralizing anti-gp120 Ab responses beyond those achievable with the wild type counterpart, indicating the

synergistic potential of N-glycan removal and immune complex formation in augmenting gp120 immunogenicity. Consistent with our previous reports [21, 22], DNA priming was not required for these immune complex vaccines, although experiments are currently in progress to evaluate the contribution of DNA priming in further improving the immunogenicity of the immune complex vaccines. The mechanisms by which mAb 654 enhances gp120 immunogenicity are not yet fully understood. The binding of mAb 654 and other anti-CD4-binding site mAbs to gp120 has been shown to cause allosteric changes in the overall gp120 structures that affect exposure of distant Th and B cell epitopes [21, 22, 26, 27, 35]. Similar to CD4, anti-CD4-binding site mAbs also induce large enthalpic and entropic changes that are not seen upon gp120 interaction with other anti-gp120 mAbs to increase rigidity of the otherwise highly flexible gp120 molecule and increase resistance of the complexed gp120 to degradative enzymes including proteases and endoglycosidases [26, 27, 36]. To our knowledge this is the first study that demonstrates this effect of anti-CD4 binding site mAbs on specific gp120 epitopes. Hence, Ab reactivity to epitopes in the V3 and C2 regions were specifically enhanced when gp120 was bound to mAb 654. The C2 epitope was also better protected from proteolytic degradation in context of gp120/654 complexes than in uncomplexed gp120. Similar results were observed with V3 in the mutant complex, albeit to a lower extent, indicating that by forming immune complexes, specific gp120 epitopes are recognized better by Abs and also are more resistant to proteolytic degradation. The enhanced antigenicity and stability of these particular Ab epitopes correlated with their increased immunogenicity *in vivo*, indicating the importance of these two factors in influencing immunogenicity of Ab epitopes on gp120 and possibly on other antigens.

Contrary to V3 and C2, the CD4-binding site and the V2 loop were occluded in the gp120/654 complexes from Ab recognition, leading to failure of animals immunized with these complexes to generate Abs against these regions (Figure 3B and [22]). Most of anti-CD4 binding site Abs made during HIV infection by slow and rapid progressors have poor or no neutralizing activity and can interfere with gp120 antigen processing that result in suppressed helper CD4 T cell responses [26, 27, 37, 38]. However, unique neutralizing epitopes such as those recognized by broadly reactive and potent neutralizing mAbs b12 and VRC01 are present in the CD4 binding site [39-42]. These epitopes are also blocked in the gp120/654 complexes (unpublished data). Similarly, Abs to V2 that have the potential to neutralize viruses [43, 44] or mediate other anti-viral functions would not be induced by the gp120/654 complexes. Therefore, further improvements of the current prototypic complexes are needed. A number of anti-gp120 mAbs that enhance the antigenicity of epitopes in the CD4 binding site and the V2 loop have been identified ([45] and Hioe et al., unpublished data) and can be utilized to produce additional immune complexes to enhance Ab responses against these epitopes. In conjunction with the complexes studied here, a cocktail of immune complex vaccines may then be employed to address the difficult challenge of increasing the breadth of anti-viral Ab responses elicited by immunization. Moreover, we demonstrated that a broader neutralizing anti-V3 Ab response effective against heterologous HIV-1 isolates could be induced by immune complexes made of gp120 JRFL, rather than gp120 LAI [21]. Because gp120 LAI has an unusual V3 sequence due to a two amino acid insertion and is antigenically distinct from gp120s of other HIV-1 isolates, the Abs generated in response to gp120 LAI are extremely specific for LAI and its homologous virus strains (Figure 4 and [22]), and thus, constructing immune complexes with gp120s that better represent the majority of HIV-1 circulating isolates would be advantageous.

