Refocusing neutralizing antibody response by targeted dampening of an immunological epitope

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Refocusing Neutralizing Antibody Response by Targeted Dampening of an Immunodominant Epitope

Robert R. Garrity, Guus Rimmelzwaan, Anton Minassian, Wen-Po Tsai, George Lin, Jean-Jacques de Jong, Taap Goudsmit, and Peter L. Nara

Immunodominant epitopes are known to suppress a primary immune response to other antigenic determinants by a number of mechanisms. Many pathogens have used this strategy to subvert the immune response and may be a mechanism responsible for limited vaccine efficacies. HIV-1 vaccine efficacy appears to be complicated similarly by a limited, immunodominant, isolate-restricted immune response generally directed toward determinants in the third variable domain (V3) of the major envelope glycoprotein, gp120. To overcome this problem, we have investigated an approach based on masking the V3 domain through addition of N-linked carbohydrate and reduction in net positive charge. N-linked modified gp120s were expressed by recombinant vaccinia virus and used to immunize guinea pigs by infection and protein boosting. This modification resulted in variable site-specific glycosylation and antigenic dampening, without loss of gp120/CD4 binding or virus neutralization. Most importantly, V3 epitope dampening shifted the dominant type-specific neutralizing Ab response away from V3 to an epitope in the first variable domain (V1) of gp120. Interestingly, in the presence of V3 dampening V1 changes from an immunodominant non-neutralizing epitope to a primary neutralizing epitope with broader neutralizing properties. In addition, Ab responses were also observed to conserved domains in C1 and C5. These results suggest that selective epitope dampening can lead to qualitative shifts in the immune response resulting in second order neutralizing responses that may prove useful in the fine manipulation of the immune response and in the development of more broadly protective vaccines and therapeutic strategies. The Journal of Immunology, 1997, 159: 279–289.

A striking disparity exists between the small number of diseases currently prevented by available vaccines and the many infectious diseases for which no vaccine is available. Failure to develop many of these vaccines may be attributed to a lack of understanding of how to circumvent complex immune-evading strategies that have evolved to favor persistent pathogenic infections. One general scheme, possibly used by a wide array of viruses, bacteria, protozoa, parasitic agents, and cancer cells, involves chronic presentation of immunodominant epitopes (1–3). Characteristically, the Ags that are displayed exhibit hypervariable, redundant, immunodominant epitopes that serve to decoy or dysregulate the ability of the immune system to focus on more protective targets. A similar scenario has been advanced for HIV-1 (4–7). Hypervariation around an immunodominant determinant, the so-called V34 loop (8, 9), appears to facilitate immune escape (10–12) and is involved in both isolate-restricted neutralization and limited protection. Although a number of promising HIV-1 vaccine candidates protect against homologous infection (13, 14) and intrasubtype challenge (15, 16), further studies are required to determine whether any of these can protect against a divergent range of naturally occurring viral isolates.

In HIV-1 infection, the major target for neutralizing antibody (NAb) is the envelope glycoprotein complex gp120/41. The HIV-1 envelope precursor protein gp160 is cleaved into gp120 and gp41 protein subunits that noncovalently associate on the surface of the viral membrane. Computer analysis of sequences from seven viral isolates predicts five hypervariable (V1–V5) and five conserved (C1–C5) gp120 domains (17, 18). Isolate-restricted protection appears to correlate with a neutralizing immune response generally directed against one or more variable domains in gp120 (19–21). The principal neutralizing determinant, the third hypervariable domain (V3), has been implicated as the major target for NABs and the focus of a number of vaccine and therapeutic strategies (15, 22–24). V3 Abs are detected early at seroconversion, suggesting the immunodominant nature of this domain (25, 26). In addition, anti-V2-specific responses resulting from acute phase viral exposure may lead to suppression of a more protective immune response through clonal restriction (27, 28) and/or idiotypic dysregulation via mimicry of the variable Ig heavy chain III (29).

Serum fractionation studies suggest that Abs capable of neutralizing a more diverse array of HIV-1 isolates are generated, however, at lower titer and occur later during natural infection (30, 31).
These broadly neutralizing Abs are largely composed of group-specific Abs directed against non-V3 epitopes (32-34). Many of these NABs inhibit viral entry through the blocking of gp120 and its T cell receptor ligand, CD4 (35-37). Although group-specific Abs have broader virus-inhibiting properties, their neutralizing titer against independent isolates is usually lower than the V3-related, type-specific neutralizing titer against homologous virus (38-40). In addition, combinatorial library approaches used to select for gp120/CD4-blocking Abs also suggest Abs with broad neutralization profiles are rare in HIV-1 infection relative to those directed against V3 (41).

