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Refocusing Neutralizing Antibody Response by Targeted Dampening of an Immunodominant Epitope[1]

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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 V3[4] 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 determinants 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-V3-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).

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[4] Abbreviations used in this paper: V1–V5, hypervariable domains in gp120; gp, glycoprotein; NAb, neutralizing Ab; C1–C5, conserved domains in gp120; NL, asparagine-linkage for carbohydrate; WT, wild type; sCD4, soluble CD4.

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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 glycosylation, 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 sequons were introduced into a 162-nucleotide fragment containing the V3 loop of the HXB2 isolate by PCR overlap extension mutagenesis. Briefly, complementary primer pairs were synthesized carrying exact V3 sequences and a desired NL mutation. Two partially complementary halves of the V3 region of HXB2 were amplified in two separate reactions. Reaction 1 included 5′ primer AAT AGTACAGCTGAACACAT (nucleotides 7075–7094), which overlapped a unique, naturally occurring *Pvu*II site just proximal to the N-terminal cysteine in the V3 loop; and a 3′ primer including an *N*-linked, in-frame V3-domain mutation. Reaction 2 included a 5′ inverted complement to the 3′ primer used in reaction 1, and a 3′ primer (5′-CCATTTTGCTCTAGAAATGTTACA) encoding a silent *Xba*I mutation just proximal to the C-terminal cysteine of the V3 loop (nucleotides 7212–7237). Both reactions were electrophoresed on 1% agarose, excised from the gel, and the gel slices were centrifuged for 15 min in 1.5-ml Costar tubes (Amicon, Beverly, MA). Three microliters of supernatants from reactions 1 and 2, the 5′ primer from reaction 1, and the 3′ primer from reaction 2 were used as substrate and primers in a third PCR amplification. The resulting amplified product included a *Pvu*II to *Xba*I V3 loop containing fragment carrying one or more NL mutations. 5′ *N*-linked primers used to generate single NL mutations include: (NL-1), 5′-ATACAAGAAAAAA CATCAGTATCCAGAGAG; (NL-2), 5′-TCCGTATCCAGAATGGAT CAGGGAGAGCAT; (NL-3), 5′-CCAGGGAGAGCAAATGTTACAAT AGG; and (NL-4), 5′-ATAGGAAATATGAGTCAAGCACATTGT. 5′-primers used to generate combination NL mutations were: NL-1:4, include primer NL-1 on substrate NL-4; NL-2:4, include primer NL-2 on substrate NL-4; NL-1:3, include primer NL-1 on substrate NL-3; NL-1:2:4, include primer NL-1:2, 5′-TCAGTATCCAGAATGGATCAGGGAGAGCAT, on combination substrate NL-1:4; and NL-1:2:3:4, include primer NL-2:3, 5′-TCAGGGAGAGCAAATGTTACAATAGG, used on combination substrate NL-1:2:4. Modified NL-V3 sequences were also used to generate complete HIV-1 genomes as described previously (42). These plasmids were CsCl purified and electroporated into SupT1 cells to generate HIV-1 NL-modified viruses. In addition, identical NL-modified V3 sequences were subcloned into vaccinia expression plasmid pSC65 (43). All NL substitutions were confirmed by dideoxy sequence analysis.

Generation of recombinant vaccinia virus

Recombinant vaccinia viruses were selected as described by Earl and Moss (43). Briefly, 1×10^6 CV-1 cells were infected with vaccinia viral strain WR at a multiplicity of infection of 0.05. These cells were transfected 2 h after infection with 20 μg of each of the NL-rgp160 vaccinia expression plasmids using Lipofectin reagents (Life Technologies, Rockville, MD) as described by the manufacturer. Infected cells were pelleted, resuspended in 0.5 ml MEM (Life Technologies), freeze thawed three times, and serially diluted to give single plaques in subsequent infection of human TK^- cells. These cells were overlaid in 2% soft agar containing 2× MEM and 0.25 μg/ml deoxybromouridine (Sigma Chemical Co., St. Louis, MO) and incubated at 37° in 5% CO_2 for 48 h. A second soft agar overlay containing 1/200 volume of 4% Xgal, 5′-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside; (Boehringer Mannheim, Indianapolis, IN) and 1/100 volume 10 mg/ml neutral red (Life Technologies) was used to select for recombinant candidates after overnight incubation. Blue plaques were selected and resuspended in 0.5 ml MEM, then processed through two additional rounds of plaque purification. A recombinant $gp160^-$ vaccinia control was generated through recombination with pSC65 and TK^- B-galactosidase$^+$ selection. NL sequence modifications were confirmed through PCR dideoxy sequencing of NL-modified insert. NL rgp160s were Western blotted and visualized with an Ab specific for gp41 (Chessie 8, AIDS Research and Reference Reagent Program, Rockville, MD) as described below.