Immune complexes have been used as vaccines to augment immune responses to hepatitis B antigens in humans [46, 47] as well as to infectious bursal disease virus, equine herpesvirus 1 and porcine parvovirus in animals [48-50]. Enhanced immunogenicity of immune complex vaccines has been attributed mainly to their Fc-mediated activity, as the Fc interaction with FcRs or complement receptors on dendritic cells and other professional antigen presenting

cells (APCs) facilitates targeting and uptake of antigen by APCs and mediates their activation, resulting in enhanced antigen presentation to helper CD4 T cells and development of a more effective T cell-dependent Ab response [51-53]. The specific involvement of the Fc fragment in influencing the immunogenicity of gp120/654 complexes was not evaluated in this study. Human mAb 654 (IgG1 subtype) was utilized to make the immune complexes and the Fc fragment of human IgG1 might not engage murine FcRs efficiently. The association constant of human IgG1 Fc for mouse splenic macrophages has been reported to be ~30x lower than that to human peripheral monocytes, and human IgG also displays distinct binding modes for mouse vs. human Fc receptors [54, 55]. Moreover, we have previously observed that an adjuvant is critical for immunization of mice with the gp120/654 complex, since administration of the complex in PBS induces much lower levels of neutralizing Abs [21], indicating that the adjuvant effect of Fc-FcR interaction may not play a substantial role in augmenting immunogenicity of gp120/654 complexes in mice. Rather, we postulate that in this *in vivo* model the enhanced immunogenicity of the complexes is mediated mainly by Fab-mediated activity that alters gp120 conformation and modulates exposure and stability of specific Ab epitope. Further studies to directly test this hypothesis are needed, but in support of the idea, we have demonstrated that this activity is not displayed by all anti-gp120 mAbs but it is unique to anti-CD4 binding site mAbs and is determined by the specificity of mAbs used to form the complexes [21, 22].

In summary, the capacity of HIV-1 gp120 to induce potent neutralizing anti-gp120 Ab responses was enhanced by a combination of select N-glycan removal and immune complex formation with a specific anti-gp120 mAb. Further improvements of this vaccine platform are needed to increase the breadth of the Ab responses in order to confer protection against a broader array of HIV-1 isolates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Immune complex vaccines made of HIV gp120 mutants elicit neutralizing Ab response
- The mutant complexes are more potent than the wild type complex
- Uncomplexed gp120 (mutant or wild type) elicits no neutralizing Ab response
- Immunogenicity of gp120 is improved by glycan removal and immune complex formation

Figure 1. Ab responses induced in C57BL/6 mice immunized with wild type (wt) or N448Q gp120s by DNA prime-protein boost vaccination

(A) Animals received DNA prime and protein boost of the same gp120 antigens (wt or N448Q mutant). Sera from the immunized mice were collected two weeks after the last immunization and pooled, diluted serially, and tested for Ab reactivity to gp120 in ELISA. Alkaline phosphatase-conjugated secondary Abs against total IgG, IgG1, IgG2a, IgG3, and IgA were used. Data showing Ig reactivity to wild type gp120 are presented, but comparable results were obtained with N448Q gp120 (not shown). (B) Sera from immunized mice were tested for reactivity to V3 peptide in ELISA. Secondary Abs against mouse total IgG were used. (C) Sera were tested for neutralization activity in the single-round infection assay with TZM-bl target cells and pseudovirus with homologous HIV-1 HXB-2 Env. Means and standard deviations were derived from duplicate wells. Results from one of two experiments are shown.

Figure 2. T cell responses of C57BL/6 mice immunized with wild type (wt) or N448Q gp120 by DNA prime-protein boost vaccination

Splenocytes collected two weeks after the last immunization were pooled and tested for proliferation and cytokine production. Lymphoproliferation and secretion of IFN- γ , IL-10 and IL-6 were measured after *in vitro* stimulation for 72 hours with wild type gp120 (1 μ g/ml) (A) and peptides 40, 41 or 18 (1 μ g/ml each) (B). Proliferation was measured by [3 H] thymidine uptake. The results are expressed as delta counts per minute (Δ cpm), which were calculated by subtracting cpm of antigen-stimulated cells with background cpm from cells treated with medium only. The amounts of cytokines in the supernatant were measured by multiplex analyte detection. The background responses of cells in medium alone, which

were below detection thresholds for most cytokines, were subtracted from the antigen-specific responses. Mean and SD values from triplicate wells are shown. The results are from one of two repeated experiments. *, $p < 0.05$ by Student's *t*-test.