We have hypothesized that the V3 domain may be one of a number of immune decoy epitopes “decotopes” that keep the immune response somewhat fixed and limited by suppressing maturation of the later pool of broadly neutralizing, low-titered Abs. To test this hypothesis and overcome problems associated with V3-related isolate-restricted protection, we have attempted to epitope mask the V3 domain through site-directed targeting of N-linked (NL) carbohydrate and reduction in net positive charge. We report here on the effects of selective NL mutagenesis on glycossylation, antigenic dampening, CD4 binding, virus replication, and functional Ab responses.

Materials and Methods

N-linked mutagenesis, HIV-1 molecular clone construction, and sequencing

Four single and several combinations of NL consensus sequences were introduced into a 162-nucleotide fragment containing the V3 loop of the HXB2 isolate by PCR overlap extension mutagenesis. Briefly, complementarily primer pairs were synthesized carrying exact V3 sequences and a desired related isolate-restricted protection, we have attempted to epitope NL carbohydrate and reduction in net positive charge. We report number of immune decoy epitopes “decotopes” that keep the immune response somewhat fixed and limited by suppressing maturation of the later pool of broadly neutralizing, low-titered Abs. To test this hypothesis and overcome problems associated with V3-related isolate-restricted protection, we have attempted to epitope mask the V3 domain through site-directed targeting of N-linked (NL) carbohydrate and reduction in net positive charge. We report here on the effects of selective NL mutagenesis on glycossylation, antigenic dampening, CD4 binding, virus replication, and functional Ab responses.

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The combinant proteins were lentil lectin purified from infected HeLa NL carbohydrate (52). Assuming that all of the NL sequons in one-half of the apparent m.w. of gp120 is due to the presence of cells. The dominant product purified was gp160. Approximately from cysteine to cysteine of introduced NL mutations was confirmed by dideoxy sequence HXB2 gp120 are glycosylated by Western blot analysis (data not shown). Presence of the introduced NL sequons were glycosylated (Fig. 2). Receptor mapping whether added NL sequons were glycosylated (Fig. 2). No change in electrophoretic mobilities was sufficient to resolve gel mobility differences between WT and single NL recombinants 3 and 4 (Fig. 2A), suggesting glycosylation at these sites. No change in electrophoretic mobilities was observed between WT and single NL recombinants 1 and 2 (Fig. 2A and B). The extent of glycosylation in NL-1:2:3:4 was not determined due to the lack of reactivity with the V3-specific serologic reagents (Fig. 3). Glycosylation of the NL mutants were corroborated by a more complete V8 digestion, resulting in smaller V3 fragments with a m.w. of approximately 30,000, by removing SDS and denaturing in the presence of guanidine-HCl and urea (data not shown).

To confirm that NL carbohydrate was responsible for observed mobility differences, V8-digested fragments were treated with N-glycosidase F (Fig. 2, C, D, and E), which cleaves asparagine bound N-glycans. Upon removal of NL carbohydrate, all V8 digestion fragments shifted back to the same electrophoretic mobility as WT (Fig. 2, D and E), suggesting that differences in electrophoretic mobilities observed between WT- and NL-modified V8 digestion fragments 3 and 4 were due to NL carbohydrate added to their V3 loops, not to charge dampening introduced by site-directed amino acid changes.

**V3 loop can be glycosylated through N-linked sequon mutagenesis**

A V8 protease-N-glycosidase F gel shift assay was used to determine whether added NL sequons were glycosylated (Fig. 2). Receptor mapping whether added NL sequons were glycosylated (Fig. 2). No change in electrophoretic mobilities was sufficient to resolve gel mobility differences between WT and single NL recombinants 3 and 4 (Fig. 2A), suggesting glycosylation at these sites. No change in electrophoretic mobilities was observed between WT and single NL recombinants 1 and 2 (Fig. 2A and B). The extent of glycosylation in NL-1:2:3:4 was not determined due to the lack of reactivity with the V3-specific serologic reagents (Fig. 3). Glycosylation of the NL mutants were corroborated by a more complete V8 digestion, resulting in smaller V3 fragments with a m.w. of approximately 30,000, by removing SDS and denaturing in the presence of guanidine-HCl and urea (data not shown).

