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Evaluation of Envelope Vaccines Derived from the South African Subtype C Human Immunodeficiency Virus Type 1 TV1 Strain

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Human immunodeficiency virus type 1 (HIV-1) subtype C infections are on the rise in Sub-Saharan Africa and Asia. Therefore, there is a need to develop an HIV vaccine capable of eliciting broadly reactive immune responses against members of this subtype. We show here that modified HIV envelope (env) DNA vaccines derived from the South African subtype C TV1 strain are able to prime for humoral responses in rabbits and rhesus macaques. Priming rabbits with DNA plasmids encoding V2-deleted TV1 gp140 (gp140TV1ΔV2), followed by boosting with oligomeric protein (o-gp140TV1ΔV2) in MF59 adjuvant, elicited higher titers of env-binding and autologous neutralizing antibodies than priming with DNA vaccines encoding the full-length TV1 env (gp160) or the intact TV1 gp140. Immunization with V2-deleted subtype B SF162 env and V2-deleted TV1 env together using a multivalent vaccine approach induced high titers of oligomeric env-binding antibodies and autologous neutralizing antibodies against both the subtypes B and C vaccine strains, HIV-1 SF162 and TV1, respectively. Low-level neutralizing activity against the heterologous South African subtype C TV2 strain, as well as a small subset of viruses in a panel of 13 heterologous primary isolates, was observed in some rabbits immunized with the V2-deleted vaccines. Immunization of rhesus macaques with the V2-deleted TV1 DNA prime/protein boost also elicited high titers of env-binding antibodies and moderate titers of autologous TV1 neutralizing antibodies. The pilot-scale production of the various TV1 DNA vaccine constructs and env proteins described here should provide an initial platform upon which to improve the immunogenicity of these subtype C HIV envelope vaccines.

Human immunodeficiency virus type 1 (HIV-1) subtype C is the most prevalent strain in the HIV epidemic and is mainly distributed in Sub-Saharan Africa, India, and parts of China (6, 16, 17, 25). Together, these areas comprise the majority of the global HIV-infected population. Among the 37.8 million HIV-1-infected individuals worldwide, an estimated 25 million live in Sub-Saharan Africa, and another 7.4 million in Southeast Asia. All seven countries in southern Africa, where subtype C HIV strains are predominant, report prevalence rates above 17%, with Botswana and Swaziland reporting prevalence rates above 35% (25). India has also experienced a rapid spread of HIV-1 subtype C infections, which are predicted to increase in the coming years (20). It is thus critical to design a safe and effective prophylactic vaccine to control the spread of HIV-1 subtype C infections in Sub-Saharan Africa and Asia.

An effective vaccine against HIV may require blocking or limiting HIV infection by virus-neutralizing antibodies and other immune mechanisms of protection in addition to cytotoxic T lymphocytes (2, 14). The induction of broadly reactive neutralizing antibodies to primary HIV-1 strains may prevent HIV infection by blocking the initial stage of infection (8). Although it is difficult to elicit broadly neutralizing antibodies against primary HIV or simian immunodeficiency virus strains in primates (11, 16), there are reports that prime/boost vaccine regimens can completely prevent immunodeficiency virus infection by inducing effective neutralizing antibody responses. For example, priming chimpanzees with replication-competent adenovirus-HIV gp160 recombinants, followed by boosting with HIV-1 SF2 gp120 in MF59 adjuvant, elicited high titers of serum antibodies capable of neutralizing homologous and heterologous primary HIV-1 isolates in vitro. This finding correlated with in vivo protection of the animals against multiple, intravenous viral challenges (19, 31). Thus, the induction of broadly reactive neutralizing antibody responses holds promise as a mechanism for blocking immunodeficiency virus infections in primates.

We have pursued a modified-envelope HIV vaccine approach based on deleting the second hypervariable region (V2) from HIV envelope immunogens. This approach is based on several observations that suggest that the V2 loop may mask conserved regions of the envelope involved in viral entry and susceptibility to neutralization (10, 22, 23, 27, 30). For instance, Stamatatos and Cheng-Mayer have shown that a partial deletion of the V2 loop from the subtype B HIV SF162 virus

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renders the resulting mutant virus, HIV SF162ΔV2, susceptible to neutralization by monoclonal antibodies whose epitopes are located within the CD4-binding site and other conserved regions of gp120 (22). Perhaps more importantly, HIV SF162ΔV2 was shown to be more susceptible to neutralization than the wild-type SF162 by sera collected from patients infected with subtype B and non-subtype B HIV-positive sera, presumably by exposing neutralization epitopes which may otherwise be masked by the V2 loop (22). Further, Wyatt et al. have shown that deleting the V1 and V2 loops enhances virus entry, a phenomenon that is particularly significant upon deletion of the V2 loop (30). Moreover, binding studies with the CD4-inducible-epitope-specific monoclonal antibodies, 17b and 48d, indicate that the V1/V2 loops mask the CCR5 coreceptor binding region of the envelope (23, 30). Similar results were obtained by Johnson et al., who demonstrated that envelopes without V1 and V2 loops can mediate fusion with CCR5+ cells in the absence of the CD4 receptor; this finding also suggests that the V1/V2 loops may mask coreceptor binding sites in the envelope (10).

The V1 and V2 loops also contain several potential N-linked glycosylation sites which may help HIV escape neutralization by masking the conserved neutralizing antibody epitopes, as recently described in the "glycan shield" model proposed by Wei et al. (27). Thus, we also reasoned that removal of N-linked glycosylation sites by deletion of the V2 loop or deletion of both V1 and V2 loops would result in improved env immunogens with increased exposure of epitopes that may be relevant in vaccination against HIV. Previous studies have shown that priming rhesus macaques with SF162ΔV2-envelope-encoding DNA vaccines, followed by boosting with o-gp140SF162ΔV2, confers partial protection against a pathogenic SHIVSF162P challenge (1, 3, 4). Whereas deletion of the V2 loop does not seem to enhance the immunogenicity of envelope antigens derived from T-cell-line-adapted, X4-tropic HIV strains such as HXB2 (13), we consider V2-loop deletions of R5-tropic derived envelopes to be a highly promising approach for designing improved immunogens for HIV vaccine development.

We describe here the production and immunogenicity of wild-type, V1- and V2- deleted subtype C HIV envelope immunogens derived from the South African R5-tropic subtype C TV1 primary HIV isolate. We show that priming with a DNA vaccine expressing subtype C gp140 with the deleted V2 region, followed by boosting with the corresponding oligomeric protein, elicits the highest env-specific antibody titers among the tested immunogens. We further show that the subtype C TV1 immunogens elicit homologous neutralizing antibodies and some indication of cross-reactive neutralizing activity against a panel of heterologous subtype B and subtype C primary isolates. The production of these TV1 immunogens provides an initial platform upon which to improve the immunogenicity of subtype C HIV envelope vaccines through further engineering and improvements in production and delivery and through the use of more effective adjuvants.