V8 protease N-glycosidase F gel shift assay

Recombinant proteins were lentil lectin affinity purified (44) and digested with V8 protease (Boehringer Mannheim) in 25 mM ammonium carbonate, pH 7.8, 0.05% SDS, and 0.005% β-mercaptoethanol for 6 h at 28°C. Digests were electrophoresed on 7.5% SDS-PAGE; transferred to nitrocellulose at 65 V for 45 min in 25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS; blocked in 10% dried milk and PBS in 0.01% Tween-20 (PBS-T) for 30 min at room temperature; incubated with primary V3-specific Ab for 2 h at 4°C; rinsed four times in PBS-T; incubated with secondary Ab, goat anti-mouse peroxidase (1:2000) and/or anti-rabbit peroxidase (1:3000) in 1% milk/PBS-T for 1 h at room temperature; developed with enhanced chemiluminescence detection reagents (ECL; Amersham, Arlington Heights, IL); exposed on Kodak XAR film. V8 digests treated with *N*-glycosidase F (Boehringer Mannheim) in 100 mM sodium phosphate, pH 7.2, 25 mM EDTA, 0.1% SDS, 1% β-mercaptoethanol, and 1% Nonidet P-40 at 37°C overnight; and Western blotted as described above.

Antigenicity profiles of NL-rgp160

Antigenicity profiles of wild-type (WT) and *N*-linked rgp160s were visualized by Western blot analysis using Abs for gp41, Chessie 8; HIV-1 IIIB V3-specific mAbs F58/H3, H902 (AIDS Research and Reference Reagent Program, Rockville, MD), 9284 (45), 0.5 β (46); and rabbit polyclonal V3-specific antisera, anti-RP135 (kindly provided by Dr. Al Profy, Repligen Corp., Cambridge, MA).

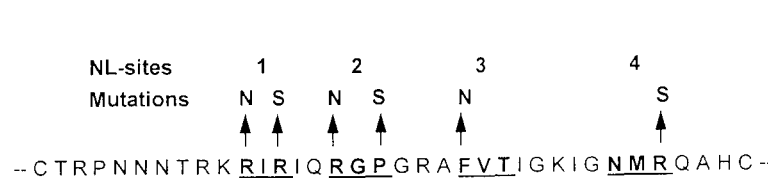
CD4, gp120, and p24 ELISA

Gp120 concentrations were determined by Ag capture ELISA with Ab to the C terminus of gp120 (anti-carboxy-terminal sheep polyclonal Ab, International Enzymes, Fallbrook, CA). Coating Ab was diluted 1:500 in 0.1 M sodium bicarbonate buffer, pH 9.5, and 100 μl adsorbed to microtiter wells (Immulon II, Dynatech Ltd., Chantilly, VA) overnight at 4°C. Plates were washed three times in PBS-0.5% Tween-20 (PBS-T5), then blocked in PBS-T5 plus 1% BSA for 30 min at room temperature. Lentil lectin purified recombinant protein was serially diluted 10-fold in PBS-T5, and 100 μl/sample was captured for 2 h at room temperature. Heat-inactivated pooled human HIV-1 serum served as the primary binding Ab. This was diluted 1:1000, and 100 μl/well was adsorbed for 1 h at room temperature, washed three times in PBS-T5, and blocked in PBS-T5 plus 10% normal goat serum (Life Technologies) for 1 h at room temperature. Anti-human horseradish peroxidase conjugate and substrate (Boehringer Mannheim) was added as described by the manufacturer and visualized at 450 nm. Concentrations were determined by comparison with a standard curve generated from commercially available gp120 (Celltech, Slough, Berkshire, U.K.). CD4 binding was determined by first adsorbing 100 μl of 7.5 μg/ml CD4 in 0.1 M Tris, pH 7.5, per well of Immulon II plate, washing, blocking, incubating with the NL sample, and visualizing as described above. A relative CD4 binding index was determined by taking the ratio of each of two capture methods (i.e., soluble CD4 (sCD4) and C-terminal anti-gp120) for a specific NL-recombinant protein over a similar ratio determined for WT gp120/160. The CD4 binding index for WT was defined as one, and the binding index for each individual NL protein was compared with this value.

Viral infectivity was measured by p24 ELISA, done with Dupont NEK-060B kits as described by the manufacturer (Wilmington, DE).

Transfection and HIV-1 detection

Transfection of HIV-1 molecular clones into SupT1 cells was described previously (42). Virus production was initially determined by p24 ELISA on culture supernatants. Virus infection was confirmed by taking cellfree



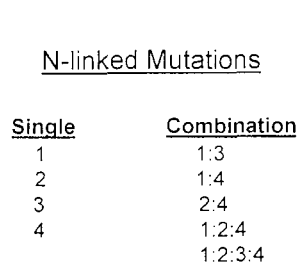


FIGURE 1. *N*-linked glycosylation sites introduced into HXB2 V3 loop. *A*, Amino acid sequence from cysteine to cysteine of HXB2 V3 loop. Underlined triplet amino acids indicate sites where amino acid changes were made. Arrows point away from WT sequence to amino acid change that resulted in NL glycosylation consensus sequon Asn-X-Thr or Ser. Numbers 1–4 designate position of NL sites. *B*, Vaccinia virus gp160 recombinants and full length HIV-1 molecular clones were constructed carrying each of four single and various combination NL V3 mutations. Numbers indicate sites and combination mutants made. Control vaccinia virus was generated by recombining vaccinia expression plasmid minus gp160 into vaccinia virus genome.

supernatants and infecting SupT1, H9, and CemSS cells and was scored for syncytia in an infectivity assay as described below.