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**N-linked Mutations**

<table>
<thead>
<tr>
<th>Single</th>
<th>Combination</th>
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<tbody>
<tr>
<td>1</td>
<td>1:3</td>
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<tr>
<td>2</td>
<td>1:4</td>
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<tr>
<td>3</td>
<td>2:4</td>
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<tr>
<td>4</td>
<td>1:2:4</td>
</tr>
<tr>
<td></td>
<td>1:2:3:4</td>
</tr>
</tbody>
</table>

**Immunization and HIV-1 neutralization**

To test the neutralizing capacity of the antigenically altered immunogens, guinea pigs were immunized with two infectious doses of 5 × 10⁷ vaccinia plaque-forming units 4 wk apart. This was followed by subunit boosts of approximately 10 μg of recombinant protein in CFA. Two animals were used for each immunogen and all immunogens were tested. Antisera were collected by cardiac puncture 2 wk after each of three subunit boosts and tested for neutralizing activity against HIV-1 IIIB and HIV-1 MN in a quantitative infectivity assay (47).

**Results**

**Site-directed introduction of N-linked sequons into V3 domain of HXB2 gp120**

The minimum requirement for NL glycosylation is the amino acid sequon asparagine-X-threonine or serine, where X is any amino acid other than proline or aspartate (48). This consensus triplet was introduced into the V3 domain of gp120 from the HXB2 isolate (49) by PCR overlap extension mutagenesis (50). Figure 1 shows the positioning of NL sites labeled 1 through 4. NL sequons were inserted into areas of high positive charge, since these were considered important antigenic determinants within V3 and subsequent targets for immune dampening. Different combinations of NL sites were made, recombined into vaccinia virus, and expressed as recombinant gp160 in HeLa cells. Full length gp160 was expressed to preserve potential neutralizing epitopes in gp41 (51). Glycosylation and proteolytic processing of gp160 into gp120 and gp41 were confirmed by Western blot analysis (data not shown). Presence of the introduced NL mutations was confirmed by dideoxy sequence analysis.

**V3 loop can be glycosylated through N-linked sequon mutagenesis**

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FIGURE 2. V8 protease gel shift assay to determine presence of introduced NL carbohydrate on recombinant gp160. A, V8 protease-treated samples include undigested (Uncut) WT gp160; V8-digested gp160− vaccinia recombinant control (C); and V8-digested gp160s labeled as wild-type (WT) and NL mutants (1, 3, and 4). A mix of V3-specific mAbs 9284, F58/H3, and H902 was used to visualize fragments. B, Samples labeled as in A plus gp160 from NL mutant 2. Blot was incubated in anti-RP135 and visualized as described in Materials and Methods. NL gp160 V8 protease digests were treated with N-glycosidase F. C, N-glycosidase F-treated samples include control (C), untreated WT gp160; and N-glycosidase F-treated WT gp160 (N-GF). D, V8-digested, N-glycosidase F-treated samples as in A. E, V8-digested, N-glycosidase F-treated samples as in B.

FIGURE 3. Antigenicity profiles of WT and N-linked gp160s. Western blot panels of gp160s visualized with control Ab specific for gp41 (top panel) or HIV-1 IIIB V3-specific mAbs F58/H3, 0.5B, 9284, H902, and anti-RP135 (lower five panels). Recombinant proteins are designated as wild-type (WT) and numbered by position of NL mutation in V3. Abs map to boxed positions underneath V3 loop. Dashed line indicates ambiguity in mapped domain.
FIGURE 4. Transfection of NL molecular clones into SupTl cells. NL V3 sequences were introduced into plasmids carrying full length HIV-1 genomes, electroporated into SupTl cells, and scored for p24 activity over a 2-wk period. NL molecular clones tested were: WT (○); NL-1 (●); NL-2 (□); NL-3 (■); NL-4 (▲); NL-1:3 (●); NL-1:4 (△); NL-2:4 (▲); NL-1:2:4 (▼); and NL-1:2:3:4 (▼).