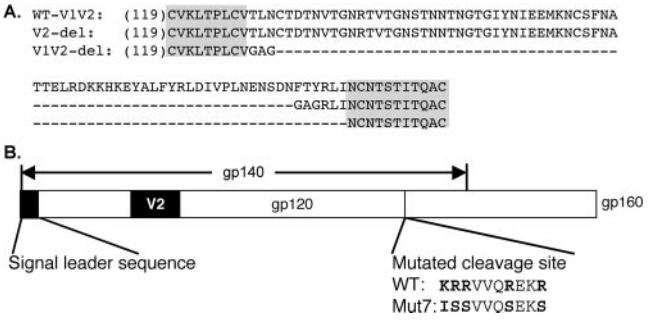

FIG. 1. Construction of the subtype C envelope immunogens. (A) Amino acid sequences of full-length and modified subtype C envelope immunogens. ΔV2 and ΔV1V2 have 32- and 76-amino-acid deletions, respectively, compared to the full-length sequence. The larger ΔV1V2 deletion removes seven additional potential N-linked glycosylation sites. GAG amino acid sequences were inserted into the deleted sites. (B) Diagram of gp140 and gp160. gp140 is a secreted form. The gene sequence, including the signal leader, was sequence modified. The cleavage site mutation was introduced for production of the oligomeric gp140 protein immunogens.

MATERIALS AND METHODS

Amplification of native subtype C HIV envelope DNA sequences. Native subtype C HIV envelope DNA sequences were amplified from eight full-length, HIV-1 molecular clones derived from the peripheral blood of subtype C HIV-1-infected individuals at Tygerberg Hospital, University of Stellenbosch, in South Africa. These infectious clones have been previously shown to utilize the CCR5 coreceptor for virus entry into host cells (24). Based on the reported proviral DNA sequences (32), PCR primers were designed to amplify and clone the native gp140 and gp160 envelope DNA sequences (primer sequences are available upon request). All primers were used at 40 pmol per reaction. The amount of proviral DNA template was 300 ng, and deoxynucleoside triphosphates at 250 μM each. For gp160 the PCR conditions were 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 65°C for 30 s, and 72°C for 5 min. For gp140 the conditions were 95°C for 2 min for the initial cycle, followed by 30 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 4 min using *Pfu* polymerase (Stratagene), and a final extension of 72°C for 7 min.

Criteria for selecting the TV1 subtype C HIV envelope DNA sequence. In order to select a functional subtype C HIV envelope sequence for further HIV immunogen design, the amplified native subtype C HIV envelope sequences were first screened for high gp140 protein expression and for functional gp160 cell fusion activity and CD4 binding. For this purpose, PCR products were digested with EcoRI/XhoI and subsequently cloned into the Chiron pCMVlink vector. The pCMVlink vector is described in detail below, together with the methods for transient transfection, protein quantitation and functional assays. Plasmids encoding native gp140 were transiently transfected into 293T cells, and gp140 protein expression was quantitated 48 h posttransfection in cell lysates and in supernatants. Plasmids encoding gp160 were transiently transfected into 293T cells, and the transfectants were used in a subsequent gp160 cell fusion assay to corroborate envelope function. When possible, gp140 binding to CD4 was also assessed. Based on these criteria, the TV1c8.2 clone was chosen as the best functional native envelope sequence to derive further subtype C envelope immunogens. The TV1c8.2 envelope sequence is hereafter referred to as "TV1."

Cloning full-length or V2-modified subtype C HIV envelope variants. The native TV1 gp160 DNA sequence was next modified by using sequence changes that included the substitution of native codon usage with that of highly expressed human genes (9). These sequence-modified gp160 genes were then synthesized either in full-length form or by incorporating a V2-deletion or a larger V1/V2 deletion, as shown in Fig. 1A. These full-length or V2-modified gp160 variants were then used as the PCR templates to derive the shorter, synthetic gp140 and gp120 variants, as illustrated in Fig. 1B. For the PCR, the 5′ primer was 5′-AGCACGGAATTCATGCGCGTGATGGGCACCCAG-3′, and the 3′ primers were 5′-AGCACGCTCGAGTTAGATGTACCAGGGCCAGTTGCT GATG-3′ for gp140 and 5′-AGCACGCTCGAGTTAGCGCTTCTCGCGCTG CACCACGCG-3′ for gp120. The PCR conditions were 95°C for 3 min, followed by 30 cycle reactions (95°C for 30 s, 56°C for 30 s, and 72°C for 3 min for gp120 and 4 min for gp140), and a final extension of 72°C for 7 min. Then, 250 μM concentrations of each deoxynucleoside triphosphate and 40 pmol of both end primers were used in a 100-μl reaction.

The synthetic PCR products were then digested with EcoRI/XhoI and cloned into the pCMVlink vector for DNA vaccine delivery or into the pCMVIII vector for protein production. The pCMVlink vector contains the cytomegalovirus (CMV) immediate-early enhancer and promoter, a polylinker for multiple clon-

TABLE 1. Immunization groups for the rabbit study[a]

Category	Group	Plasmid DNA prime at wk 0, 4, and 20	Protein boost at wk 20, 49, and 59
Subtype B gp140	1	pCMVgp140ΔV2.SF162	o-gp140ΔV2 SF162
Subtype C gp160	2	pCMVgp160.TV1	o-gp140 TV1
	3	pCMVgp160ΔV2.TV1	o-gp140ΔV2 TV1
	4	pCMVgp160ΔV1V2.TV1	o-gp140ΔV1V2 TV1
Subtype C gp140	5	pCMVgp140.TV1	o-gp140 TV1
	6	pCMVgp140ΔV2.TV1	o-gp140ΔV2 TV1
	7	pCMVgp140ΔV1V2.TV1	o-gp140ΔV1V2 TV1
Combined subtypes B and C	8	pCMVgp140ΔV2.TV1	o-gp140ΔV2 TV1
		pCMVgp140ΔV2.SF162	o-gp140ΔV2 SF162
	9	pCMVgp140ΔV1V2.TV1	o-gp140ΔV1V2 TV1
		pCMVgp140ΔV2.SF162	o-gp140ΔV2 SF162

[a] TV1 here refers to the modified genes derived from TV1c8.2.

ing sites, a bovine growth hormone poly(A) termination signal, a kanamycin resistance gene, and an *Escherichia coli* origin of replication. The pCMVIII vector contains the same promoter and termination signal as the pCMVlink vector. In addition, it contains Mdhfr (dihydrofolate reductase) and neomycin resistance genes that allow for stable transfection, selection, and amplification of the gene of interest in Chinese hamster ovary (CHO) cell lines. This vector also contains the ampicillin resistance gene and a simian virus 40 origin of replication. Similar methods were used to clone an envelope gene (E0975) derived from published subtype C sequences from Botswana (data not shown). The E0975 envelope was used in the fusion assay.