Immunization and HIV-1 neutralization

To test the neutralizing capacity of the antigenically altered immunogens, guinea pigs were immunized with two infectious doses of 5×10^7 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 aspargine-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

A V8 protease-*N*-glycosidase F gel shift assay was used to determine whether added NL sequons were glycosylated (Fig. 2). Recombinant proteins were lentil lectin purified from infected HeLa cells. The dominant product purified was gp160. Approximately one-half of the apparent m.w. of gp120 is due to the presence of NL carbohydrate (52). Assuming that all of the NL sequons in HXB2 gp120 are glycosylated (53), then on average glycosylation of one additional site would increase the apparent m.w. by 2,000 to 2,500. NL V3-modified and WT gp120/160s were digested with V8 protease and analyzed by SDS-PAGE to identify glycosylation of the introduced site(s). To visualize the V8 digestion fragments, three V3 monoclonals and one V3-specific polyclonal antisera were used. A mixture of the three monoclonals resulted in optimal visualization of NL fragments 1, 3, and 4 (Fig. 2*A*). Polyclonal antisera RP135 was used by itself because this was found to be the most sensitive way to resolve V8 digestion fragment V3 NL-2 (Fig. 2*B*). V8 protease cleavage in the presence of SDS resulted in incomplete V3 digestion fragments with a m.w. of ~70,000. This was sufficient to resolve gel mobility differences between WT and single NL recombinants 3 and 4 (Fig. 2*A*), suggesting glycosylation at these sites. No change in electrophoretic mobilities was observed between WT and NL recombinants 1 and 2 (Fig. 2, *A* 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.

NL mutagenesis dampens V3 antigenicity without affecting CD4 binding or homologous neutralization

Western blot analysis using five V3-specific Abs was done to determine the effects NL mutagenesis had on altering antigenicity of the V3 domain (Fig. 3). These generally correlated the loss of V3-specific Ab binding with the presence of an NL mutation in that specific Ab binding domain. As noted above, NL-1:2:3:4 failed to bind to any of the V3-specific Abs used in this study.

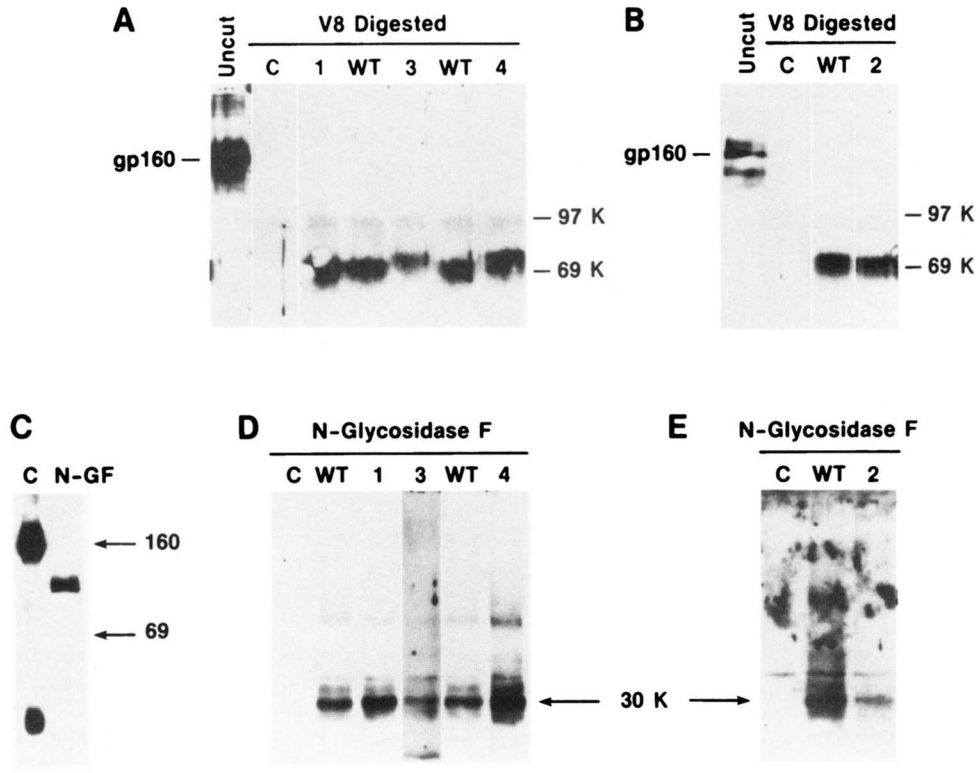


FIGURE 2. V8 protease gel shift assay to determine presence of introduced NL carbohydrate on recombinant gp160. *A*, V8 protease-treated samples include undigested (Uncut) WT gp160; V8-digested gp160$^-$ vaccinia recombinant control (C); and V8-digested gp160s labeled as wild-type (WT) and NL mutants (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*.