Table I. Syncytia-inducing and infectious capacity of NL molecular clones

<table>
<thead>
<tr>
<th>NL Virus</th>
<th>Syncytia in SupTl Cells (Days Post-Transfection)</th>
<th>p24 Production After Cellfree Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+++ 7 14</td>
<td>+++ 4 b</td>
</tr>
<tr>
<td>1</td>
<td>+ 7</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+ 7</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+ 7</td>
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<td>4</td>
<td>+++ 7 14</td>
<td>+++ 4 b</td>
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<tr>
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</tr>
<tr>
<td>1:4</td>
<td>- 7</td>
<td>ND</td>
</tr>
<tr>
<td>2:4</td>
<td>- 7</td>
<td>ND</td>
</tr>
<tr>
<td>1:2:4</td>
<td>- 7</td>
<td>ND</td>
</tr>
<tr>
<td>1:2:3:4-</td>
<td>- 7</td>
<td>ND</td>
</tr>
</tbody>
</table>

*SupTl cells infected with cellfree supernatants from 4; 7; and 14-day post-transfections of NL molecular clones and scored for presence of syncytia (†) and p24 positivity.

b For p24 assay >3 SD above backgrounds.

CD4 binding index was calculated from two quantitative ELISA determinations of lentil lectin-purified NL-gp120/160s by Ag capture with Ab to envelope glycoprotein and Ag capture with sCD4.

Table II. CD4 binding measured in Ag capture ELISA

<table>
<thead>
<tr>
<th>NL protein</th>
<th>gp 120/160 Capture (ng/ml)</th>
<th>CD4 Capture (ng/ml)</th>
<th>CD4 Binding Index a</th>
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<tbody>
<tr>
<td>WT</td>
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<td>8.3</td>
<td>1.00</td>
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<tr>
<td>1</td>
<td>51.5</td>
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<td>48.1</td>
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<td>46.0</td>
<td>13.0</td>
<td>1.26</td>
</tr>
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</table>

a CD4 binding index = [(CD4 capture)/gp120/160 capture] NL protein

These profiles were confirmed by Ag capture ELISA, live cell immunofluorescence of recombinant vaccinia virus-infected cells, and dot blot analysis (data not shown).

The effects of NL mutations on HIV-1 infectivity was determined by introducing these mutations into plasmids carrying full length HIV-1 genomes, transfecting them into SupTl cells, and determining virus replication by the presence of soluble core protein p24 (Fig. 4). Molecular clone NL-4 showed p24 kinetics similar to WT virus, suggesting that this mutation is tolerated and does not interfere with viral replication. Other single NL substitutions 1, 2, and 3, as well as combination NL mutant 1:3, also showed positive increases in p24. To confirm viral replication, p24 cellfree supernatants were collected at day 4, 7, and 14, incubated with fresh, uninfected SupTl cells, and assayed for syncytia production (Table I). NL-4 matched the WT pattern, showing the earliest and strongest indications of virus growth.
ANTIGENIC ATTENUATION BY SITE-DIRECTED MUTAGENESIS

**Table III. Neutralizing capacity of WT and NL 1:2:3:4 antisera against homologous virus HIV-1 IllB and MN**

<table>
<thead>
<tr>
<th>Antisera</th>
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<th>IllB</th>
<th>MN</th>
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<tbody>
<tr>
<td>WT</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>NL-1:2:3:4</td>
<td>1</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>256</td>
<td>8</td>
</tr>
</tbody>
</table>

*Neutralization titers of WT and NL 1:2:3:4 antisera against HIV-1 IllB and MN. Antisera collected after priming by two infections with vaccinia recombinants and three recombinant protein boosts. Neutralization titers given as reciprocal dilution that neutralizes 90% of infecting virus.*

were tested for Ab reactivity against a limited set of overlapping peptides, representing gp120 constant (C) and variable (V) domains (Fig. 6). Background values are shown in negative control antisera raised by immunization with a recombinant vaccinia virus. As previously observed in other gp120/160 immunogens, WT antiserum at a dilution of 1/1000 showed significant binding to a V3 peptide containing amino acids from the middle of the V3 loop (i.e., amino acids 302-321). Interestingly, Ab binding changes within V3 appear to correlate with the positioning of a specific NL mutation. When the middle of the V3 loop is charged dampered at site N1-2, significant Ab reactivities occur relative to a WT increase on both the N- and C-terminal sides of V3. Ab reactivity to the N-terminal side of the loop increases when the C-terminal side of V3 is glycosylated (NL-4). When NL-sites 2 and 4 were introduced, Ab reactivities to the N-terminal side increased. In contrast to V3, the highest Ab reactivities consistently observed in all antisera were to two overlapping peptides, representing the first variable region of gp120.

