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# Antigenic and Immunogenic Study of Membrane-Proximal External Region-Grafted gp120 Antigens by a DNA Prime-Protein Boost Immunization Strategy▽†

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**The membrane-proximal external region (MPER) of human immunodeficiency virus type 1 (HIV-1) gp41 is a target of broadly neutralizing monoclonal antibodies (MAbs) 2F5, 4E10, and Z13. Here we engrafted the MPER into the V1/2 region of HIV-1 gp120 to investigate the ability of the engineered antigens to elicit virus-neutralizing antibodies (NAbs). To promote the correct folding and presentation of the helical 4E10 epitope, we flanked the epitope with helical domains and manipulated the helix by sequential deletion of residues preceding the epitope. Binding of the recombinant proteins to MAb 4E10 increased four- to fivefold with the deletion of one or two residues, but it returned to the wild-type level when three residues were deleted, suggesting rotation of the 4E10 epitope along the helix. Immunization of mice and rabbits by electroporation-mediated DNA priming and protein boosting with these constructs elicited high levels of gp120-specific antibodies. A consistent NAb response against the neutralization-resistant, homologous JR-FL virus was detected in rabbits but not in mice. Analysis of the neutralizing activity revealed that the NAbs do not target the MPER or the V1, V2, or V3 region. Through this study, we learned the following. (i) The 4E10 epitope can be manipulated using a "rotate-the-helix" strategy that alters the helix register. However, presentation of this epitope in the immunogenic V1/2 region did not render it immunogenic in mice or rabbits. (ii) DNA vaccination with monomeric gp120-based antigens can elicit a consistent NAb response against the homologous neutralization-resistant virus by targeting epitopes outside the V1, V2, and V3 regions.**

An ideal human immunodeficiency virus type 1 (HIV-1) vaccine should elicit strong cellular and humoral responses against a broad spectrum of viral variants. Broadly neutralizing antibodies (NAbs) against HIV-1 do exist but are rare, and only a few such human monoclonal antibodies (MAbs) have been described (reviewed in references 12 and 75). MAb b12 targets the CD4 binding site (CD4bs) on gp120 (11). MAb 2G12 recognizes a glycan moiety on the silent face of gp120 (13, 66). MAb 447-52D targets the variable region 3 (V3) of gp120 (19, 25), and MAbs 2F5 (45, 51), 4E10, and Z13 (65, 77) are specific to the membrane-proximal external region (MPER) of gp41. MAbs b12 and 2G12 recognize discontinuous epitopes, whereas 447-52D, 2F5, 4E10, and Z13 recognize continuous epitopes of gp120 or gp41. These epitopes have been studied intensively in the last few years, providing critical structural information for the understanding of antibody neutralization of HIV-1 (13, 15, 48, 50, 64, 72). Harnessing molecular and structural information on neutralizing epitopes for the rational design of vaccines has proven to be extremely challenging, and no breakthrough in eliciting NAbs against diverse primary HIV isolates has been reported so far.

The MPER of gp41 is a prime target of NAbs because of its relatively conserved amino acid sequence among all HIV-1 subtypes (5, 43). The structures of peptides containing the 2F5 epitope vary from a $3_{10}$ helix (6, 7) to an extended structure with a type I β turn at the DKW core sequence of the epitope when in complex with MAb 2F5 (48, 49). These observations indicate that this region may undergo structural changes during different stages of virus entry. The core of the 4E10 epitope contains a WFX(I/L)(T/S)XX(L/I)W motif (14). Structures of MAb 4E10 in complex with peptides have been determined recently, and they consistently show that the 4E10 epitope adopts an α-helical conformation (14, 15). Numerous attempts to elicit 2F5-like NAbs by using conjugated peptides or mimics of the 2F5 epitope have so far not been successful, although in some cases the mimics could elicit high levels of binding antibodies (24, 30, 32, 38, 42, 44). In contrast, the potential of the 4E10 epitope, which has a more defined helical structure, in eliciting NAbs has not been investigated systematically (39).

In this study, we tested whether grafting the 2F5 and 4E10 epitopes, with an emphasis on the helical structure of the 4E10 epitope, into an exposed and immunogenic region of an immunogen would facilitate the induction of specific antibodies. We selected monomeric gp120 as a scaffold for this grafting strategy because it contains several defined immunogenic regions suitable for engineering. Furthermore, the simultaneous induction of gp120- and gp41-specific NAbs will likely improve the breadth of anti-HIV antibody responses via vaccination. The V1, V2, and V3 regions of gp120 are highly accessible on monomeric gp120. V3 is a well-known immunodominant region in gp120 and is also the principal neutralizing determinant of neutralization of T-cell line-adapted virus (26). V1 and V2 are extremely variable in sequence due to frequent insertions/

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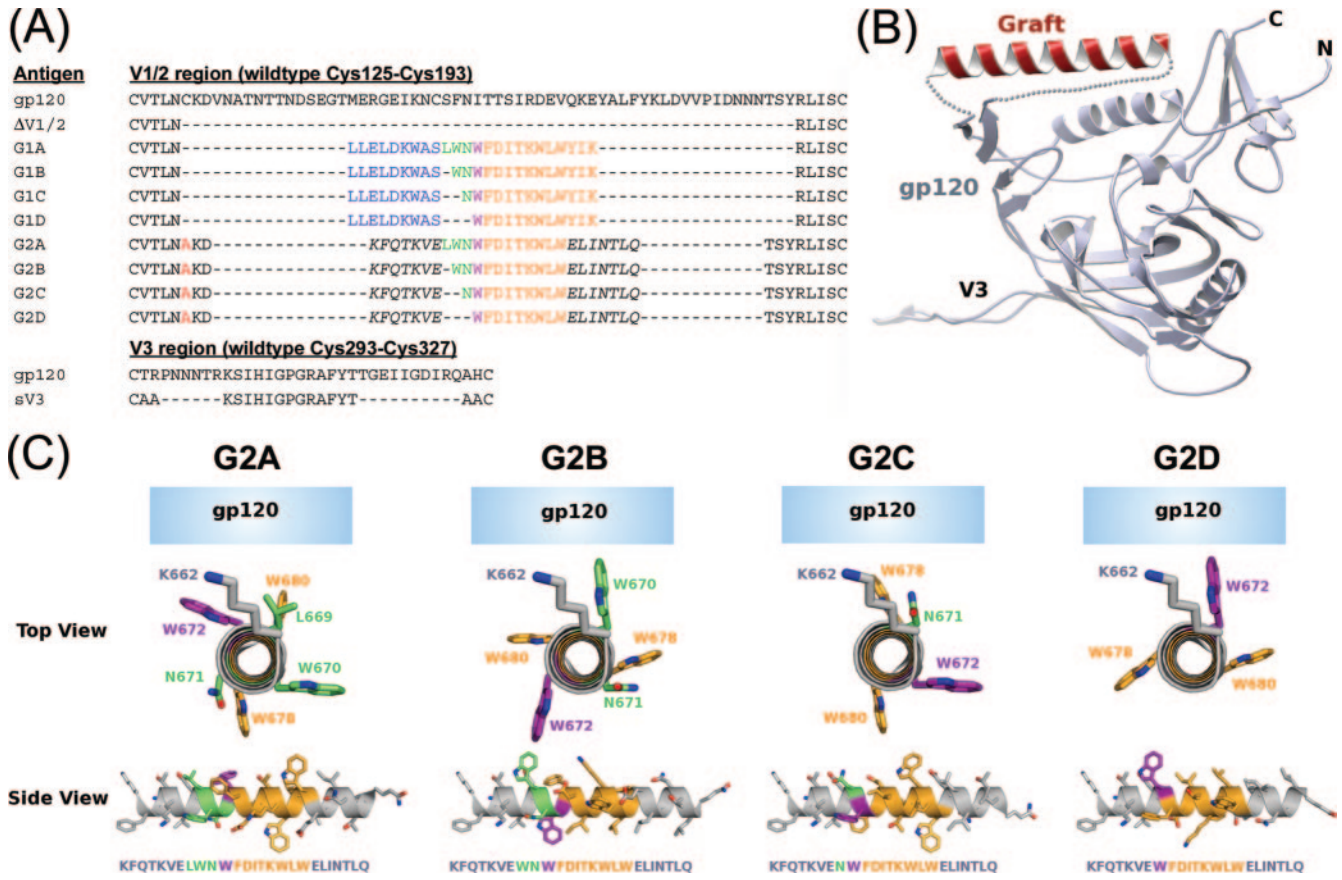


FIG. 1. (A) MPER-grafted gp120 antigens. The antigens were designed using JR-FL gp120 as a template. Amino acid sequences of the modified V1/2 and V3 regions are shown, with the MPER sequence divided into the 2F5 (blue) and 4E10 (orange, with the key epitope residue W672 shown in magenta) regions. The three residues (LWN) deleted sequentially are labeled in green. Dashes indicate deleted residues. The two flanking cysteines of V1/2 shown are expected to form a disulfide bridge (35). In the Graft2 antigens, the remaining cysteine in the N-terminal part of V1 is replaced by alanine (red). Flanking residues from CMP are shown in italics. (B) Model of MPER-grafted gp120. The predicted helical MPER (red) is joined to gp120 (gray) by amino acid residues (gray dotted line) of the gp120 V1/2 loop. The model was built using the CD4-bound gp120 structure (PDB entry 2B4C) (31) and the G2A sequence. (C) Side and top views of the various Graft2 helices. In the Graft2 antigens, the 4E10 epitope (orange, with the key epitope residue W672 shown in magenta) is flanked by amino acid sequences of the coiled-coil domain of CMP (gray), which should promote helicity of the epitope region. The MPER residues LWN (green) were removed sequentially to create different versions of Graft2 antigens, namely, G2A, G2B, G2C, and G2D. Theoretically, these deletions will rotate the helical epitope through 300 degrees and expose the different faces of the 4E10 epitope region on the grafted gp120. Such rotation is better identified in the top view of the helix, where the side chains of W672, W678, and W680, residues located C-terminal to the deletion region (L669, W670, and N671), change position in reference to the postulated gp120 interface (blue box) and K662, a residue located N-terminal to the deletion. Similar manipulation was performed for the Graft1 antigens, although the epitope is not flanked by a CMP sequence and the deletion occurs sequentially within the interface of the 2F5 and 4E10 epitopes.