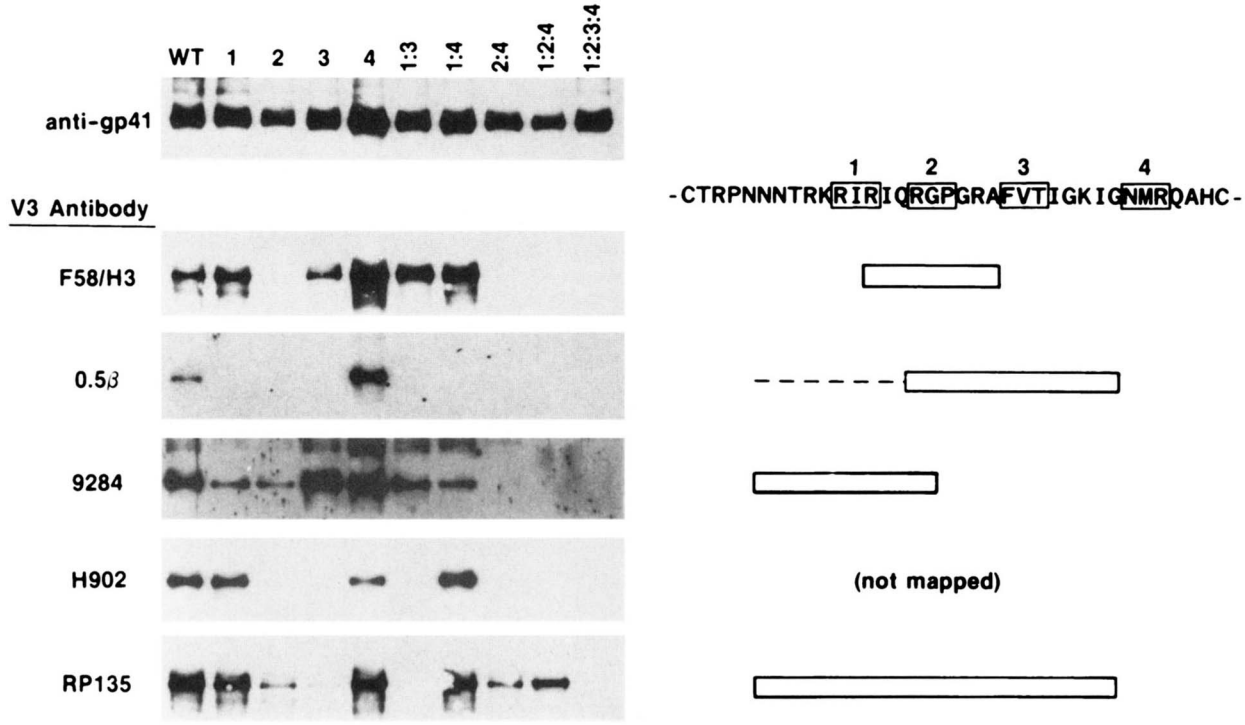


FIGURE 3. Antigenicity profiles of WT and *N*-linked gp160s. Western blot panels of gp160s visualized with control Ab specific for gp41 (*top panel*) or HIV-1 IIIB V3-specific mAbs F58/H3, 0.5*B*, 9284, H902, and anti-RP135 (*lower five panels*). Recombinant proteins are designated as wild-type (WT) and numbered by position of NL mutation in V3. Abs map to boxed positions underneath V3 loop. Dashed line indicates ambiguity in mapped domain.