Different Ab reactivities between WT- and NL-modified antisera were observed outside of V3 as well. Ab reactivities to C1 were increased in antisera NL-2, NL-4, NL-2:4, and NL-1:2:4. Ab reactivities to C5 were increased in NL-1, NL-4, and NL-1:2:4.

Solution phase competition assays were used to map the neutralizing activities of WT and NL-1:2:3:4 antisera. As expected, WT neutralizing activity could be competitively inhibited by a homologous V3-specific peptide (Fig. 7A). This peptide had no effect on neutralizing activity in antisera NL-1:2:3:4. In contrast, a peptide homologous to the V3 loop of NL-1:2:3:4 did not competitively inhibit activity in antisera NL-1:2:3:4 (Fig. 7B). Screening all ELISA-reactive peptides against antisera NL-1:2:3:4 revealed a unique, non-V3 neutralizing activity that mapped to the first variable domain of gp120 (Fig. 7B). This is in marked contrast to WT antiserum, which, despite equivalent V1 ELISA binding reactivity (Fig. 5), was not neutralizing.

To rule out any V1 neutralizing activity in WT antiserum, WT and NL-1:2:3:4 antisera were mixed in equivalent volumes and assayed for neutralization over a range of dilutions (Fig. 8A). This mixed antiserum was compared with the neutralizing activity of each of the two individual antisera. When mixed together, WT and NL-1:2:3:4 antisera appear to have an increased or additive effect in neutralizing homologous virus, suggesting that V1 and V3 NAb act in an independent manner. This hypothesis was corroborated with V1 peptide competition studies (Fig. 8B) showing that V1 NAb can be competed out in the presence of V3 NAb.

**Discussion**

The major goal of this study was to determine whether an attenuated immune response to V3 would result in a shift in the NAb
response to less dominant non-V3 epitopes that provide broader protection. Site-directed NL natagenesis was investigated as a mechanism to selectively glycosylate and charge dampen immunodominant epitopes within the third hypervariable domain of HIV-1 gp120. The V3 domain was targeted for study because of its hypothesized role in decoying the immune response through immunodominant presentation and immune dysregulation.

We hypothesized that negative selection against NL carbohydrate, occurring in the major neutralizing determinant of the V3 loop, is part of a viral adaptation to maintain immune visibility. The beta-turn secondary conformation predicted for the V3 domain (54) makes an ideal context for NL carbohydrate addition (55). However, except for a consensus NL sequon found on the amino terminus of V3, it is extremely rare to find an NL sequon in this domain. Selective absence of NL carbohydrate in V3 is surprising in lieu of the infidelity of reverse transcriptase and the ability of HIV-1 to tolerate hypervariation in this domain. We believe the lack of potentially shielding carbohydrate, consistent selection of charged amino acids, and high degree of rotational freedom (56) all favor immunodominance of the V3 epitope. In addition to neutralizing B cell epitopes, V3 encodes helper T cell, cytotoxic T cell and Ab-dependent cellular cytotoxicity epitopes (57). As some form of V3 structure is necessary for viral infectivity (58, 59), it is somewhat paradoxical that HIV-1 would have coevolved V3 to facilitate immune escape through hypervariation, while focusing a large part of the immune repertoire onto a functional domain. An equally likely adaptation would have been to evolve the V3 epitope away from immune recognition and more toward self-mimicry, as has been reported for a number of domains on gp120/41 (60, 61). Since viruses were observed to replicate in the