deletions in addition to mutational substitutions normally found in V3 (www.hiv-web.lanl.gov). V1/2 has been suggested to serve to mask and protect the more conserved neutralizing epitopes on the HIV envelope spike (55, 63). In several immunization studies, including a clinical trial, V1 was shown to be highly immunogenic and, in some cases, induced homologous NAbs (3, 21, 37, 46, 61, 71, 74). Using peptides matching the sequence of the infecting virus, Pincus et al. showed that V1 could be as immunogenic as V3 in HIV-infected laboratory workers and that the anti-V1 antibodies were responsible for part of the neutralizing activity in the sera (53). Although the neutralizing activities of anti-V2 antibodies are relatively less established (54), V2 has also been found to be immunogenic in several immunization studies using gp120 or gp140 (3, 33, 38, 61, 74). Liang et al. previously showed that the 2F5 epitope inserted into V1, V2, and V3 of gp120 was immunogenic in mice and that the V2 insertional mutant was most effective in guinea pigs (38). Since the V3 region harbors a somewhat broadly neutralizing epitope recognized by MAb 447-52D (75), we decided to retain V3 and utilize the V1/2 region for epitope display.

We hypothesized that the grafted MPER would be constrained by the adjoining disulfide bridge at the base of the V1/2 loop and that the grafts would be highly exposed on the recombinant gp120. The design and hypothetical structures of these constructs are illustrated in Fig. 1. We named the constructs that contain the entire MPER sequence "Graft1" or "G1" antigens. In another set of constructs, "Graft2" or "G2" antigens, the 4E10 epitope was flanked by the amino acid sequence of a well-characterized helical domain of the carti-

lage matrix protein (CMP; residues 462 to 475 [GenBank accession no. P05099]) (70) in order to promote helicity in this grafted epitope. Assuming that grafting into the immunogenic V1/2 region would allow correct folding of the MPER sequences, we speculated that this may not necessarily present the correct face of the neutralizing epitopes to the immune system. To increase our chances of success, we removed the residues preceding the 4E10 epitope sequentially in order to turn the helical 4E10 epitope stepwise by approximately 100°. We speculated that one of these constructs would better expose the hydrophobic, neutralizing face of the 4E10 epitope to the immune system. Two other constructs, ΔV1/2 (V1/2-deleted gp120) and sV3 (gp120 with a shortened V3 region), were included in this study as controls. The immunogenicities of the Graft1 and Graft2 antigens were assessed using the DNA prime and protein boost immunization strategy.

## MATERIALS AND METHODS

**Materials.** The MAbs used in this study were 447-52D, provided by S. Zolla-Pazner (19, 25); 2F5, 4E10, and 2G12, provided by H. Katinger (45, 65, 66); and FDA2 serum, obtained from the NIH (52). MAbs b6, b12, and $gp120_{JR-FL}$ were produced in-house (2, 11, 60), and gp41 (isolate IIIB) was purchased from Viral Therapeutics. The sheep antibody D7324, raised against the C5 peptide APTK AKRRWQREKR, was purchased from Cliniqa (Fallbrook, CA), and soluble CD4 (sCD4; extracellular domains 1 and 2, amino acids 1 to 183) and CD4-immunoglobulin G2 (CD4-IgG2) were provided by Progenics Pharmaceuticals (NY). The plasmids for the HIV-1 Env genes of isolates JR-FL, SF162, HxB2, JR-CSF, and ADA have been described previously (60), and the MN isolate was provided by S. Beddows and J. P. Moore (4). The peptides CMP1 (TLNAKDK FQTKVENWFDI), CMP2 (TKWLWELINTLQTSYRL), V4a (TWNNNTE GSNNTEGNTIT), and V5a (TRDGGINENGTEIFR) were purchased from Synthetic Biomolecules. The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program: U87.CD4.CCR5 and U87.CD4.CXCR4 cells (contributed by H. Deng and D. Littman) (8), TZM-bl cells (contributed by J. C. Kappes, X. Wu, and Tranzyme Inc.) (22, 56, 68), plasmids pNL4-3.Luc.R-E- (contributed by N. Landau) (20, 28), pCAGGS SF162 gp160 (contributed by L. Stamatatos and C. Cheng-Mayer) (18, 62, 63), $pSG3^{\Delta env}$ (contributed by J. C. Kappes and X. Wu) (68, 69), and pHEF-VSVG (contributed by L. J. Chang) (17), a standard reference panel of subtype B HIV-1 Env clones (contributed by D. Montefiori, F. Gao, M. Li, B. H. Hahn, J. F. Salazar-Gonzalez, X. Wei, G. M. Shaw, D. L. Kothe, J. C. Kappes, and X. Wu) (36, 68, 69), and an HIV-1 consensus subtype B Env overlapping peptide library (15-mer; complete set no. 9480 [the peptide sequences can be found at www.aidsreagent.org]).

**DNA vaccine constructs.** The pCMV-Tag4A-$tpa_{JR-FLgp120}$ plasmid (50), encoding a codon-optimized gp120 of the primary clade B isolate JR-FL, was used as a template for PCR. The modifications shown in Fig. 1A were introduced into the plasmid by overlapping extension by PCR. All clones were verified by DNA sequencing, and PCR errors were amended using a QuikChange mutagenesis kit (Stratagene). The plasmids, primer sequences, and protocols for PCR are available upon request.

**Protein production using a vaccinia virus expression system.** Recombinant vaccinia viruses expressing ΔV1/2, sV3, G1A, G1C, G2A, G2B, G2C, and G2D were generated as previously described for wild-type JR-FL gp120 (60). Briefly, modified gp120 genes were amplified by PCR from DNA vaccine constructs by using the primers PoxF2 (5′-TTTAAGATCTGCAGGAATTCGCCGCC-3′ [BglII site is underlined]) and PoxR2 (5′-TTTACCCGGGCTCGAGTCAGCG CTTCTCC-3′ [XmaI site is underlined]) and were inserted into pSC65 (16). The recombinant plasmids were used for generation of recombinant viruses by standard methods (16, 23). Recombinant viruses were screened, and the production of the designed proteins was confirmed by capture enzyme-linked immunosorbent assays (ELISAs) (see below).

Recombinant proteins were produced by infecting HEK 293T cells with the corresponding recombinant vaccinia viruses at a multiplicity of infection of 5 in Dulbecco's modified Eagle's medium supplemented with penicillin-streptomycin and incubated at 37°C for 48 h. Supernatants were clarified by low-speed centrifugation and filtration through a 0.22-μm filter. Triton X-100 (0.1%) was added to inactivate residual vaccinia virus prior to purification. The highly glycosylated recombinant proteins were purified using *Galanthus nivalis* lectin cross-linked to agarose beads (Vector Laboratories). The lectin column was equilibrated in phosphate-buffered saline (PBS), and the clarified supernatants were applied by gravity flow. Nonspecifically bound proteins were washed away with 500 mM NaCl in PBS, and bound proteins were eluted with 1 M methyl alpha-D-mannopyranoside (Sigma) in PBS. The eluants were concentrated to 2 ml, and monomers of modified gp120 were purified by size-exclusion chromatography using a Superdex 200 16-cm by 60-cm column (Amersham Biosciences). The purified proteins were evaluated by nonreducing 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by the Bradford method (9) (Quick Start Bradford dye reagent; RioRad) and by optical density measurements (calculated extinction coefficients were as follows: gp120, 1.2; ΔV1/2, 1.3; sV3, 1.3; G1A and G1B, 1.8; G1C, G1D, G2A, and G2B, 1.7; and G2C and G2D, 1.6).

**Immunization of mice.** Eight-week-old female BALB/c mice were immunized intramuscularly with four doses of DNA vaccine (40 μg/dose) and boosted twice with subcutaneous injections of the corresponding protein antigen (10 μg/injection) in RIBI adjuvant (monophosphoryl lipid A [MPL] plus synthetic trehalose dicorynomycolate emulsion; Sigma). DNA vaccines were delivered by in vivo electroporation using a TriGrid delivery system (for rodents) developed by Ichor Medical Systems (40).

**Immunization of rabbits.** New Zealand White female rabbits (3 kg) were used for the different immunization protocols. For the DNA prime/protein boost regimens, four doses of 0.5 to 4 mg of DNA vaccines were delivered intramuscularly by in vivo electroporation using Ichor's TriGrid delivery system (for rabbits) (40), and three doses of corresponding protein antigens (50 μg) in RIBI adjuvant (MPL plus synthetic trehalose dicorynomycolate plus cell wall skeleton emulsion; Corixa) were administered by the intradermal and subcutaneous routes.

Animal housing and immunization procedures adhered to the protocols of the Institutional Animal Care and Use Committee.