Figure 3. Reactivity of serum Abs from mice immunized with wild type (wt) gp120/654 complex or mutant gp120/654 complexes

(A) gp120, V3 peptide, gp120 core, gp120 core+V3, and gp120 outer domain (OD1) were coated on ELISA plates and reacted with serially diluted sera. Sera were collected from mice immunized with gp120/654 complexes in MPL/DDA adjuvant two weeks after the last boost and pooled. Serum IgG reactivity was detected using alkaline phosphatase-conjugated anti-mouse IgG as a secondary Ab. Sera from animals injected with PBS in the presence of MPL/DDA adjuvant were used as control. (B) Epitope mapping was done using overlapping peptides representing the entire gp120 HXB-2 sequence. Sera from animals immunized with the complexes were reacted (at a 1:200 dilution) with pools of peptides (see Material and Methods) coated on ELISA plates at 10 µg/ml. Reactivity of control sera from animals receiving no antigen (PBS) was also shown. Mean and SD values are shown. *, $p < 0.01$. **, $p < 0.001$, compared to wt gp120/654 and calculated by Student's t test.

Figure 4. Neutralizing activity of sera from mice immunized with wild type (wt) or mutant gp120/654 complexes

(A) Sera collected after the final immunization were serially diluted and tested for neutralizing activity in the single-round infection assay with TZM-bl cells against pseudovirus with HIV-1 HXB-2 Env. Similar results were obtained with the infectious LAI isolate (see panel C). (B) To detect heterologous neutralization, sera were also tested for neutralization against pseudovirus expressing HIV-1 SF162 Env. (C) To measure the contribution of anti-V3 Abs to neutralization, serially diluted sera were pre-incubated with V3_{HXB-2} peptide and tested for neutralization assay against LAI. A scrambled control peptide was also tested and caused no change in neutralization (not shown). (D) To evaluate the contribution of Abs against C1 and C2 to neutralization, sera from N448Q gp120/654-immunized mice were pre-incubated with C1/C2 peptides and tested for neutralization assay against LAI. Representative results from one of two experiments are shown.

Figure 5. Alterations of mAb reactivity to V3, C2 and V2 epitopes by formation of gp120/654 immune complexes

Wild type (wt) or mutant gp120s, either alone or as complexes with mAb 654, were captured on ELISA plates with Ab to the C terminus of gp120, and reacted with biotinylated mAbs specific for V3 (694) (A), C2 (1006) (B) and V2 (697) (C). Binding of the biotinylated mAbs were determined using alkaline phosphatase-conjugated streptavidin. Means and standard deviation from duplicate wells are presented. Data from one of three repeated experiments are shown. The bars in each set represent ten-fold serial dilutions of each of the biotinylated mAbs tested. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. by Student's *t* test.

A

B

Figure 6. Susceptibility of V3 and C2 epitopes on gp120/654 complexes versus uncomplexed gp120 to cathepsins L, S, and D

Wild type (wt) and mutant gp120, either alone or in complex with mAb 654, were treated individually with cathepsins L, S or D for 1 hr, captured onto the microtiter plates with anti-C terminal Abs, and reacted with biotinylated mAbs specific for V3 (694) (left panel) or C2 (1006) (right panel). The binding of biotinylated mAbs were then determined by alkaline phosphatase-conjugated streptavidin. Means and standard deviation were calculated from duplicate wells. Results in (A) show the remaining mAb reactivity after digestion as compare to the respective undigested controls, which were normalized to 100%. **, $p < 0.01$; ***, $p < 0.001$ by Student's *t*-test. Examples of data showing the C2 reactivity (OD₄₀₅) to

wild type (wt) or N448Q gp120, as uncomplexed antigens or in immune complexes, with or without cathepsin L digestion are shown in (B).