![Anti-gp120 Abs of NL immune sera measured by ELISA. BH10 gp120 peptides were obtained from Harvey Holmes, U.K. Medical Research Council AIDS Directed Program Reagent Repository. Peptides are sequentially numbered according to amino acids as indicated in the Human Retroviruses and AIDS database. Serum samples were diluted 1:1000, added to peptide prebound to plate, and visualized with anti-guinea pig IgG conjugated to peroxidase. Peptides carrying designated amino acids and gp120 constant and variable domains are listed on the x-axis. Values on the y-axis are in OD units at 495 nm. Values represent geometric means ± SEM, n = 4. Guinea pig prebleed represents background minus vaccinia virus. All other NL antisera are represented based on the site of NL mutation in V3.](image-url)
FIGURE 7. Neutralization-competition assay. Equivalent dilutions of WT and NL-1:2:3:4 antisera giving approximately 90% inhibition of HIV-1 IIIB (y-axis) in the presence of increasing concentrations of competitor V3 and V1 peptides (x-axis). Values represent geometric means ± SEM, n = 4. Control antiserum was raised against vaccinia recombinant minus gp160. Concentrations of competitor peptides are given in pg/ml. Control antiserum, ▲; WT antiserum, ■; and NL-1:2:3:4 antiserum, ○. Amino acid sequences of peptide competitors are: A, WT V3 peptide NNTRKRIRIQRGPGRAFVTIGKIC; B, NL-1:2:3 V3 peptide NNTRKNISIQNGSGRANVTIGKIC; C, V1-1 peptide 131-151 CTDLKNDTNTNSSSCRMIMEK; and D, V1-2 peptide 142-161 SSSCRMIMEKGEIKNCSFNI.

presence of introduced NL sequons, it is less likely that the absence of naturally occurring NL carbohydrate in V3 plays as important a role in infectivity as it plays in the role of V3 as an immunodominant decoy.

Not all parts of the V3 loop of the IIIB isolate are accessible to NL glycosylation. Translocation of gp160 into the endoplasmic reticulum results in folding, potential dimerization, addition of NL carbohydrate, transport to the golgi, NL carbohydrate trimming, proteolytic processing, and finally, localization at the surface membrane. NL glycosylation requires access to amino acids on the nascently folded molecule. Even though all of the naturally occurring sites in gp120 are glycosylated, and V3 is in an appropriate context for NL glycosylation, the outcome as to whether any of the introduced NL sequons would be accessible for glycosylation was uncertain. Therefore, NL mutagenesis was coupled to a strategy involving site-directed charge dampening, since charge preservation is an apparent V3 adaptation and humoral recognition component. To do this, positively charged arginines were changed to polar amino acids, which was intended to keep the local conformation in a hydrophilic state and preserve external access to this site for glycosylation. There are other conformational requirements, however, in addition to primary NL sequon mutagenesis that are necessary for glycosylation to occur, since only two of the four introduced NL sequons were glycosylated. The two nonglycosylated sites were localized near the N-terminal side and center of V3. Primary amino acid changes resulting from site-directed mutagenesis have created either an incorrect context for glycosylation or glycosylation at the naturally occurring NL site slightly amino terminal of NL-1 and NL-2 (Fig. 1), which is known to interfere with Ab binding (62), is a preferred site for NL carbohydrate, and sterically interferes with additional glycosylation in this area. We are generating more NL-scanning mutants in V3 to better determine correlates required for NL glycosylation.

Despite the sequence variation in the gp120/CD4 binding domain, HIV-1 entry via the CD4 receptor suggests this domain is potentially conserved among viral isolates. One of the most broadly neutralizing Abs to date has been mapped to this domain (41). Preservation of this site in our NL immunogens makes this a potential candidate for broader NAb. It appears, however, that other hypervariable epitopes may be involved in interfering with recognition of this domain. Deletion of both the V1 and V3 domains has been reported to improve Ab recognition of the gp120/CD binding region (63). Since V1 and V3 flank a conserved region in C2 that is part of the discontinuous
FIGURE 8. Additivity characteristics of V3 and V1 NAb. A, Reciprocal serum dilutions (x-axis) of WT, NL:1:2:3:4, and an equivalent mixture of both (Mix) were assayed for % neutralization (y-axis) of homologous HIV-1 IIIb virus. Starting dilutions were approximated to give 90% virus neutralization. WT antisera, ■; NL A1:2:3:4 antisera, ○; and equivalent mix of WT and NL-1:2:3:4 antisera, ▲. B, V1 peptide competition of WT and mixed antisera. Increasing concentrations of V1 peptide in ng/ml (x-axis) were added as competitor peptide to a 1:256 dilution of 1:1 mix of WT and NL-1:2:3:4 antisera. A competition index (y-axis) was calculated from the ratio of % neutralization in the absence of peptide to % neutralization in the presence of peptide. WT antisera, ■; and mixed antisera, ▲.