Subtype C envelope protein expression. Transient transfection of the pCMVlink DNA vaccine delivery constructs described above was performed in 293T cells. Cells were seeded in six-well plates at a density of 5×10^5 cells/well. A 100-μl transfection mixture containing 10 μl of transIT-LT1 transfection reagent (Mirus Corp., Madison, Wis.) and 2 μg of DNA was added into each of six wells per construct. The plates were incubated at 37°C for 4 to 6 h. Transfection medium was then aspirated and complete Dulbecco modified Eagle medium was added. After 48 h of incubation, the supernatant from the six wells was recovered, pooled, and passed through a 0.45-μm-pore-size filter. Cells in each well were also lysed in 40 μl of 1% NP-40, and the lysates were pooled. The amount of HIV envelope proteins in the supernatants and in cell lysates was quantitated by using the format of an HIV gp140 antigen capture enzyme-linked immunosorbent assay (ELISA) that has previously been described in detail (21), with known concentrations of gp120TV1 proteins used to plot a standard curve. Oligomeric, intact gp140 proteins or gp140 proteins with V2 deletions were produced and purified as described elsewhere in detail (21).

Cell fusion and CD4-binding assays. gp160-mediated cell-to-cell fusion was assayed by using a fluorescent dye transfer system. Briefly, 293T cells were transfected with 2 μg of gp160-expression vectors using FuGENE-6 (Roche Molecular Biochemicals) in 100-mm culture dishes. Envelope-expressing 293T cells were replated at 6 h posttransfection onto coverslips in growth medium at 37°C. At 12 h posttransfection, the cells were labeled with 2 μM CMTMR (5-(and -6)-(((4-chloromethyl)benzoyl)amino)tetramethyl rhodamine; Molecular Probes, Inc., Eugene, Oreg.) for 30 min at 37°C. Separately, PM-1 cells (provided by Paolo Lusso) were labeled with 2 μM CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes) for 30 min at 37°C. The labeled cells were washed, resuspended in fresh medium, incubated for 30 min at 37°C, washed twice, and resuspended in growth medium. CMFDA-labeled PM-1 cells and CMTMR-labeled env-expressing 293T cells were cocultured at a ratio of 10:1. After 8 h of cocultivation, the cells were examined by using a Nikon Eclipse E800 epifluorescence microscope equipped with a charge-coupled device camera. CMTMR was visualized with an EF-4 G-2E/C TRITC (tetramethyl rhodamine isothiocyanate) filter cube (excitation, 528-553 nm; excitation, 600-660 nm). CMFDA was visualized with an EF-4 B-2E filter cube (excitation, 450-490 nm; excitation 520-560 nm). Cells were also viewed under bright-field microscopy to assess overlapping cells. For each experimental condition, an entire coverslip was examined with the ×10 objective lens to assess the distribution of green and red fluorescence. Representative fields were then captured by using the ×20 or ×40 objective lens. For each field, three images (bright field, CMTMR fluorescence, and CMFDA fluorescence) were captured directly into Photoshop software.

Binding of oligomeric gp140 or gp160 to the CD4 molecule was assessed by using an in-house high-pressure liquid chromatography-based assay that uses fluorescein isothiocyanate-conjugated CD4. CD4 binding is scored as a shift in migration of the fluorescein isothiocyanate-labeled CD4 protein peak. This method has been previously described in detail to assess the CD4 binding of subtype B envelope proteins (21)

Immunization of rabbits. Nine groups of four rabbits each were used in this immunogenicity study. All animals were primed with the indicated sequence-modified envelope DNA vaccines and boosted with oligomeric (o-) envelope gp140, as outlined in Table 1. Because of our previous results with the subtype B V2-modified envelope immunogens (1, 3, 4, 21), group 1 animals were used as a reference control and were primed with the subtype B gp140SF162ΔV2 DNA and boosted with the o-gp140SF162ΔV2. Animals in groups 2 through 7 were immunized with various subtype C envelope immunogens, whereas animals in groups 8 and 9 were immunized with a combination of subtype B and C immunogens. Of the subtype C-immunized animals, groups 2, 3, and 4 were primed with gp160 variants, whereas groups 5, 6, and 7 were primed with gp140 variants. All DNA priming immunizations were delivered in the Chiron pCMVlink vector.

Three doses of DNA, at 1 mg of DNA per animal per immunization, were administered by needle injection at two sites in the quadriceps of each rabbit. Immunizations were given at 0, 4, and 20 weeks. After each DNA injection, electroporation was performed by using a six-needle circular array with 1-cm diameter, 1-cm needle length. Six electroporation pulses were given at 23.3 V/mm, with a 50-ms pulse length and 1 pulse/s, using electroporation methods described in detail elsewhere (18, 28). The first protein boost was administered intramuscularly, in the gluteus, at week 20, which was concurrent with the third DNA immunization. Two additional boosts containing 50 μg of oligomeric proteins formulated in MF59 were administered at weeks 47 and 59 at two sites on the gluteus. Sera were collected before each immunization; 4 weeks after the first immunization; 2, 4, 8, 12, and 16 weeks after the second immunization; and then 2 and 4 weeks after each additional immunization.

Envelope-specific antibody titers in rabbit sera. Three different ELISAs were run to measure titers of envelope-specific antibodies in rabbit sera. The 96-well plates were coated with either subtype C monomeric TV1 gp120, subtype C oligomeric TV1 ΔV2 gp140, or subtype B oligomeric SF162ΔV2 gp140 at a concentration of 1 μg/ml. Serially threefold-diluted rabbit sera were then added, and peroxidase-conjugated goat anti-rabbit (1:20,000; Sigma) was used for detection. Tetramethyl benzidine (KPL) was used as the substrate, and the antibody titers were read at 450 nm. Titers are reported as the highest serum dilution yielding an optical density of 0.5 absorbance units.

Immunogenicity of subtype C TV1ΔV2 variants in rhesus macaques. DNA vaccines encoding TV1ΔV2 gp140 were tested in combination with DNA vaccines encoding subtype C-derived *gag* and *pol* in rhesus macaques (*Macaca mulatta*). Three groups of macaques were included in this nonhuman primate study. Group 1 animals ($n = 4$) received three immunizations with 2 mg of pCMVgp140TV1ΔV2 and 3.5 mg of pCMVgagSF2 at 0, 4, and 8 weeks, followed by two boosts with 100 μg o-gp140TV1ΔV2 protein in MF59 adjuvant at 30 and 45 weeks. Groups 2 ($n = 3$) and 3 ($n = 4$) did not receive the env DNA primes but instead received three DNA immunizations with 3.5 mg of pCMVgag plus 4.2 mg of pCMVpol or 5.0 mg of pCMVgagpol, respectively; these groups also received two intramuscular immunizations with 100 μg o-gp140TV1ΔV2 protein

in MF59 adjuvant at 30 and 45 weeks. DNA injections were always followed by electroporation, as described above for the immunization of rabbits.