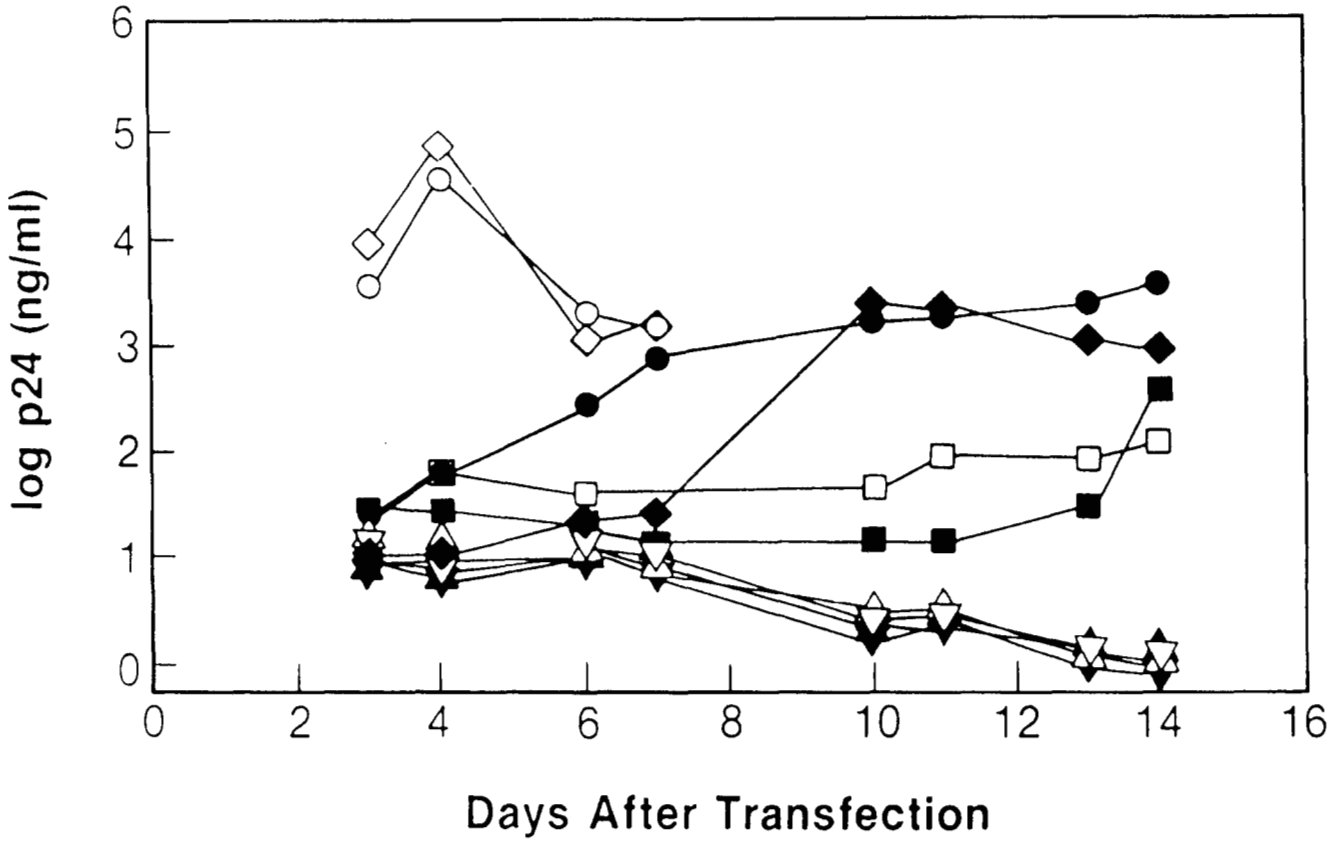


FIGURE 4. Transfection of NL molecular clones into SupT1 cells. NL V3 sequences were introduced into plasmids carrying full length HIV-1 genomes, electroporated into SupT1 cells, and scored for p24 activity over a 2-wk period. NL molecular clones tested were: WT (○); NL-1 (●); NL-2 (□); NL-3 (■); NL-4 (◇); 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*[a]

NL Virus	Syncytia in SupT1 Cells (Days Post-Transfection) 4	7	14	p24 Production After Cellfree Infection
WT	+++	+++	+++	+[b]
1	+	++	+	+
2	−	−	+	+
3	−	−	++	+
4	+++	+++	+++	+
1:3	−	++	+++	+
1:4	−	−	−	ND
2:4	−	−	−	ND
1:2:4	−	−	−	ND
1:2:3:4−	−	−	ND	

[a] SupT1 cells infected with cellfree supernatants from 4; 7; and 14-day post-tranfections of NL molecular clones and scored for presence of syncytia (±) and p24 positivity.

[b] + for p24 assay >3 SD above backgrounds.

Table II. *CD4 binding measured in Ag capture ELISA*

NL protein	gp 120/160 Capture (ng/ml)	CD4 Capture (ng/ml)	CD4 Binding Index[a]
WT	36.9	8.3	1.00
1	51.5	11.4	0.98
2	48.1	12.0	1.11
3	11.2	3.9	1.55
4	48.7	8.5	0.78
1:3	ND	ND	ND
1:4	44.9	10.6	1.05
2:4	47.1	12.0	1.13
1:2:4	52.1	11.4	0.97
1:2:3:4	46.0	13.0	1.26

[a] CD4 binding index $= \frac{[(\text{CD4 capture})/(\text{gp120/160 capture})]\ \text{NL protein}}{[(\text{CD4 capture})/(\text{gp120/160 capture})]\ \text{WT protein}}$

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.

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 SupT1 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 SupT1 cells, and assayed for syncytia production (Table I). NL-4 matched the WT pattern, showing the earliest and strongest indications of virus growth.

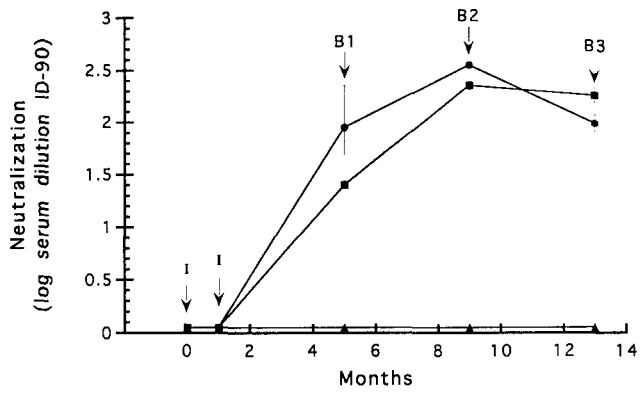


FIGURE 5. Immunization regimen. Guinea pigs were immunized with two infectious doses 4 wk apart of 10^7 plaque-forming units of recombinant vaccinia virus. These were followed by 3 subunit boosts of 10 μg each of matching gp120/160 recombinant protein. Antisera were collected by cardiac puncture and assayed for HIV-1 inhibition in a quantitative infectivity assay. The *x*-axis indicates the time in months of the first two vaccinia infections (I) and three subunit boosts (B1–B3). The *y*-axis is the log serum dilution that neutralizes 90% of HIV-1 IIIB virus. Data are given as mean calculated from duplicate means ± SE, $n = 4$. Vaccinia virus control, ▲; WT, ■; and NL-1:2:3:4, ●.

NL-1 produced early signs of syncytia, but appeared to grow more slowly. NL-2, 3, and 1:3 showed evidence of infection at 14 days post-transfection. These data suggest that NL sequons introduced in the V3 domain can be tolerated and that they result in infectious virus.

Binding of NL-modified gp120s to sCD4 was measured by Ag capture ELISA to further study the receptor ligand interaction between gp120 and CD4 (Table II). This was an important consideration, since the gp120/CD4 binding domain is a candidate epitope for group-specific NAb. Given that the WT CD4 binding index is defined as one, the greatest range of binding differences, which was less than a factor of two, was observed in NL 3 and 4 (Table II). Since infectious virus was recovered when these NL sequons were subcloned into full length HIV-1 genomes and transfected into SupT1 cells (Fig. 4 and Table I), we concluded that all NL-modified recombinant molecules bind sCD4 to the same extent as WT.