CD4 binding domain (64, 65), it is interesting to speculate that the immunodominance of V1 as well as V3 interferes with Ab maturation in this area. Our findings are consistent with other studies characterizing V1 as an immunodominant epitope that is rarely neutralizing (66, 67). In the presence of V3 dampening, V1 becomes a primary neutralizing epitope with broader neutralizing properties. We believe our study is the first to demonstrate a V1-specific NAb response raised by a recombinant immunogen.

Additional Ab binding changes observed in a number of the NL antisera suggest that sequential site-directed NL mutagenesis results in an intradomain refocusing of the Ab response within V3. A characteristic Ab response was observed to the middle of the V3 loop in antisera raised by the WT immunogen. This middle domain carries the GPGR motif, characteristic of North American and European B-subtype viruses, and a potent target for NAb (68). Although site-directed NL alterations in GPGR did not result in glycosylation, it did induce an increased Ab binding response to the N- and C-terminal sides of V3 (Fig. 6, NL-2). This altered immunogenicity could be due to localized charge dampening, resulting from an arginine to asparagine change. Similarly, an N-linked mutation on the C-terminal side of V3, in NL-4, which was glycosylated, resulted in an increased response to the N-terminal side of V3 (Fig. 6, NL-4). Immune dampening of the middle and C-terminal side of V3, NL-2:4, results in an enhanced Ab binding response in the N-terminal side. Dampening NL sites 1, 2, and 4 apparently leaves enough of the C-terminal side of V3 to be antigenic, since an Ab response to the C-terminal side increases. Glycosylation at site NL-3 may shield a large part of V3 and sterically inhibit Ab binding, since no shift to another part of the loop was observed in antisera raised to this immunogen. Ab binding differences were also observed in conserved domains, most notably in the C1 domain. Although the significance of enhanced C1 Ab reactivities correlating with an NL-2 site mutation are as yet undefined, it is interesting to note that the C1 domain has been reported to be a conserved immunorecessive neutralizing epitope (69, 70).
A consideration in any mutagenesis strategy is the conformational effect that any amino acid change can have on a distant site. Although antigenic differences in V3 Ab binding were observed, most NL mutations correlate the loss of V3-specific Ab binding with the presence of an NL mutation somewhere in the Ab binding domain. This leads to uncertainty as to whether loss of Ab binding is due to amino acids changes resulting from NL mutagenesis, or whether lack of Ab binding is due to the steric influence of carbohydrate. Although the mechanism involved in induction of V1-mediated neutralization in antisera NL-1:2:3:4 is uncertain, the relevance of a NAb shift from V3 to a qualitatively different V1 Ab response raised by the recombinant subunit immunogen is underscored by the fact that it neutralizes infectious virus. This makes it less likely that changes induced by NL mutagenesis affected conformation at a distant site on the Ag and are nonrelevant, since for virus neutralization to occur, similar antigenic targets have to be available on the virus. Another possibility is that attenuation or dampening of an immunodominant anti-V3 response has allowed V1 Abs to undergo a more complete polyclonal selection, and affinity maturation as has been reported in the selective down-regulation of immunodominant epitopes on multistep mutants (71, 72). Regardless of the mechanism, as stated previously, the V1 domain is also immunodominant, more variable than V3, and therefore an unlikely target for broadly neutralizing Ab. We are in the process of dampening an immune response to V1 to establish whether a further gradient or hierarchy of neutralizing responses can be revealed. In addition, we have observed similar immunodominant responses in gp41 (73) and are applying this strategy to refocusing the response to less dominant broadly neutralizing epitopes.

B cell dysfunction was one of the earliest abnormalities reported in progressive HIV-1 disease (74). Early B cell activation can be followed by a disproportionate increase in activated and immature B cells (75). Our model suggests that part of the B cell dysfunction is due to an early immune response to immunodominant decay epitopes such as the V3 loop. This strategy may be central to viral persistence in a hostile immune environment since it can delay and suppress the kinetics of the broadly neutralizing pool of non-V3 Abs. Subsequent immune escape can occur through changes in V3 or outside in other variable epitopes. The resultant effect is an apparent fixing or freezing of the B cell response by escape variants that induce "original antigenic sin" by cross-reacting and stimulating the dominant B cell clones (76). This early freezing or imprinting is evidenced by the binding affinities and neutralization titers that autologous kinetics of the broadly neutralizing pool of non-V3 Abs. Subsequent development of more broadly protective vaccines and therapeutics.

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