HIV neutralizing antibody assays. (i) GFP-reporter cell assay. Green fluorescent protein (GFP)-reporter cell assays were performed to assess neutralizing antibody responses elicited by DNA priming immunizations. Neutralizing activity was assessed against peripheral blood mononuclear cell-grown SF162ΔV2 and the heterologous TV2 HIV isolate using an in-house GFP-reporter cell line assay that uses the 5.25.EGFP.Luc cell line developed by N. Landau. This cell line expresses CD4, CCR5, CXCR4, and BONZO receptor/coreceptors on its cell membrane and is therefore susceptible to infection by R5-tropic HIV (N. Landau, unpublished data). The gene encoding green fluorescence protein (GFP) is under the control of the HIV-long terminal repeat, so that HIV-infected cells can be detected by flow cytometry using a FACSCalibur. For the virus neutralization assay, 50 μl of titrated virus (the selective virus dilution was based on the linearity of the positive control titer, as well as the value of the negative control), and 50 μl of diluted immune or preimmune serum (1:20 dilution) were incubated at 37°C temperature for 1 h. This mixture was added into wells containing 10^4 of cells/ml plated in a 24-well plate and incubated at 37°C for 5 to 7 days. The cells were then fixed with 2% of formaldehyde after being washed with phosphate-buffered saline. Fifteen thousand events were collected for each sample on a Becton Dickinson FACSCalibur using CellQuest software. The data presented are the mean of the triplicate wells. Controls were virus incubated without rabbit sera (virus control) and cells incubated without virus and without sera (cell control). The percent virus inhibition was calculated by using the following equation: percent virus inhibition = [(GFP in virus control − GFP in experimental sera)/(GFP in virus control − GFP in cell control)] × 100. Any background virus inhibition observed in the prebleed has been subtracted for each individual animal. Values of ≥50% are considered significant virus inhibition. Animals whose sera yielded significant inhibition were categorized as "positives."

(ii) M7-luc assay. Neutralizing antibody titers against TV1 and SF162 were assessed at the centralized NIAID-NIH-sponsored laboratory facility, Duke University, by using an M7-luciferase-based neutralization assay (15). Briefly, the assay used 5.25.EGFP.Luc.M7 cells and reductions in luciferase reporter gene expression as a measurement of neutralization. The cells express CD4, CXCR4, CCR5, and GPR15/Bob and contain Tat-responsive luciferase reporter genes and GFP. A total of 5,000 50% tissue culture infective doses of virus were used. Test sera were added in triplicate serial dilutions in 96-well flat-bottom culture plates. After incubation for 1 h at 37°C, cells were added. The virus control contained only cells plus virus and no sera, while the background control contained cells only. After 3 days of incubation, 10% of the cells in the virus control wells were positive for GFP expression by fluorescence microscopy. At this time, a 100-μl suspension of cells was transferred to a 96-well white solid plate (Costar) for the measurement of luminescence using Bright Glo substrate solution (Promega) and a model Victor 2 luminometer (Perkin-Elmer, Shelton, Conn.). Neutralization titers are the highest serum dilution at which the relative luminescence units (RLU) were reduced 50% compared to virus control wells (no serum sample) after subtraction of background RLU.

(iii) TZM-bl assay. The breadth of neutralizing antibodies in sera was assessed by using a panel of 13 molecularly cloned pseudoviruses and a luciferase reporter gene assay in TZM-bl cells (15). The gp160 envelope sequences of the cloned pseudoviruses were derived from six subtype B and seven subtype C, R5-tropic, sexually transmitted, primary isolates from the United States (6101.10, 3988.25, 6535.3, and BG1168.1), Trinidad and Tobago (QH0692.42), Italy (PVO.4), South Africa (Du123.6, Du151.2, Du156.12, Du172.17, and Du422.1) and Zimbabwe (ZM18108M.6 and ZM14654M.7). All parental viruses were isolated by peripheral blood mononuclear cell coculture and are from early seroconverters that lacked a detectable homologous neutralizing antibody response at the time of virus isolation. Pseudovirus stocks were prepared by cotransfection of 293T cells with the pSG3.delta.env plasmid and the indicated gp160 env constructs. Serum samples were screened at the centralized NIAID-NIH-sponsored laboratory facility at Duke University by using a 1:15 serum dilution in triplicate. Reported values are the percent reduction in RLU relative to a 1:10 dilution of corresponding preimmune serum (in the case of rabbits) or preprotein immunization serum (in the case of rhesus macaques). Values of ≥50% are considered positive for virus neutralization. To check for non-HIV specific neutralizing antibody activity in some of the rhesus macaque immune sera (Table 3), we tested threefold serial dilutions of immune sera against murine leukemia pseudotyped viruses (murine leukemia virus [MuLV] gp70 was derived from the 4070A amphotropic MuLV strain [provided by N. Landau]).

Statistical analysis. Analysis of variance (ANOVA) was used to compare the treatment group means within a time point and to compare mean responses between time points, with two exceptions (see Fig. 6 and 7C) that used a nonparametric test. The statistical tests were performed in PC SAS version 9.1 (SAS Institute, Inc., Cary, NC). The ANOVA F-statistic was used to assess significance at the 5% level. If the overall F-statistic was significant, pairwise comparisons between two groups or two time points were conducted (within the ANOVA model) using linear contrasts and *P* values obtained from a "*t* distribution" (referred to as *t* test here). For the neutralization data against HIV-1 TV1 strain on the macaques (Fig. 7C), some observations were left-censored by titer 20, the lowest dilution of the assay. Here a nonparametric method, the Kruskal-Wallis test, was used to compare the three groups.

For each of the analyses, the number of subjects within each group is small (three to four animals). Thus, there is low statistical power to observe small differences between groups or time points. Throughout the present study, two-tailed *P* values are cited. A result is considered significant if the *P* value is <0.05 (5% level of significance). However, if the *P* value is between 0.05 and 0.10 (10% level of significance), the results are interpreted as an indication of a possible difference in treatment group means.

RESULTS

Suitability of TV1 as a functional subtype C envelope sequence. Subtype C HIV envelope sequences were amplified from R5-tropic viruses derived from the peripheral blood of HIV-infected patients in South Africa. The gp140 and gp160 proviral sequences were cloned and expressed in transiently transfected 293T cells. Expression levels of gp140 were highest for the TV1c8.2, TV1c8.5, and TV2c12.1 clones, with the TV1c8.2 clone yielding microgram quantities of gp140 in both the cell lysate and the supernatant (data not shown). However, of these three highest-envelope expressing candidate sequences, only the TV1c8.2 sequence was able to mediate cell-to-cell fusion (green and red dye colocalized) when gp160 was cloned and transfected into 293T cells (Fig. 2G to I). This indicates that the other two envelope sequences were not similarly functional. CD4 binding of gp140 and gp160 proteins derived from these clones also indicated that the TV1c8.2 clone encoded a functional subtype C envelope sequence (I. Srivastava and S. Barnett, unpublished). Phylogenetic analysis of the TV1c8.2 DNA sequence placed this clone firmly in the cluster of subtype C HIV sequences; thus, TV1c8.2 is not an intersubtype recombinant form (5). This *env* gene exhibits amino acid sequence identity of ca. 80% (range, 76 to 82%) with other cloned and sequenced *env* genes from Africa and Asia (5). Based on these phylogenetic properties and functional criteria, we chose TV1c8.2 as a subtype C envelope sequence for subsequent experiments and modifications of subtype C envelope immunogens. For brevity, we refer to this sequence as "TV1."