To further test our hypothesis of immune refocusing, candidate NL immunogens were used to raise antisera and tested in viral neutralization assays. The first subunit boost of WT and all NL-modified recombinant proteins resulted in nearly equivalent 90% neutralization titers. Since the NL-1:2:3:4 immunogen represented the most antigenically dampened V3 NL mutant, neutralization titers were followed through subsequent boosts (Fig. 5). The second subunit boost with WT and NL-1:2:3:4 proteins increased both of the 90% neutralization endpoint titers to 256 and 512, respectively (Fig. 5 and Table III). Interestingly, NL-1:2:3:4 antisera showed a qualitatively different response, since these antisera neutralized a more divergent HIV-1 strain, MN. Although a tendency toward broader neutralization was observed, other T cell line-adapted viruses, including RF and CC, and primary virus MR452, which was isolated and passaged in PBMCs, were not neutralized (data not shown). Since neutralization of homologous virus is known to involve Abs to V3, it appeared that neutralization in antisera NL-1:2:3:4 was occurring through a non-V3 epitope(s).

Shift in NAb response from V3 to V1 domain

Solid phase peptide ELISA was used to determine the extent of gp120 Ab binding reactivities. WT- and NL-modified antisera 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 antisera 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 charge dampened at site NL-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.

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

Antisera	Boost	Neutralization Titer IIIB	Neutralization Titer MN
Wt	1	32	
	2	256	
	3	256	
NL-1:2:3:4	1	32	
	2	512	16
	3	256	8

[a] Neutralization titers of WT and NL-1:2:3:4 antisera against HIV-1 IIIB 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.

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 reactivity could be competitively inhibited by a homologous V3-specific peptide (Fig. 7*A*). This peptide had no effect on inhibiting 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 reactivity in antisera NL-1:2:3:4 (Fig. 7*B*). 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. 7, *C* and *D*). This is in marked contrast to WT antisera, which, despite equivalent V1 ELISA binding reactivity (Fig. 5), was not neutralizing. To rule out any V1 neutralizing activity in WT antisera, WT and NL-1:2:3:4 antisera were mixed in equivalent volumes and assayed for neutralization over a range of dilutions (Fig. 8*A*). 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 NAbs act in an independent manner. This hypothesis was corroborated with V1 peptide competition studies (Fig. 8*B*) 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