**ELISA.** For capture ELISA, microplates (Corning Costar 3960) were coated overnight with the anti-gp120 antibody D7324 at 250 ng/well in PBS. Wells were washed four times with PBS containing 0.05% Tween 20, using an automated plate washer (Molecular Devices), and were blocked with 3% bovine serum albumin (BSA). Variants of recombinant gp120 were diluted in PBS containing 1% BSA and 0.02% Tween 20 and captured by the D7324 antibody for 2 h at room temperature. For MAb 4E10, PBS containing Tween 20 and 4% or 1% nonfat milk was used as a blocking or binding buffer, respectively, to minimize the background. The captured antigens were detected by the specified primary antibodies for 1 h, followed by phosphatase-conjugated or peroxidase-conjugated goat anti-human IgG $F(ab')_2$ secondary antibody (diluted 1/1,000; Pierce) and disodium *p*-nitrophenyl phosphate (Sigma) or tetramethylbenzidine substrate (Pierce). Absorbances were measured at 405 or 450 nm using a microplate reader (Molecular Devices).

For indirect ELISA to determine end-point titers (EPTs) of sera, vaccinia virus-expressed JR-FL gp120 (60) was used to coat wells at a saturating concentration (150 ng/well). Serially diluted mouse or rabbit sera were added to the washed and blocked ELISA plates. Bound antibodies were detected by phosphatase-conjugated secondary antibodies specific to the species [goat anti-mouse or anti-rabbit IgG $F(ab')_2$, diluted 1/1,000; Pierce] and *p*-nitrophenyl phosphate as described above. The EPT of each serum was defined as the reciprocal of the dilution giving a threefold higher titer than the background titer. A control positive serum was included in each ELISA plate for the normalization of data between plates. For detection of antibody reactivity against peptides, ELISA plate wells were coated overnight with peptides (2.5 μg/well) in sodium bicarbonate buffer (pH 9.6). The control proteins gp120, gp41, and ovalbumin were used at 25 ng, 50 ng, and 100 ng, respectively. Rabbit antisera were tested at 1/100, and specific binding was determined as above.

**CD4bs competition assay.** ELISA plate wells were coated with 100 ng/well gp120 overnight at 4°C, followed by blocking with 3% BSA for 1 h at room temperature. Serially diluted rabbit antisera were added to the wells for 30 min before an equal volume of biotinylated sCD4 (diluted 1/10,000) was added and incubated for another hour. Biotinylated sCD4 was produced using EZ-Link-sulfo-NHS-biotin reagent (Pierce) at 1 mg/ml according to the manufacturer's instructions. The plates were washed, and specific signals were detected using peroxidase-conjugated streptavidin and the tetramethylbenzidine substrate (Pierce).

**Pseudotyped virus neutralization assay, with or without V3 loop peptides.** The assay to determine antibody neutralizing activities against HIV Env-pseudotyped virus particles (SF162, JR-FL, JR-CSF, MN, HxB2, ADA, and vesicular stomatitis virus as a negative control) has been described previously (60). Briefly, serially diluted mouse or rabbit heat-inactivated sera were incubated with virus for 1 h at 37°C, and then 100 μl of the antibody-virus mixture was added to

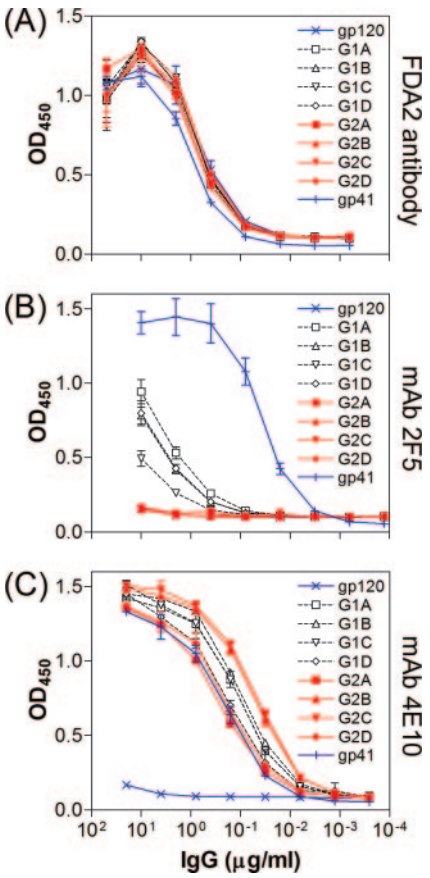


FIG. 2. Binding of MAbs 2F5 and 4E10 to Graft1 and Graft2 antigens. Saturating levels of recombinant proteins expressed by 293 cells were captured by the D7324 anti-gp120 antibody and detected with serially diluted antibodies. Directly coated gp41 (10 ng/well) was used as a positive control. The binding of FDA2 antibody (A), MAb 2F5 (B), and MAb 4E10 (C) to the antigens is illustrated. The apparent affinities of G2B, G2C, and G2D relative to that of G2A are increased 4.4-, 4.8-, and 1.2-fold, respectively. Data shown are the means of two repeats and are representative of at least two experiments. $OD_{450}$, optical density at 450 nm.

U87.CD4.CCR5 or U87.CD4.CXCR4 cells in a 100-μl volume and incubated for 3 days before assaying for luciferase activity. The inhibitory concentration for 50% neutralization ($IC_{50}$) of a given serum was reported as the reciprocal of the serum dilution in the virus-antibody mixture that caused a 50% reduction of viral infectivity in comparison to that of an antibody-free control. For mouse serum titration, equal volumes of sera from the same group were pooled in order to provide a sufficient volume for the neutralization assays. For neutralization of the NIH standard reference panel of subtype B HIV-1 strains, the pseudotyped viruses were generated using the $pSG3^{\Delta env}$ plasmid and a panel of reference Env clones. The assays were performed using TZM-bl cells.

To adsorb V3-specific antibodies in the antisera, a pool of five overlapping V3 peptides (peptides 8837 to 8841) covering the entire V3 region was incubated with diluted antisera at 50 μg/ml (each peptide) for 30 min at 37°C prior to the addition of an equal volume of virus. The mixture was incubated for 1 h at 37°C, and the rest of the assay was carried out as described above. The V3 peptides did not significantly affect the virus infectivity in repeated experiments. MAb 447-52D was used as a control to validate the inhibitory activities of the peptides.

## RESULTS

**MPER grafts are well tolerated in the V1/2 region of gp120, and the 4E10 epitope is highly accessible on MPER-grafted gp120.** The antigenic properties of the MPER-grafted gp120, Graft1, and Graft2 antigens (Fig. 1) were studied using a panel of MAbs recognizing discontinuous epitopes (b6, b12, and 2G12), continuous epitopes (447-52D, 2F5, and 4E10), and the soluble CD4-IgG2 fusion protein (1). Except for b6, all MAbs target broadly neutralizing epitopes on the HIV-1 envelope spike. The antigens were produced by transient transfection of 293T cells and captured by D7324 antibody in ELISA plate wells. The D7324 antibody recognizes the C terminus of gp120, and therefore this capture ELISA monitors antigens that are fully translated and secreted. A human polyclonal IgG with broad neutralizing activity (FDA2) (52) was used to titrate the antigens to ensure that a saturating level of each antigen was captured for the subsequent experiments (Fig. 2A).

The Graft1 antigens contain the whole gp41 MPER. However, the reactivities of MAb 2F5 to Graft1 antigens, particularly to G1C, were much weaker than that to gp41 (Fig. 2B). The interactions were specific because neither gp120 nor any of the Graft2 antigens reacted with MAb 2F5. The results suggest that the 2F5 epitope might be hidden or folded incorrectly.

The 4E10 epitope appeared to fold correctly in Graft1 and Graft2 antigens (Fig. 2C). The relative apparent affinities (half-maximal binding titers) of MAb 4E10 binding to the different Graft1 antigens were similar and within an approximately twofold range. A greater range was observed for the Graft2 antigens. Deletion of one or two residues immediately preceding the 4E10 epitope (G2B and G2C) increased the apparent antibody affinity four- to fivefold. Interestingly, the deletion of all three residues preceding the epitope (G2D) gave an apparent affinity similar to that of the original antigen (G2A). The data for the Graft2 antigens suggest that the sequential deletion of residues N-terminal to the epitope results in a better presentation of the 4E10 epitope, likely because of rotation of the helical epitope and the stepwise exposure of its neutralizing face, as illustrated in Fig. 1.

Table 1 summarizes the changes in relative apparent affinities of MAbs and CD4 binding to the Graft1 and Graft2 antigens in comparison to wild-type gp120, ΔV1/2, and sV3. The deletion of V1/2 (antigen ΔV1/2) did not significantly

TABLE 1. Relative binding levels of grafted gp120 to human Abs and CD4[a]

| Antigen | % apparent affinity relative to wildtype gp120 | | | | | |
|---|---|---|---|---|---|---|
| | FDA2 | CD4-IgG2 | b6 | b12 | 447-52D | 2G12 |
| ΔV1/2 | 124 | 94 | 97 | 104 | 91 | 91 |
| sV3 | 79 | 79 | 91 | 121 | 143 | 6 |
| G1A | 65 | 24 | 55 | 41 | 100 | 69 |
| G1B | 63 | 36 | 55 | 53 | 91 | 91 |
| G1C | 67 | 38 | 55 | 58 | 91 | 96 |
| G1D | 63 | 38 | 59 | 58 | 91 | 91 |
| G2A | 65 | 42 | 60 | 60 | 87 | 100 |
| G2B | 65 | 42 | 63 | 62 | 105 | 100 |
| G2C | 58 | 42 | 75 | 62 | 67 | 100 |
| G2D | 65 | 63 | 71 | 67 | 91 | 120 |

[a] Saturating levels of recombinant proteins expressed by 293 cells were captured by the D7324 anti-gp120 antibody and detected by sCD4 and the indicated antibodies. Apparent affinity was defined as the concentration of the detecting reagent that resulted in 50% of the maximal binding level. Reductions in apparent affinity of 2- to 5-fold and >10-fold are highlighted in gray and black, respectively.