Expression of sequence-modified TV1 envelope gp140 sequences. We previously demonstrated that removal of the V2 loop enhanced the immunogenicity of subtype B SF162ΔV2 envelope immunogens (1). Therefore, the V2 loop of the subtype C TV1 sequence was removed. In addition, TV1 immunogens were constructed by deleting both the V1 and the V2 loops. Figure 1 shows the predicted amino acid sequences and schematic representations of the wild-type TV1 envelope, as well as the variant forms, ΔV2 and ΔV1V2, with 32- and 76-amino-acid deletions, respectively. Whereas the TV1ΔV2 deletion removes an amino acid sequence region similar to that of SF162ΔV2 as well as two predicted N-linked glycosylation sites, the larger ΔV1V2 deletion removes seven additional potential N-linked glycosylation sites. Based on this immunogen design, the native TV1 coding sequence was modified by using codons from highly expressed mammalian genes, and

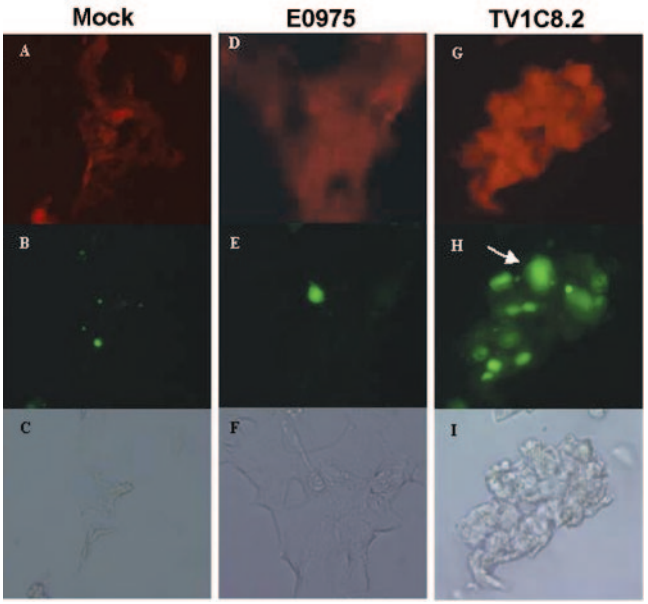

FIG. 2. Fusogenicity of gp160. Red fluorescence (A, D, and G), green fluorescence (B, E, and H), and bright-field (C, F, and I) images were obtained 8 h after coculturing PM-1 cells with 293T cells expressing no *env* gene (mock) or the *env* genes derived from HIV-1-CAR402 or HIV-1 subtype C TV1c8.2. For subtype C gp160TV1c8.2 (G to I), the green fluorescence observed in lymphocytes spreads, forming larger areas of diffuse green fluorescence. The arrowhead indicates an example of colocalization of green and red fluorescence. Controls were PM-1 cells incubated with mock-transfected 293T cells (A to C) or PM-1 cells cultured with consensus-subtype C envelope-E0975-transfected 293T cells (D to F). In these control cocultures, the CMFDA-labeled lymphocytes maintained a uniform, rounded morphology and showed little adherence to the 293T cells. Hardware: Macintosh G4; software: Adobe Photoshop 7.0.

TV1 envelope variants were cloned into the Chiron-pCMVlink vector. Expression in transiently transfected 293T cells showed that the secretion of the sequence-modified gp140 TV1 was approximately an order of magnitude higher than that of the native gp140 sequence and that the mutation of the gp140 cleavage site (mut7) did not abrogate ΔV2gp140 secretion (data not shown). Mutation of a similar cleavage site on SF162ΔV2 allows for the expression of oligomeric gp140 envelope structures (21). Therefore, a similar mutant was constructed for production and purification of subtype C TV1 gp140 oligomers (I. Srivastava and S. Barnett, unpublished).

Immunogenicity of TV1 DNA prime/protein boost regimen in rabbits. The immunogenicity of the various subtype C TV1 env antigens was tested in nine groups of New Zealand White rabbits (Table 1). Animals immunized with HIV-1 subtype B env were included for comparison. All animals were primed with DNA vaccines and boosted with oligomeric protein. In all cases, the prime/boost protocol elicited high titers of subtype C-specific antibodies capable of binding monomeric TV1gp120 (Fig. 3A to D). However, priming with gp140 DNA forms elicited higher ELISA titers than priming with the gp160 DNA constructs (for weeks 6 to 61, groups 2, 3, and 4 versus groups 5, 6, and 7 in Fig. 3A versus B: $P < 0.0001$, *t* test within ANOVA [abbreviated as *t* test]). Notably, the strongest gp140 vaccine regimen was the ΔV2 DNA prime, followed by the ΔV2 boost (group 6), since these animals had significantly higher titers than animals primed and boosted with the ΔV1V2 gp140 immunogens (group 7) ($P = 0.0103$, *t* test) and indications of higher titers than group 5 animals primed and boosted with the intact gp140 immunogens (group 6 versus 5, Fig. 3B: $P = 0.0573$, *t* test). Group 6 animals also had higher TV1-gp120 binding antibody titers than the group 1 animals immunized with gp140ΔV2SF162 ($P < 0.0001$, *t* test). Importantly, combining subtype B SF162 and subtype C TV1 ΔV2 immunogens (Fig. 3C and D) did not lower the subtype C binding antibody titers, since we observed no significant differences between the mean responses of animals in group 6 versus those in group 8 ($P = 0.1089$, *t* test) and in group 7 versus those in group 9 ($P = 0.7742$, *t* test).

Induction of binding antibodies to oligomeric gp140. All immunogen combinations elicited high titers of oligomeric gp140-binding antibodies able to recognize the subtype B SF162 gp140ΔV2 (Fig. 4A), as well as the subtype C TV1 gp140ΔV2 proteins (Fig. 4B). As was the case for monomeric gp120-binding antibodies, higher titers of oligomeric gp140-binding antibodies were elicited by priming with the gp140 forms than with the gp160 forms (2wp2, groups 5, 6, and 7 versus groups 2, 3, and 4: $P = 0.0011$ for binding to gp140ΔV2 TV1 and $P = 0.0063$ for binding to gp140ΔV2 SF162, *t* test). In all cases, the titers of oligomeric gp140-specific binding antibodies increased after the first protein boost (2wp3 versus 2wp2; $P < 0.0001$, *t* test). However, the highest TV-1 gp140-binding titers were detected in group 6 animals immunized with the ΔV2 TV1 variants among the gp140 forms (Fig. 4B, group 6 versus 5 and 7; $P = 0.0183$, *t* test). Priming and boosting with a combination of SF162 and TV1 ΔV2 variants did not reduce the oligomeric TV1 binding titers, since no significant differences were observed between animals in groups 6 and 8 ($P = 0.6712$, *t* test) and in groups 7 and 9 ($P = 0.6840$, *t* test).