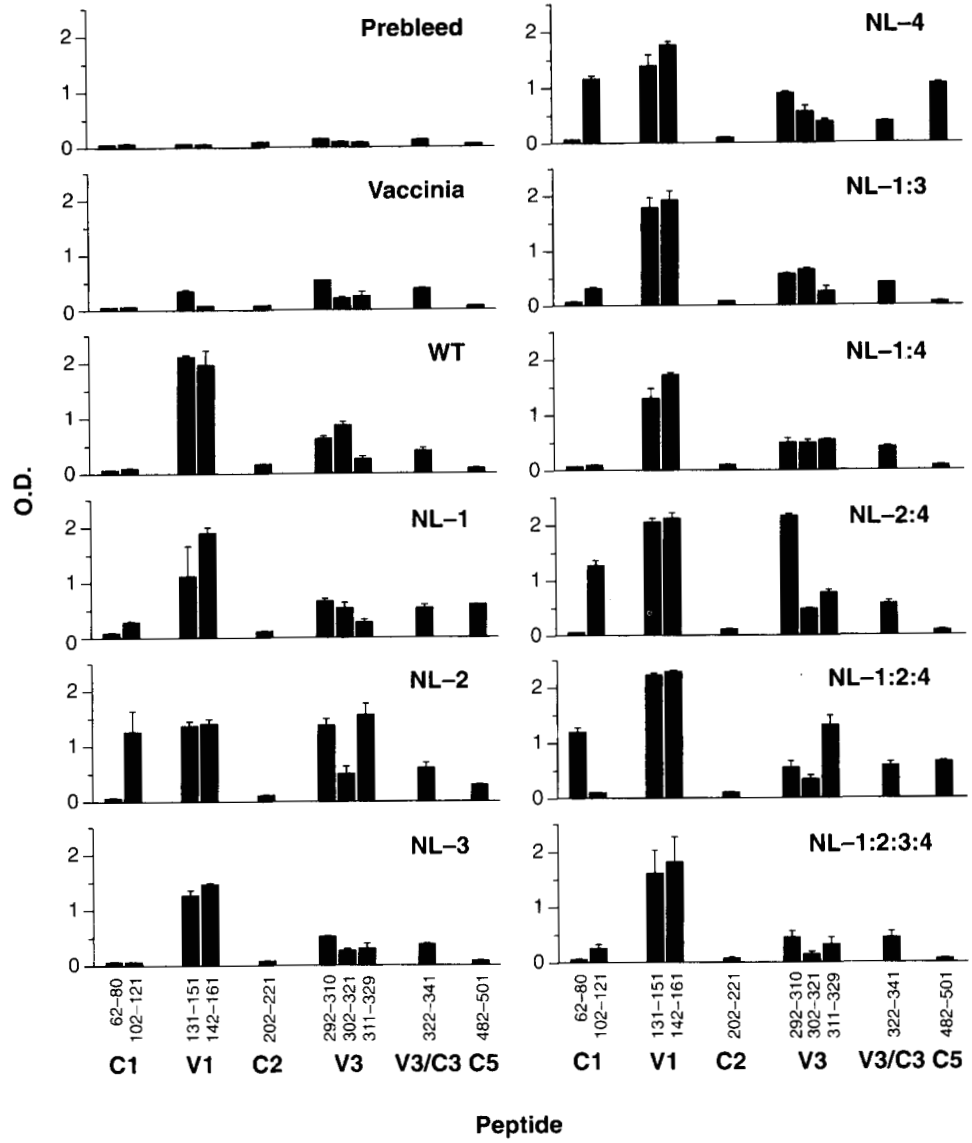


FIGURE 6. Anti-gp120 Abs of NL immune sera measured by ELISA. BH10 gp120 peptides were obtained from Harvey Holmes, U.K. Medical Research Council AIDS Directed Program Reagent Repository. Peptides are sequentially numbered according to amino acids as indicated in the Human Retroviruses and AIDS database. 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. Antisera raised to vaccinia control carry all plasmid transfer sequences except gp160. All other NL antisera are represented based on the site of NL mutation in V3.

response to less dominant non-V3 epitopes that provide broader protection. Site-directed NL mutagenesis 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 β-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 paradoxic 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

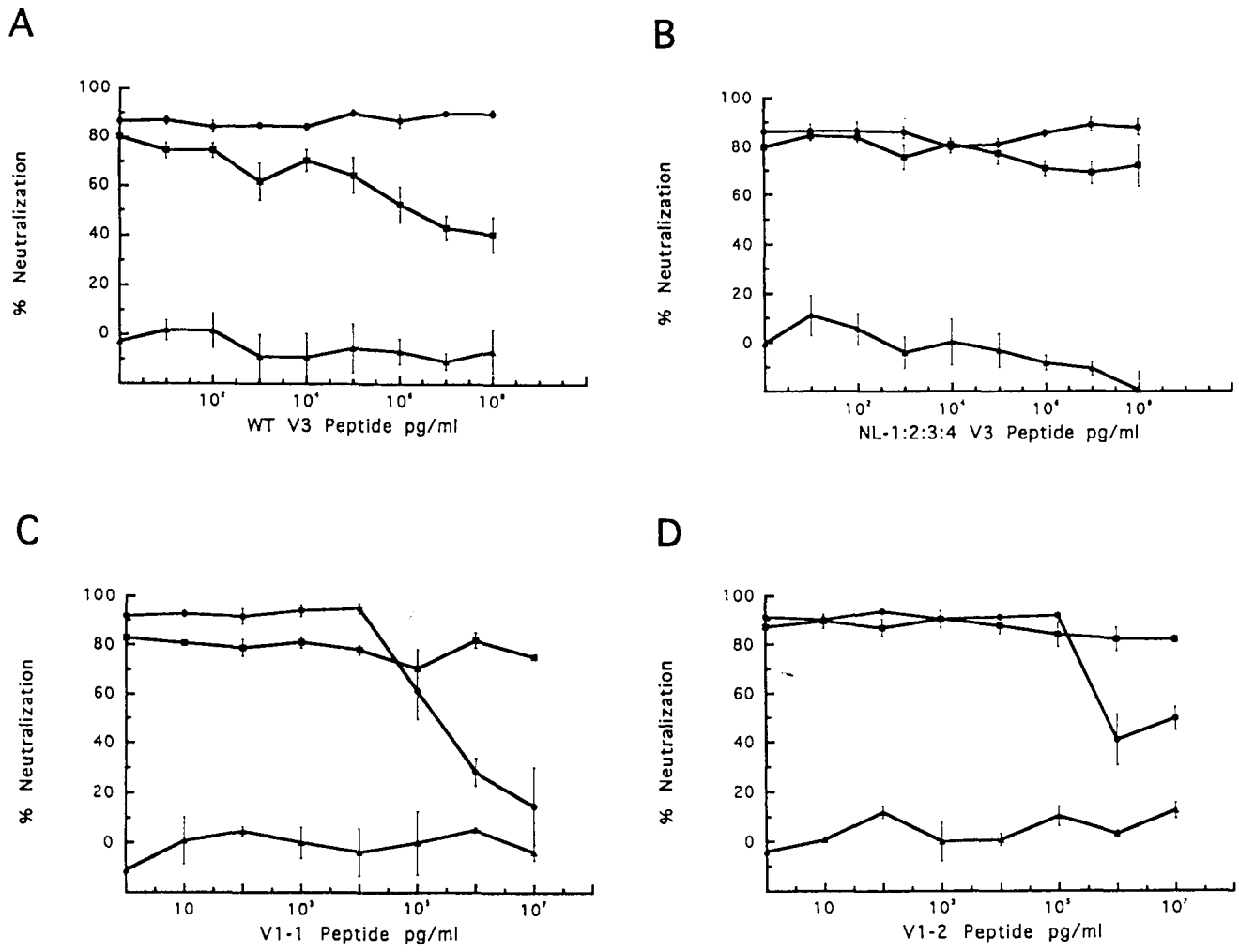


FIGURE 7. Neutralization-competition assay. Equivalent dilutions of WT and NL-1:2:3:4 antisera giving approximately 90% inhibition of HIV-1 IIIB (*y*-axis) in the presence of increasing concentrations of competitor V3 and V1 peptides (*x*-axis). Values represent geometric means ± SEM, $n = 4$. Control antiserum was raised against vaccinia recombinant minus gp160. Concentrations of competitor peptides are given in pg/ml. Control antisera, ▲; WT antisera, ■; and NL-1:2:3:4 antisera, ●. Amino acid sequences of peptide competitors are: *A*, WT V3 peptide NNTRKRIRIQRG PGRAFVTIGKIG; *B*, NL-1:2:3 V3 peptide NNTRKNISIQNGSGRANVTIGKIG; *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 argininines 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