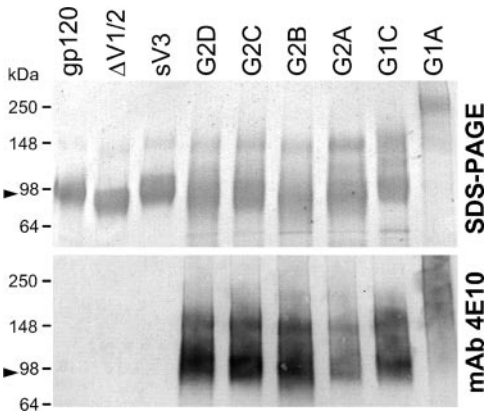


FIG. 3. Graft1 and Graft2 antigens expressed by vaccinia virus. The panel of modified gp120 antigens were expressed using recombinant vaccinia virus and purified by affinity and size-exclusion chromatography, except for G1A, which was affinity-purified G1A because of poor yields in the usual method. The proteins were mixed with Laemmli loading buffer, resolved by 4 to 15% gradient nonreducing SDS-PAGE (top) (5 μg protein/lane), and transferred to a polyvinylidene difluoride membrane for detection by MAb 4E10 (bottom) (0.5 μg protein/lane). Arrowheads indicate the approximate locations of the monomers.

affect binding to any of the antibodies. The replacement of V1/2 with G1 and G2 grafts did not greatly affect the binding of the broadly neutralizing MAbs 2G12 and 447-52D (<2-fold difference). However, the grafts appeared to have some deleterious effects on the CD4bs, resulting in reduced affinities for the anti-CD4bs MAbs b6 and b12 (~30 to 60% reduction) and for CD4-IgG2 (~40 to 80% reduction). In antigen sV3, the shortening of the N and C termini of the V3 loop enhanced the relative apparent affinity of MAb 447-52D to 140% that for wild-type gp120 but nearly abolished the binding of MAb 2G12. Overall, the grafts were tolerated in the V1/2 region of gp120, with some deleterious effects on the CD4bs. The panel of engineered gp120 DNA constructs was amplified for DNA immunization.

Recombinant vaccinia viruses expressing ΔV1/2, sV3, G1A, G1C, and the four Graft2 antigens were generated to produce the recombinant antigens for protein immunization. G1A expression in vaccinia virus appeared to be toxic, and the recombinant viruses isolated in three independent attempts grew much slower and produced a reduced plaque size (data not shown). In contrast, the expression of G1C, an antigen that differs by only two amino acids from G1A, did not affect virus growth. The yields of the recombinant proteins were about 3 to 5 mg per 500 million cells after lectin affinity column purification. A significant portion of the grafted proteins appeared to be oligomeric by size-exclusion chromatography and SDS-PAGE (data not shown). Figure 3 shows the purified monomeric proteins resolved by 4 to 15% nonreducing SDS-PAGE and in an immunoblot detected by MAb 4E10. Except for G1A, monomers of the proteins were purified successfully, and as predicted, MAb 4E10 recognized only antigens bearing the 4E10 epitope (G1A, G1C, and G2A to G2D), not wild-type gp120, ΔV1/2, and sV3. The monomeric fractions of the antigens were used for immunization. For G1A, the protein eluted from the lectin column was used instead due to its poor yield.

**Graft1 and Graft2 antigens and wild-type gp120 are equally immunogenic, but Graft1 and Graft2 antigens do not induce MPER-specific antibodies in BALB/c mice.** The immunogenicities of the panel of nine antigens (excluding G1B and G1D) were first investigated by using BALB/c mice as a small animal model for the screening of useful immunogens. Mice ($n$ = 4) were immunized four times with DNA by in vivo electropora-

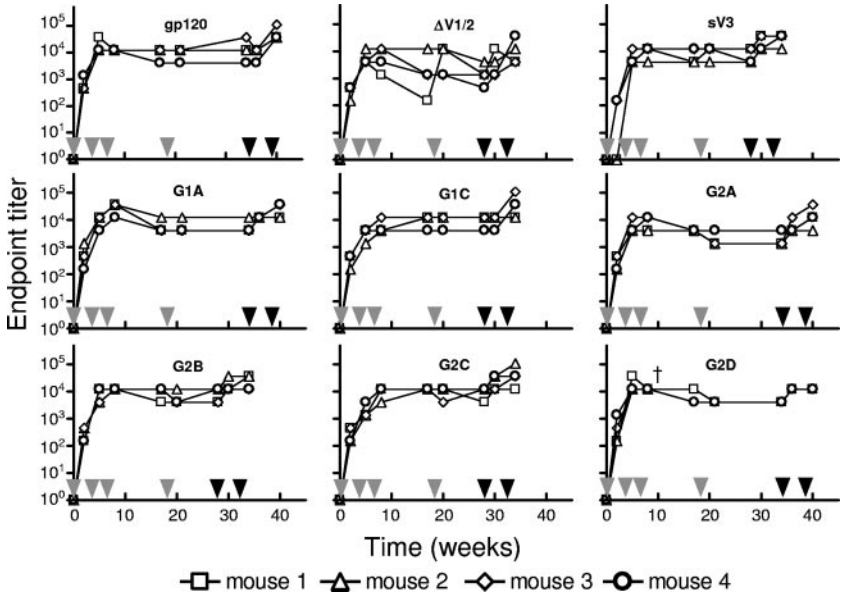


FIG. 4. End-point titration of mouse anti-gp120 antibody. Mouse sera over the course of immunization were serially diluted, and their reactivities against wild-type JR-FL gp120 were determined by ELISA. The EPTs were defined by the serum dilutions that gave readings threefold higher than the background. Gray and black arrowheads indicate the times of DNA and protein immunization, respectively. The symbol "†" indicates the time that two mice in group G2D died during the experiment.

TABLE 2. Tier 1 assessment of neutralizing activities of mouse antisera, using Env-pseudotyped virus[a]

| | Group | pre-immune | 2nd DNA | 3rd DNA | 4th DNA | 1st protein | 2nd protein |
|---|---|---|---|---|---|---|---|
| JR-FL | gp120 | – | – | – | – | – | – |
| | ΔV1/2 | – | – | – | – | – | – |
| | sV3 | – | – | – | – | – | – |
| | G1A | – | – | – | – | – | 10 |
| | G1C | – | – | 10 | – | – | 10 |
| | G2A | – | – | – | – | – | – |
| | G2B | – | – | – | – | – | – |
| | G2C | – | – | – | – | – | – |
| | G2D | – | – | – | – | – | – |
| SF162 | gp120 | – | 10 | 10 | 10 | 20 | 40 |
| | ΔV1/2 | – | 10 | 20 | 40 | 20 | 40 |
| | sV3 | – | – | – | – | – | 10 |
| | G1A | – | 10 | 10 | 10 | 20 | 40 |
| | G1C | – | 10 | 20 | 10 | 10 | 40 |
| | G2A | – | 20 | 20 | – | – | 20 |
| | G2B | – | 20 | 40 | 20 | 40 | 40 |
| | G2C | – | – | 10 | – | 10 | 10 |
| | G2D | – | 10 | 10 | 10 | 20 | 10 |

[a] Serially diluted mouse sera (10-, 20-, and 40-fold) were assayed for neutralizing activity against pseudotyped virus. Dilutions resulting in >50% virus neutralization ($IC_{50}$ titers) are shown, and dashes indicate <50% neutralization at a 10-fold dilution. Different titers are highlighted by degrees of shading.

tion, followed by two boosts with the corresponding protein antigen in RIBI adjuvant. The EPTs of the mice against wild-type gp120 over the course of immunization are shown in Fig. 4. Moderate titers were observed after the first DNA vaccination in the majority of mice, except for two animals in group sV3. The titers were boosted effectively in all animals by the second vaccination and reached a plateau (~10,000 EPTs) after the third DNA vaccination. The titers were stable in the majority of the animals over 10 weeks, and the fourth DNA vaccination did not increase the titers significantly. Protein boosting 10 or 15 weeks later increased the titers slightly, and a second protein boost increased the titers in most of the animals two- to threefold.

Virus-neutralizing activity of the sera was assessed by a single-round infectivity assay using Env-pseudotyped viruses. We employed the rationale proposed by Mascola et al. (41) that sera from vaccinated animals should first be tested against the homologous virus (JR-FL) and a neutralization-sensitive virus from the same clade (SF162) as a tier 1 screening before proceeding to a larger panel of heterologous primary viruses. To provide sufficient sera for neutralization studies, equal volumes of sera from each mouse of the same group were pooled. The data are summarized in Table 2. Homologous neutralization against JR-FL was not detected in the mice. Some borderline cases were observed for G1A- and G1C-immunized mice, but clearly these responses were not consistent among the vaccinated animals. In contrast, neutralization of SF162 virus was observed more frequently, and all groups neutralized this sensitive virus to various extents.