Comparison of antibody responses against homologous and related vaccine strains. We next compared the potency of homologous neutralizing antibody responses elicited by the various TV1 vaccine constructs. Because some groups also received the SF162 envs, we included both TV1 and SF162 in this homologous neutralizing antibody potency comparison. All prime/boosted animals, including those immunized with only TV1 immunogens, developed significant neutralizing antibody responses against the neutralization-sensitive SF162 strain (Fig. 5A). Two significant differences in potency were observed at 2 weeks after the second protein boost (2wp4). First, animals primed with the subtype C TV1 gp140 forms had higher titers of SF162 neutralizing antibodies than those primed with the gp160 forms (groups 5, 6, and 7 versus groups 2, 3, and 4, $P = 0.0102$, *t* test). Not surprisingly, animals primed and boosted with the gp140ΔV2SF162 form had higher neutralizing antibody titers to the homologous subtype B strain than those primed and boosted with gp140ΔV2TV1 (group 1 versus group 6, $P = 0.0134$, *t* test). Interestingly, rabbits immunized with the combined V2-modified SF162 and TV1 immunogens (groups 8 and 9) showed indications of higher neutralizing antibody titers than those immunized with the modified TV1 immunogens alone (groups 6 and 7) ($P = 0.0532$, *t* test). However, after the third protein boost (2wp5), the neutralization titer toward SF162 remained at a level sim-

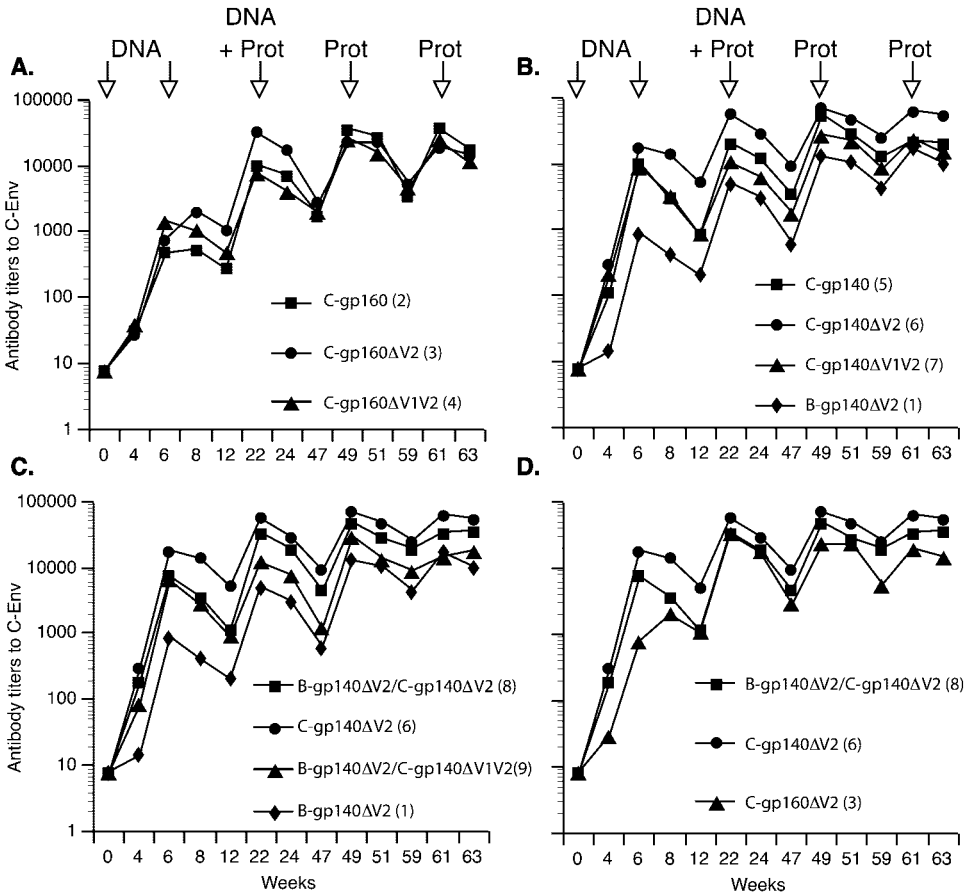

FIG. 3. Antibody-binding titers (geometric mean) to the monomeric subtype C gp120TV1 protein (C-Env). All rabbits developed high titers of subtype C gp120-binding antibodies. Immunization groups are described in Table 1 and are presented here in four panels for purposes of comparison. (A) Groups primed with subtype C gp160 forms: C-gp160 (group 2), C-gp160ΔV2 (group 3), and C-gp160ΔV1V2 (group 4). (B) Groups primed with either subtype B or subtype C gp140 forms: C-gp140 (group 5), C-gp140ΔV2 (group 6), C-gp140ΔV1V2 (group 7), and B-gp140ΔV2 (group 1). (C) Comparison between combination groups and subtype-alone groups: B-gp140ΔV2/C-gp140ΔV2 (group 8), B-gp140ΔV2/C-gp140ΔV1V2 (group 9), C-gp140ΔV2 (group 6), and B-gp140ΔV2 (group 1). (D) Comparison between the C-gp140ΔV2 (group 6), C-gp160ΔV2 (group 3), and combination B-gp140ΔV2/C-gp140ΔV2 (group 8).

ilar to that after the second protein ($P = 0.4121$, t test), and no significant differences were observed among groups ($P = 0.0550$, ANOVA).

We also noted several significant differences in potency of homologous TV1 neutralizing antibodies (Fig. 5B). At 2 weeks after the second protein boost (2wp4), priming with TV1 gp140 elicited higher neutralizing antibody titers than priming with TV1 gp160 immunogens (groups 5, 6, and 7 versus 2, 3 and 4, $P = 0.0025$, t test). Compared to group 1 animals, the subtype C gp140ΔV2TV1 rabbits (group 6) had higher neutralizing antibody titers to the homologous TV1 strain ($P = 0.0073$, t test). Priming with a combination of ΔV2 gp140s from both subtypes (group 8) elicited higher homologous neutralizing antibody titers against the TV-1 strain than priming with the subtype B gp140 alone (group 1) ($P = 0.0067$, t test). gp140ΔV2, as well as gp140ΔV1V2, showed higher titers of homologous neutralization than the intact gp140 (Fig. 6B; group 5 versus group 6, $P = 0.0548$; group 5 versus group 7, $P = 0.0598$ [t test]). However, after the third protein boost (2wp5), TV1 neutralizing antibodies were significantly reduced ($P < 0.0001$, t test), although the difference between gp140 forms and gp160 forms remained (groups 5, 6, and 7 versus groups 2, 3, and 4, $P = 0.0384$, t test). Importantly, gp140ΔV2 showed significantly higher titers of homologous neutralization than the intact gp140 (2wp5, Fig. 5B; group 5 versus group 6, $P = 0.0491$, t test).