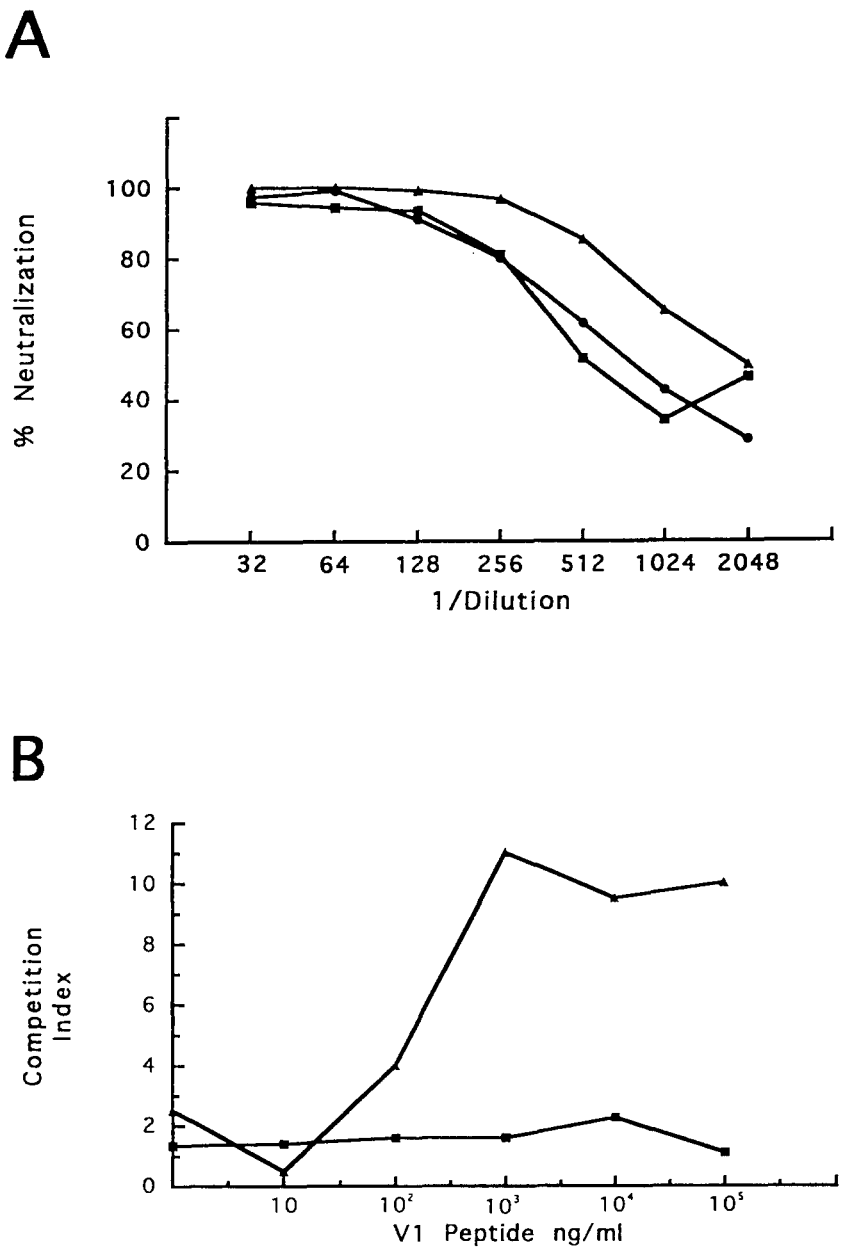


FIGURE 8. Additivity characteristics of V3 and V1 NAbs. *A*, Reciprocal serum dilutions (*x*-axis) of WT, NL:1:2:3:4, and an equivalent mixture of both (Mix) were assayed for % neutralization (*y*-axis) of homologous HIV-1 IIIB virus. Starting dilutions were approximated to give 90% virus neutralization. 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 multideterminant Ags (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 decoy 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 earliest dominant B cell clones (76). This early freezing or imprinting is evidenced by the higher binding affinities and neutralization titers that autologous HIV-1 antisera have on heterologous isolates.

We have shown that dampening an immune response to V3 can lead to a shift or refocusing of the Ab response to a previously silent, qualitatively different, second-order neutralizing epitope. Whether this strategy can be used to reveal additional neutralizing epitopes in HIV-1 envelope glycoproteins awaits further study. However, it is important to note that a number of persistent or reoccurring pathogens, in addition to HIV-1, complicate host immunity through presentation of immunodominant, hypervariable, or repeated epitopes; and many if not most of these pathogens have resisted conventional vaccine strategies. These epitopes appear to play little or no role in host protection. If the evolution of these epitopes is part of a clever pathogenic strategy to evade immune clearance, then the approach of refocusing the immune response away from these domains may have an application in the development of more broadly protective vaccines and therapeutics.

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