Reactivities of the antisera against gp41, V3, and MPER peptides were studied by ELISA (Fig. 5). The results showed that none of the antigens induced a specific antibody response to gp41 and MPER peptides after DNA and protein immunization. Interestingly, modification of V1/2 appeared to affect the immunogenicity of V3. In mice immunized with wild-type gp120, sV3, G1A, or G1C, significant levels of anti-V3 antibodies (three times above the background) were elicited in all animals. The overlapping peptides 8836 to 8843 allow the dissection of antibody specificities to the different V3 regions. The N- and C-terminal parts of V3 do not seem to be important for the immunogenicity of V3 because sV3 contains only the crown of V3 (KSIHIGPGRAFYT). The removal of V1/2 (ΔV1/2) or replacement with Graft2 residues had a more variable effect in the immunized animals. Only low levels of anti-V3 antibodies were induced in ΔV1/2-immunized mice, and the reactivity was directed mostly to the N-terminal part of V3 (peptide 8837) instead of the crown (peptide 8838). For Graft2 antigen-immunized mice, some of the animals did not produce detectable anti-V3 antibodies (e.g., three of four mice in group G2C), and the specificities towards different V3 peptides varied between animals, with more frequent reactivity towards peptide 8839. This suggests that alterations in the V1/2 loops not only have local effects but also may affect the immunogenicity of V3 in monomeric gp120, supporting the notion that V1/2 interacts with V3 and affects its conformation and sensitivity to anti-V3 NAbs (55, 58, 63, 76).

Overall, DNA vaccination by in vivo electroporation induced high gp120-specific antibody titers in all groups over the course of immunization, and protein boosting enhanced the titers severalfold in the animals. However, MPER-specific antibody was not induced in mice, and the immune sera neutralized only SF162, not JR-FL virus.

**DNA prime/protein boost immunization induces a consistent homologous NAb response against neutralization-resistant primary virus in rabbits.** The results above showed that the antigens are immunogenic in mice by DNA prime/protein boost immunization. However, no significant NAb response was observed. It has been speculated that a long heavy chain complementarity region 3 (HCDR3) is an important feature of broadly neutralizing NAbs against primary HIV isolates (12), and this feature is missing in murine immunoglobulins (59). Rabbits, in contrast, do produce antibodies with long HCDR3s (57), and therefore we immunized seven rabbits with G2C. We selected G2C because of its interesting property of binding to MAb 4E10 in the antigenicity study (Fig. 2C).

Rabbits were immunized by a DNA prime/protein boost regimen ($n = 5$; animals 7669 to 7672 and 7745) or with protein only ($n = 2$; animals 7743 and 7744). In this experiment, we applied different amounts of DNA to investigate the minimal dose of DNA required for in vivo electroporation in rabbits. Animals were immunized with 0.5, 1, or 2 mg DNA four times and then boosted three times with 50 μg of G2C protein in RIBI adjuvant. For protein immunization only, the rabbits were primed with 100 μg of G2C and then boosted with a 50-μg dose. The EPTs of the rabbits against wild-type gp120 over the course of immunization are shown in Fig. 6.

DNA vaccination by in vivo electroporation was also very effective in eliciting anti-gp120 antibody responses in rabbits. Similar to the case in the mouse experiments, moderate titers were observed after the first DNA vaccination (Fig. 6, top panel). In all animals, the titers were boosted effectively, about 10- to 20-fold, by the second DNA vaccination and reached a plateau after the third DNA vaccination (~10,000 EPTs). The titers became stable in all animals, and the fourth DNA vaccination did not increase the titers further. No significant dif-

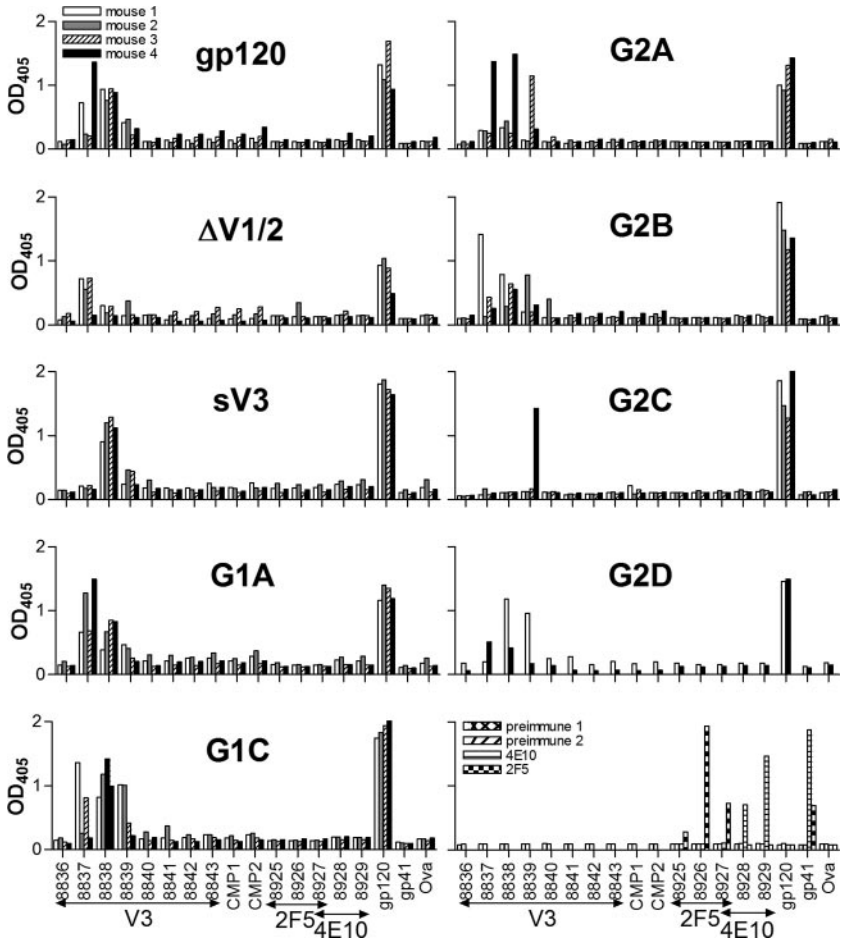


FIG. 5. Reactivities of mouse antisera to MPER peptides. ELISA plate wells were coated with peptides (2.5 μg/well), gp120 (25 ng/well), gp41 (50 ng/well), or ovalbumin (100 ng/well) overnight at 4°C. Mouse sera (preimmune sera or those obtained after the second protein boost) were tested at a 1:50 dilution with control MAbs 2F5 and 4E10 (0.2 μg/ml). The peptide sequences were as follows: 8836, EINCTRPNNNTRKSI; 8837, TRPNNNTRKSIHIGP; 8838, NNTRKSIHIGPGRAF; 8839, KSIHIGPGRAFYTTG; 8840, IGPGRAFYTTGEIIG; 8841, RAFYTTGEIIG DIRQ; 8842, TTGEIIGDIRQAHCN; 8843, IIGDIRQAHCNISRA; CMP1, TLNAKDKFQTKVENWFDI; CMP2, TKWLWELINTLQTSYRL; 8925, QEKNEQELLELDKWA; 8926, EQELLELDKWASLWN; 8927, LELDKWASLWNWFDI; 8928, KWASLWNWFDITNWL; and 8929, LWNWFDITNWLWYIK. Peptides CMP1 and CMP2 contain the flanking CMP sequences (underlined) of Graft2 antigens. Data shown are representative of two experiments.

ference in antibody titer was found with the dose of DNA given, suggesting that 0.5 mg DNA per immunization may be sufficient. Protein boosting 8 weeks later increased the titers about fourfold, and a second protein boost increased the titers another twofold. The overall trend and titers of the antibody responses in rabbits were very similar to those of mice immunized with G2C. For immunization using protein only (animals 7743 and 7744), gp120-specific antibodies were detected only after the second protein boost, and the titers only increased two- to fourfold with subsequent boosting (Fig. 6, bottom panel). After five protein immunizations, the titers were approximately twofold higher than those of animals receiving four DNA vaccinations.

The neutralizing activities of the rabbit antisera against JR-FL and SF162 viruses were studied (Table 3). Interestingly, only animals receiving DNA and protein immunizations produced NAbs against JR-FL ($n = 5$), and four of these five rabbit antisera also neutralized SF162. Neutralizing activity was observed in four rabbits after the fourth DNA immunization, and the neutralizing activity was enhanced by protein boosting up to an $IC_{50}$ titer of 160 in two animals. In contrast, the rabbit antisera generated without DNA priming neutralized only SF162 (animals 7743 and 7744). The data demonstrate a difference between mice and rabbits in the response to DNA prime/protein boost vaccination.