In some cases, we were able to assess the contribution of DNA priming to the neutralizing antibody activity against SF162ΔV2 and against the closely related heterologous subtype C TV2 strain (5). In these experiments, HIV neutralization was analyzed in sera collected 2 weeks after the second DNA immunization, when the animals had not received any protein immunizations. Figure 6 shows that all four animals in group 6, those primed with subtype C gp140ΔV2TV1 DNA, had significant serum neutralizing antibody activity against the neutralization-sensitive SF162ΔV2 strain (Fig. 6A) and against the heterologous South African TV2 strain (Fig. 6B). Among the gp140 groups, priming with the ΔV2 gp140 (group 6) was most effective at eliciting heterologous neutralizing antibody activity against TV2 (group 6 versus groups 5 and 7 combined values, $P = 0.0333$ [Kruskal-Wallis test]).

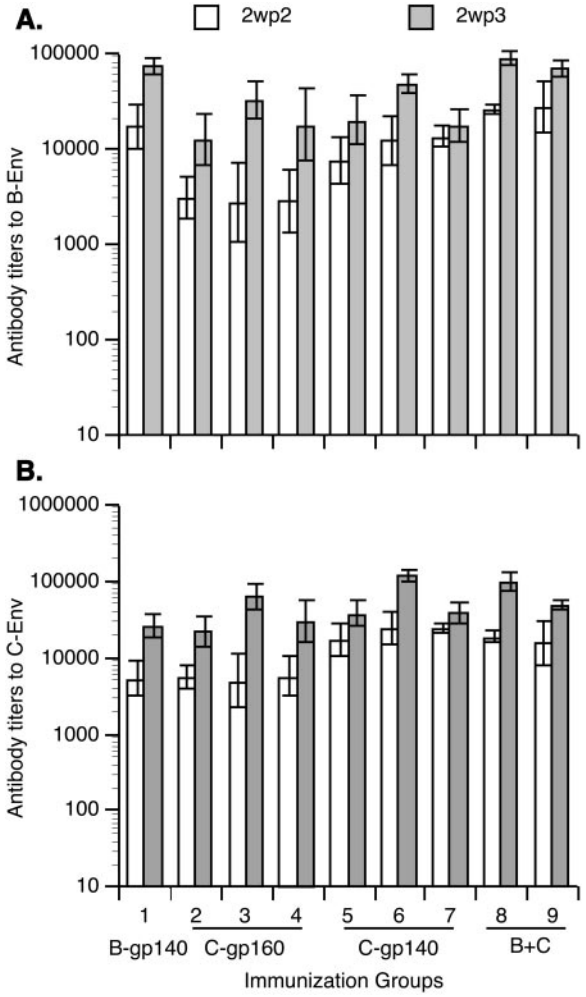

FIG. 4. Titers of vaccine-elicited antibodies (geometric mean) binding to oligomeric env proteins. Antibodies binding to both subtype B SF162 (A) and subtype C TV1 (B) oligomeric proteins were evaluated 2 weeks after the second DNA (2wp2) and 2 weeks after the third DNA and protein (2wp3) immunizations. ELISA plates were coated with either o-gp140ΔV2SF162 (B-Env) or o-gp140ΔV2TV1 (C-Env) proteins. All immunogens elicited high titers of cross-reactive, oligomer-binding antibodies.

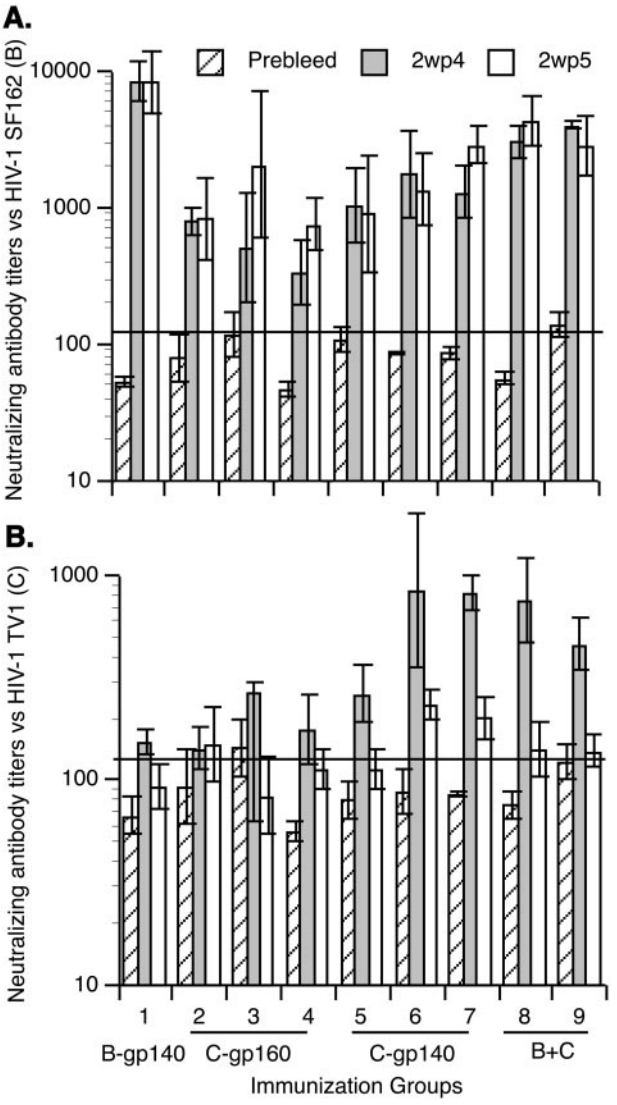

FIG. 5. Neutralizing antibody titers (geometric mean) after the fourth and fifth immunizations. Experiments were performed by using the M7-luc assay. Titers are the reciprocal of the highest serum dilution at which relative RLU were reduced 50% compared to virus control wells (no test sample). Groups are described in Table 1. (A) Neutralizing antibody titers against the neutralization-sensitive HIV-1 subtype B SF162 primary isolate. (B) Neutralizing antibody titers against homologous HIV-1 subtype C TV1 primary isolate. Neutralizing antibodies against subtype C TV1 for the C-gp140ΔV2 (group 6) appeared higher compared to C-gp140 (group 5) at 2 weeks after the second protein boost (2wp4, $P = 0.0548$). After the third protein (2wp5), a significant difference was seen for C-gp140ΔV2 versus C-gp140 ($P = 0.0491$).