Since the DNA/protein-immunized rabbit antisera neutralized both the homologous and neutralization-sensitive HIV-1 isolates and fulfilled the requirement of tier 1 assessment of NAb responses, these antisera were evaluated further by a tier 2 neutralization assay against a wider panel of HIV-1 isolates (MN, HxB2, JR-CSF, ADA, and the NIH standard reference panel of subtype B HIV-1 clones) (41) (Table 4). The results showed that immunization of rabbits with G2C by the DNA prime and protein boost strategy induced a consistent NAb response against the homologous and neutralization-sensitive viruses (SF162, MN, and HxB2), but the breadth of responses against other viruses from the same clade was limited. There was some neutralization, e.g., rabbit 7670 neutralized >50% of

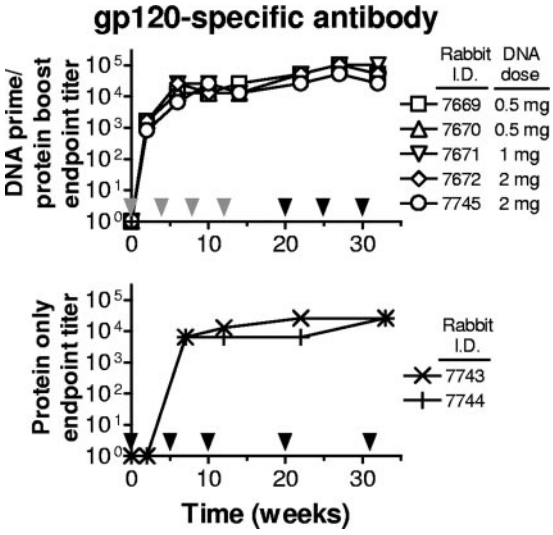


FIG. 6. End-point titration of rabbit anti-gp120 antibody. The EPTs of anti-gp120 antibody in immunized rabbits were defined by the serum dilutions that gave readings threefold higher than the background, as described in the legend to Fig. 4. Gray and black arrowheads indicate the times of DNA and protein immunization, respectively.

SVPB 8, 12, 13, and 18 viruses at a 1:10 dilution, and rabbit 7672 neutralized JR-CSF at a 1:20 dilution. However, these responses were not observed in the majority of the animals.

**Immunosilencing of 4E10 epitope in G2C antigen.** We previously immunized rabbits with wild-type and engineered JR-FL gp120, but NAb responses were poor against the homologous JR-FL virus (60). In this study, by DNA vaccination and protein boosting of G2C, we observed a consistent activity against the homologous neutralization-resistant virus. The greater volume of rabbit serum permits a more detailed dissection of the antibody responses. The specificities of responses to linear epitopes were investigated using a library of overlapping peptides (Fig. 7). Although NAbs were elicited, none of the rabbits produced antibodies specific to gp41, MPER, or CMP peptides at the end of the immunization schedule, as detected by ELISA. Interestingly, the patterns of reactive linear epitopes differed between rabbits immunized with DNA/protein and protein only. Antibodies specific to V3 and C5 peptides were detected more frequently in the former, whereas anti-C1 activity was dominant in the latter group. Antibodies specific to V5 peptides were detected in all animals. Nevertheless, the results indicate that the 4E10 epitope was not immunogenic, even though it was folded correctly and exposed on an immunogenic region of gp120. To confirm these results, competition assays using antisera from G2C-immunized rabbits to block biotinylated MAb 4E10 binding to G2C antigen or gp41 were performed, and the assays did not detect any 4E10 epitope-specific antibody in the antisera (data not shown). In addition, the most potent rabbit antiserum (from animal 7672) did not neutralize the simian immunodeficiency virus SIVmac expressing the 2F5 or 4E10 epitope (73; E. Yuste, W. Johnson, and R. Desrosiers, unpublished data). These results confirm that the G2C graft is not immunogenic in rabbits by either DNA/protein or protein immunization.

**SF162 and JR-CSF viruses, but not JR-FL virus, can be neutralized by anti-V3 antibodies.** A notable observation in this study is that homologous NAbs against the neutralization-resistant JR-FL virus were elicited in all immunized animals. The neutralization of JR-FL is not mediated by anti-V1/2 antibodies because the majority of the V1/2 region is deleted from G2C and because the rabbit antisera did not bind to peptides containing the remaining V1/2 overhangs in ELISA (Fig. 7B). We also studied the role of anti-V3 antibodies in mediating neutralization (Fig. 8A). A pool of five overlapping peptides covering the entire V3 region of JR-FL virus was used to block anti-V3 NAbs. The peptides inhibited the anti-V3 MAb 447-52D efficiently. Preincubation of the rabbit antisera with V3 peptides significantly reduced the neutralizing activity against SF162 but not that against JR-FL, suggesting that JR-FL virus was neutralized by antibodies targeting regions outside V3. In addition, all cross-neutralizing activity against JR-CSF in antiserum 7672 is mediated by anti-V3 antibodies (Fig. 8A, bottom panel). JR-CSF is a neutralization-resistant primary isolate and is resistant to the anti-V3 MAb 447-52D (5). It was isolated from the cerebrospinal fluid of a patient,

TABLE 3. Tier 1 assessment of neutralizing activities of rabbit antisera, using Env-pseudotyped virus[a]

| | Rabbit I.D. | pre-immune | 3rd DNA | 4th DNA | 1st protein | 2nd protein | 3rd protein | 4th protein | 5th protein |
|---|---|---|---|---|---|---|---|---|---|
| JR-FL | 7669 | – | – | 20 | 40 | 40 | 40 | | |
| | 7670 | – | – | – | 10 | 10 | 40 | | |
| | 7671 | – | 10 | 10 | 80 | 160 | 80 | | |
| | 7672 | – | 10 | 20 | 160 | 40 | 80 | | |
| | 7645 | – | 10 | 20 | 80 | 20 | 80 | | |
| | 7743 | – | N.A. | N.A. | N.D. | – | – | – | – |
| | 7744 | – | N.A. | N.A. | N.D. | – | – | – | – |
| SF162 | 7669 | – | – | 10 | 40 | 40 | 20 | | |
| | 7670 | – | – | 20 | 20 | 80 | 80 | | |
| | 7671 | – | – | – | – | – | – | | |
| | 7672 | – | – | 10 | 80 | 160 | 320 | | |
| | 7645 | – | 10 | 10 | 80 | 10 | 80 | | |
| | 7743 | – | N.A. | N.A. | N.D. | – | – | 160 | 10 |
| | 7744 | – | N.A. | N.A. | N.D. | – | – | 80 | 20 |

[a] Serially diluted rabbit sera (10- to 320-fold) were assayed for virus-neutralizing activity. The data are presented similarly to those in Table 2, and neutralizing activity is highlighted by the degree of shading, i.e., darker shading indicates a higher level of neutralization. N.A., not applicable; N.D., not determined.

TABLE 4. Tier 2 assessment of neutralizing activities of rabbit antisera, using Env-pseudotyped virus[a]

| Rabbit I.D. | VSV | MN | HxB2 | ADA | JR-CSF | *SVPB 5* | *SVPB 6* | *SVPB 8* | *SVPB 11* | *SVPB 12* | *SVPB 13* | *SVPB 14* | *SVPB 15* | *SVPB 16* | *SVPB 17* | *SVPB 18* | *SVPB 19* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7669 | – | 200 | 80 | – | – | – | – | 10 | – | – | – | – | – | – | – | – | – |
| 7670 | – | 200 | 80 | – | – | – | – | 10 | – | 10 | 10 | – | – | – | 10 | 10 | – |
| 7671 | – | 100 | 40 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 7672 | – | 800 | 40 | – | 20 | – | – | – | – | – | – | – | – | – | – | – | – |
| 7645 | – | 400 | 40 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| b12 | | | | | | 79% | 99% | 99% | – | – | 91% | 73% | 94% | 89% | – | 81% | – |

[a] Serially diluted rabbit sera (after the third protein boost) were assayed for virus-neutralizing activity. The reciprocal of the serum dilution that resulted in >50% virus neutralization ($IC_{50}$ titer) is shown and highlighted by shading as in Tables 2 and 3. MAb b12 was used as a positive control (100 μg/ml), and the % relative light unit reduction is shown. The NIH standard reference panel of subtype B HIV-1 clone pseudotyped viruses are shown in italics, and the assays were performed using TZM-bl cells. Other viruses were generated and assayed as in Tables 2 and 3. VSV is a control virus pseudotyped with the envelope G protein of vesicular stomatitis virus. MN and HxB2 are T-cell line-adapted neutralization-sensitive viruses.

whereas JR-FL was isolated from brain tissue (34, 47). Our results showed that JR-CSF was susceptible to anti-V3 antibodies in rabbit 7672. In contrast, JR-FL was neutralized by antibodies targeting regions outside V1, V2, and V3.

Finally, we examined whether anti-CD4bs antibodies were elicited in rabbits. In Table 1, we show that binding of CD4 and anti-CD4bs MAbs b6 and b12 to G2C was reduced (two- to fourfold). We speculated that G2C might be less effective in inducing antibodies that would block the interaction between CD4 and gp120. The ability of the rabbit antisera to inhibit biotinylated sCD4 binding to wild-type gp120 is compared to that of the broadly neutralizing human antiserum FDA2 in Fig. 8B. The sCD4 used in this assay contains only the first two extracellular domains of CD4 in order to minimize nonspecific steric hindrance. The results showed that antibodies that competed with sCD4 were produced in all immunized rabbits, and their activity was even higher than that of the broadly neutralizing FDA2 human serum. Notably, at high concentrations, the rabbit antisera inhibited nearly 100% of binding of sCD4. Therefore, G2C is capable of inducing antibodies very close to or overlapping the CD4bs. It is uncertain whether these antibodies neutralize JR-FL, and there is no facile assay currently available to correlate the function of anti-CD4bs antibodies in immune sera to virus-neutralizing activity (29).

## DISCUSSION

In this study, we aimed to design an immunogen that could induce broadly neutralizing Abs, particularly to the 2F5 and 4E10 epitopes. Although we failed to induce broadly neutralizing Abs, the following two important observations may be useful in the future engineering of MPER- and gp120-based immunogens.

**(i) Manipulation of a helical epitope and the poor immunogenicity of the 4E10 epitope.** The gp41 MPER contains the epitopes for the broadly neutralizing MAbs 2F5 and 4E10. Crystal structures of the epitopes have shown that the 2F5 epitope has an extended conformation with a type I β turn at the DKW core sequence (48), whereas the 4E10 epitope adopts a completely α-helical conformation (14, 15). We proposed that to induce broadly neutralizing Abs, it would be minimally necessary to expose the neutralizing faces, possibly to block the nonneutralizing faces, to lock the epitopes into their native antigenic conformations, and to present the epitopes in an immunogenic environment. We engrafted the 2F5 and 4E10 epitopes or the 4E10 epitope alone into the immunogenic V1/2 region of gp120 to generate a panel of Graft1 and Graft2 antigens (Fig. 1). In Graft 1 antigens, both 2F5 and 4E10 epitopes were included in the graft. In Graft2 antigens, we focused on the 4E10 epitope, which was flanked N- and C-terminally by CMP helical domains. Brunel et al. recently showed that one can increase the affinity of MAb 4E10 binding to 4E10 epitope-like helical peptides >1,000-fold by limiting the conformational flexibility of the peptides (10). We hypothesized that the flanking residues would promote an α-helical conformation in the whole grafted region, including the 4E10 epitope. We further speculated that simply presenting the 4E10 epitope as a helix might not favorably present the neutralizing face of the epitope. Since the accessibility to one face of the helical epitope would likely be limited by steric hindrance due to the carrier gp120, we used a "rotate-the-helix" strategy in an attempt to rotate the 4E10 helix over the surface of gp120. We hypothesized that the CMP-4E10-CMP graft would form a continuous helix and that sequential deletion of residues preceding W672 of the 4E10 epitope, a key residue for the binding of MAb 4E10, would force the 4E10 epitope helix to adopt a different register and expose its different faces to the immune system.

We first showed that the insertion of MPER residues into V1/2 is well tolerated in engineered gp120. The modifications had some deleterious effects on grafted antigen binding to CD4-IgG2 and to MAbs b6 and b12. Binding to the polyclonal antibody FDA2 and to MAbs 447-52D and 2G12 was not affected significantly (Table 1). These data suggest that the overall structures of the engineered gp120s are maintained, with some changes in the CD4bs. Nevertheless, these changes do not affect the induction of antibodies that can inhibit sCD4 binding to monomeric gp120 (Fig. 8B).

For Graft1 antigens, interactions with MAb 2F5 were rather weak, suggesting that the 2F5 epitope was either not exposed on the antigens or was folded differently (Fig. 2). Notably, Liang et al. inserted the 2F5 epitope LLELDKWASL into V1 or V2, and they did detect high binding activities of MAb 2F5 with the recombinant proteins, confirming the accessibility of the epitope (38). Here, in contrast, most of the V1/2 region was replaced by the MPER sequence. It is known that the region spanning the 2F5 epitope has a tendency to adopt a helical conformation (6, 7), and it may be that the inclusion of the complete MPER sequence in our Graft1 constructs promoted such helical formation. Since MAb 2F5 recognizes its epitope

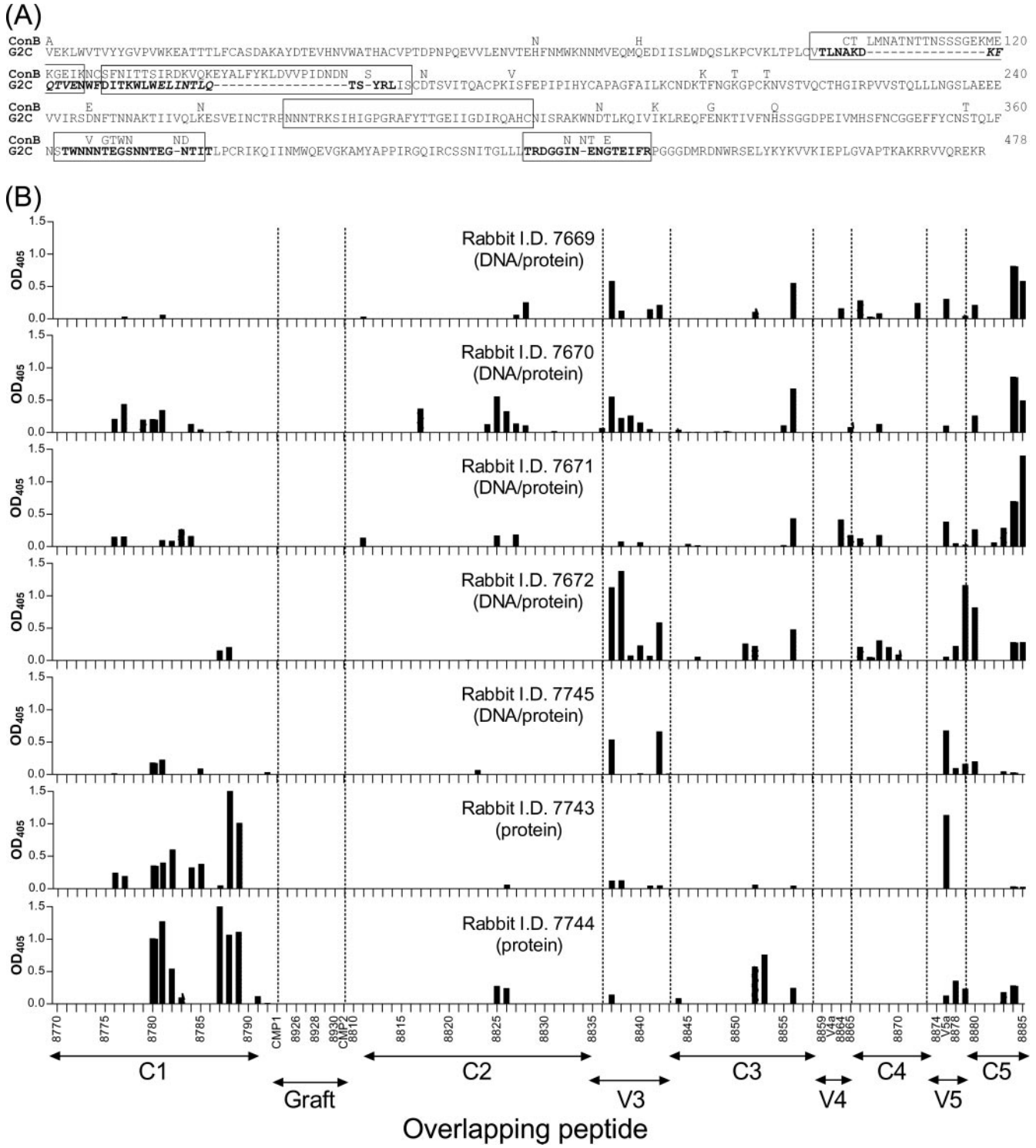


FIG. 7. (A) Alignment of G2C antigen and NIH consensus B gp120 (ConB) sequences. Only the differences between the sequences are shown. Dashes are deleted residues in G2C, and the additional peptides corresponding to the CMP, 4E10 epitope, V4, and V5 sequences are shown in bold. The five variable regions of gp120 are boxed. (B) Reactivities of rabbit antisera to overlapping peptides. The experiments were performed as described in the legend to Fig. 5, using the NIH consensus B Env peptide library and the CMP1, CMP2, V4a, and V5a peptides to cover the entire G2C sequence, as shown in panel A. Antisera collected after the third protein boost, for rabbits immunized with DNA and protein, or after the fifth protein-only immunization were tested at a 1:200 dilution. Data presented are background-subtracted optical density (OD) readings. The background is defined as twice the average reading for the five least reactive peptides in each rabbit sample.

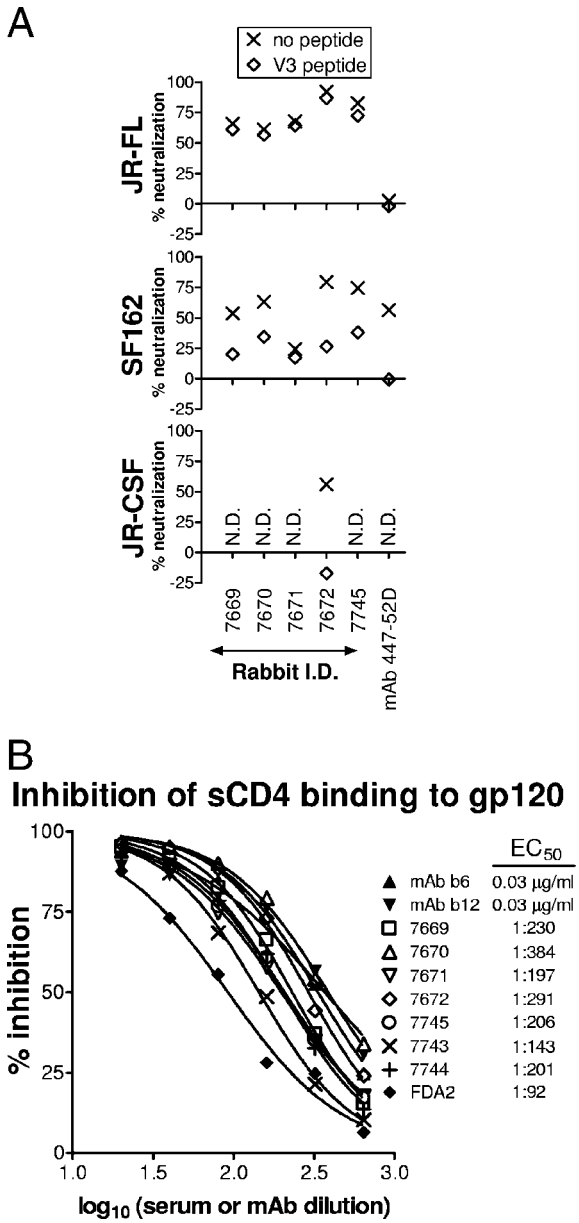


FIG. 8. (A) Anti-V3 antibody in virus neutralization. The rabbit antisera were preincubated with the V3 peptide pool for 30 min before being mixed with the pseudotyped virus JR-FL, SF162, or JR-CSF for 1 h at 37°C, and residual virus infectivity was titrated as described in Materials and Methods. The V3 peptides have one and three different residues in JF-CSF (underlined) (TRPSNNTRKSIHIGPGRAFYTTGEIIGDIRQAH; GenBank accession number AAB03749) and SF162 (TRPNNNTRKSITIGPGRAFYATGDIIGDIRQAH; GenBank accession number P19550), respectively. The viruses were neutralized at a final serum dilution of 1:10. For JR-CSF virus, only antiserum 7672 was tested because the other antisera did not neutralize JR-CSF. N.D., not determined. (B) Anti-CD4bs antibody. The ability of the rabbit antisera to compete with sCD4 binding to monomeric gp120 was studied by ELISA. gp120-coated ELISA wells were preincubated with serially diluted rabbit sera for 30 min before the addition of an equal volume of biotinylated sCD4. Bound sCD4 was detected with peroxidase-conjugated streptavidin. The level of inhibition was defined as the % reduction in optical density readings relative to that without rabbit sera. MAbs b6 and b12 and the broadly neutralizing human antiserum FDA2 were positive controls for anti-CD4bs activity. The 50% effective concentration ($EC_{50}$) values for the antibodies in inhibiting sCD4 binding to gp120 were calculated by nonlinear curve fitting using Prism 4 software.

in an extended rather than helical conformation (48), helix formation may explain the lack of MAb 2F5 binding to our constructs. In contrast to MAb 2F5, MAb 4E10, recognizing an epitope immediately adjacent to the 2F5 epitope, bound to Graft1 antigens efficiently, suggesting that the 4E10 epitope is expressed and exposed.

For Graft2 antigens, the sequential deletion of the first two residues (L or LW) preceding the key residues WF of the 4E10 epitope enhanced the antigen-antibody interaction (Fig. 1 and 2). Deletion of three residues (LWN), which should alter the helix register and hence turn the 4E10 helix almost a complete cycle (300°), returned the binding affinity to a value similar to that of G2A. Among the panel of antigens, G2C had the highest affinity for MAb 4E10 and, therefore, the most favorable properties for 4E10 epitope presentation.

We then used BALB/c mice as a screening tool to study the immunogenicities of the panel of antigens. Despite the efficient induction of gp120-specific antibodies, the murine antisera did not neutralize the homologous JR-FL virus, and anti-MPER antibodies were not elicited (Table 2 and Fig. 5). Broadly neutralizing MAbs, including MAb 4E10, typically have a long HCDR3 (12). It is speculated that mice may not be a suitable animal model for eliciting HIV NAbs because they do not make antibodies with long HCDR3s (59), while antibodies with long HCDR3s are found in rabbits (57). Therefore, we immunized rabbits with G2C, our most promising antigen. Rabbits immunized by DNA prime/protein boost vaccination, but not those immunized by protein vaccination alone, produced NAbs against SF162 and JR-FL viruses (Table 3). Further analysis of the rabbit NAbs demonstrated that they are specific to JR-FL and do not significantly neutralize other neutralization-resistant primary HIV isolates (Table 4).

It is surprising that none of the graft antigens elicited MPER-specific antibodies in mice, nor did G2C in rabbits (Fig. 5 and 8B). Yuste et al. screened 92 HIV-positive plasma samples from 47 different patients in neutralizing 4E10 epitope-engrafted SIV and found that only 4 samples possessed anti-4E10 epitope activity (73). This study highlights the fact that the 4E10 epitope is poorly immunogenic in natural infections. One possible explanation is that MAb 4E10 is an autoantibody to cardiolipin and that elicitation of 4E10-like antibodies is inhibited by tolerance mechanisms (27). However, we have not observed that MAb 4E10 has an unusual affinity for cardiolipin (E. Scherer and D. Burton, unpublished data). Furthermore, the crystal structure of MAb 4E10 reveals that it has an extremely hydrophobic antibody-combining site (14, 15) and, hence, some appreciable affinity for hydrophobic molecules. It is possible that this hydrophobicity presents problems in terms of the elimination of potentially antigen-reactive clones through tolerance mechanisms.

However, if tolerance is not an explanation, why is antibody to the 4E10 peptide not induced by the grafted antigens? One possibility is that the highly hydrophobic 4E10 epitope may bind to other hydrophobic molecules upon introduction into an animal by immunization, thereby masking the epitopes from interaction with B-cell receptors. We noted that a larger fraction of oligomers were present in the Graft1 and Graft2 antigens than in the control proteins after affinity purification. Wild-type gp120, ΔV1/2, and sV3 produced ~30 to 40% monomers in size-exclusion chromatography, whereas fewer mono-

mers, often <10%, were found in the grafted antigens (data not shown), suggesting that the grafted region might be involved in oligomerization. It will be interesting to investigate whether reducing the hydrophobicity of the 4E10 epitope will overcome its poor immunogenicity.

**(ii) DNA vaccination versus protein immunization in eliciting NAbs against primary HIV isolates.** Another important observation here is that it is possible to elicit a consistent NAb response against a neutralization-resistant virus with a monomeric gp120-based immunogen by the DNA prime/protein boost approach. We previously immunized rabbits by multiple inoculation with purified monomeric gp120 protein in RIBI adjuvant but were unable to elicit a potent NAb response against the homologous virus JR-FL (60). A similar failure was also noted for rabbits immunized only with the G2C antigen formulated in RIBI adjuvant. These results are in agreement with two other recent studies that demonstrate the induction of NAbs against JR-FL virus after priming of animals with DNA followed by boosting with proteins (4, 67). Beddows et al. primed rabbits twice with 1 mg of DNAs encoding different versions of gp140 engineered to promote the association and oligomerization of gp120 and gp41 by in vivo electroporation and observed better JR-FL neutralization after five boosts with 30 μg of the antigens in QS-21 adjuvant (4). Wang et al. did not observe NAbs in animals immunized with monomeric gp120 proteins in Freund's incomplete adjuvant, while NAbs were found in rabbits primed with DNA encoding unmodified gp120 or gp140 by use of a gene gun (four immunizations with 36 μg of DNA) and boosted with gp120 protein (100 μg in Freund's incomplete adjuvant) (67). The data from our laboratory and the other two laboratories strongly suggest a difference in the quality of antibodies elicited by DNA prime and protein booster immunizations and favor the prime/boost vaccination approach.

Most of our animals reached plateau antibody titers against gp120 after two or three DNA immunizations, and subsequent boosting with corresponding antigens did not increase the titers greatly, demonstrating that in vivo electroporation is an efficient means of DNA delivery. Low titers of JR-FL NAbs were detected readily during DNA vaccination (Table 3), suggesting that DNA vaccination is better than protein immunization in eliciting NAbs, while subsequent protein immunization boosts the quantity of these NAbs. It is unclear why there is a difference. The inference is that DNA immunization presents an epitope(s) on gp120 in a conformation that is found on the envelope trimer more favorably than does protein immunization. This makes sense since NAbs are directed to the envelope trimer. This neutralizing epitope appears to be largely isolate specific, since JR-FL virus was neutralized but other primary isolates were not. However, the epitope does not involve the V1/2 loops, since they were replaced by the MPER in the immunogen used here. Furthermore, competition experiments appeared to eliminate the V3 loop, at least as the sole contributor to the JR-FL neutralizing epitope recognized by the sera. This leaves the V4 and V5 regions as the best candidates for the isolate-specific neutralizing epitope. We performed competition assays with V4 and V5 peptides matching the JR-FL sequence, and at 50 μg/ml, the peptides did not reduce the neutralizing activity of the rabbit antisera against JR-FL virus (data not shown). It is uncertain whether the NAbs are directed to previously undescribed conformational epitopes in V4 and V5 that cannot be blocked by linear peptides, to epitopes requiring the presence of sugar residues that would not be present on the synthetic peptides, or to epitopes outside the variable regions. However, if the neutralizing epitope is part of the relatively conserved regions of gp120, why are the NAbs so isolate specific? One possible explanation is that each neutralization-resistant primary isolate has a unique pattern of N-linked glycosylation (69) on the envelope spike which influences the exposure or folding of the epitope, thus allowing the isolate to escape antibodies targeting conserved regions. In any case, protein immunization does not display this neutralizing epitope as well as DNA immunization. Presumably, preparation of the protein disturbs the epitope or glycosylation of the recombinant protein adversely affects epitope presentation.

In summary, we have illustrated that monomeric gp120 can tolerate major modifications, including the replacement of the V1/2 region with the highly hydrophobic MPER sequence, without losing its overall immunogenicity. Therefore, monomeric gp120 has potential as a scaffold for further engineering to present broadly neutralizing epitopes. The 4E10 epitope is one of the most promising broadly neutralizing epitopes for engineering. However, its poor immunogenicity has yet to be overcome and may be associated with its hydrophobicity. Finally, DNA vaccination appears to be the most direct and reproducible method for eliciting homologous NAbs against neutralization-resistant viruses. Further investigation to understand the humoral responses elicited by DNA vaccination is warranted.

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