Subtype C DNA prime/protein boost immunogenicity study in rhesus macaques. To determine whether the proposed ΔV2TV1 vaccines were immunogenic in nonhuman primates, rhesus macaques were immunized with the HIV subtype C DNA prime/oligomeric protein boost regimen described in Materials and Methods. Figure 7A shows that priming with ΔV2TV1 envelope-encoding DNA vaccines (group 1) elicited TV-1-specific envelope-binding antibodies that were boosted to higher titer after oligomeric ΔV2TV1 protein immunizations in MF59 adjuvant. The potency of neutralizing antibody responses against SF162 and TV1 was also examined after DNA immunizations (week 28) and after env protein immunizations (week 47) (Fig. 7B and C). At the end of the vaccination regimen, animals that had been primed with the envelope gene showed higher neutralizing antibody titers against the neutralization-sensitive SF162 isolate than animals that had been primed with the gag-C-pol DNA ($P = 0.0253$, t test). Although homologous TV1 neutralizing antibody titers were substantially lower (Fig. 7C) than those seen against SF162, TV-1 envelope-primed animals displayed significantly higher TV-1 neutralizing antibodies than those primed with gag and pol ($P = 0.0323$, Kruskal-Wallis test) or with the gag-C-pol DNA vaccines ($P = 0.0132$, Kruskal-Wallis test).

Neutralizing antibody activity against heterologous HIV-1 strains. To determine whether the subtype C ΔV2TV1 immunogens could elicit cross-reactive neutralizing antibodies

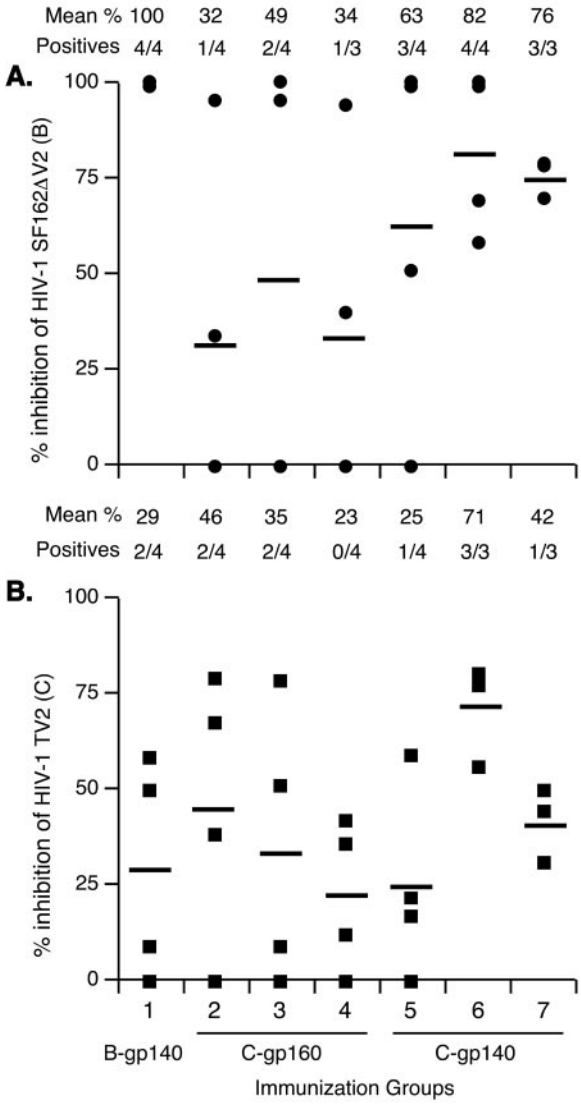

FIG. 6. Neutralizing antibody activity in sera after priming with DNA. Experiments were performed by using the GFP-reporter cell assay. The percent virus inhibition indicates the percent reduction in GFP-expressing cells at a 1:20 serum dilution as described in Materials and Methods. (A) Neutralization activity against the subtype B mutant SF162 ΔV2 virus at 2 weeks after the second DNA immunization. (B) Neutralization activity against the heterologous subtype C TV2 isolate. Priming with the C-gp140ΔV2 (group 6) was more effective at eliciting TV2-neutralizing antibodies (group 6 versus groups 5 and 7: $P = 0.0333$).

against heterologous HIV-1 strains, we screened sera from rhesus macaques and selected groups of rabbits against a panel of 13 HIV-env pseudotyped viruses derived from sexually transmitted, R5-tropic, geographically diverse, primary isolates. Table 2 shows that the ΔV2 TV1 DNA prime/protein boost approach used here elicited low levels of neutralizing activity against heterologous strains in rhesus macaques. An apparent exception was macaque 1.3, whose serum had neutralizing activity against three subtype B and five subtype C HIV strains. However, further testing revealed that this activity could be attributable to non-HIV-specific antiviral activity, since it was also observed against MuLV gp70-pseudotyped viruses (Table 3). Background antiviral activity in these neutralization assays could be demonstrated at titers of >15 in three of four rhesus macaques when ID50 (50% reduction in RLU) values were used as the cutoff and in one of four macaques when ID80 (80% reduction in RLU) values were used. The presence of this nonspecific activity made it impossible to determine whether any specific activity against heterologous HIV-1 isolates was induced in these macaques.

In the rabbit study, animals immunized with the TV1 gp140ΔV2 were able to neutralize the heterologous TV2 strain (Fig. 6B) but showed limited breadth against a panel of 13 additional heterologous primary strains (Table 2). Interestingly, heterologous neutralizing activity was observed in rabbit 38, which received a combined immunization with both the SF162 and TV1 ΔV2 gp140 antigens. Neutralizing antibody activity was often observed against HIV 6535.3, an early isolate from Washington, D.C., that is unusually sensitive to neutralization by V3-specific monoclonal antibodies. The pilot studies described here were performed with early process DNA vaccine and oligomeric env protein preparations. The results obtained thus far suggest that further enhancements in immunogen engineering, production, adjuvant formulation, and delivery will be necessary to provide more consistent induction of neutralizing activity against non-vaccine-related HIV-1 strains.

DISCUSSION

This study illustrates our strategy for selecting HIV-1 envelope immunogens for advancement. Ideally, envelope immunogens should resemble those of the ongoing HIV epidemic, be derived from primary isolates using the CCR5 coreceptor, show high expression and functionality in vitro, and be able to elicit high titer, durable, and functional antibody responses, ultimately, against the diverse heterologous (nonvaccine) HIV-1 strains that characterize the epidemic in the population to be vaccinated. As is the case with several HIV envelope vaccines in the research and development pipeline, the TV1 env vaccines described here meet most of the above criteria, except for the generation of breadth in virus neutralization assays. Cross-reactive neutralizing antibody responses were infrequently observed in animal studies (Table 2), suggesting that these subtype C immunogens may require additional modifications in vaccine design to reach a consistent pattern of elicitation of broad neutralizing antibody responses. Production and purification of the TV1 env protein from stable mammalian cell lines, the use of improved adjuvants and delivery systems, and the construction of recombinant TV1-adenovirus and alphavirus vectors are some of the approaches we are currently pursuing to address the present limitations of breadth in terms of heterologous neutralizing antibody responses.

The global diversity of HIV strains is a major challenge in anti-AIDS vaccine development. There have been many attempts to identify an effective envelope sequence based on ancestral sequences (12), consensus sequences (7), or circulating sequences (26, 29). In theory, a subtype consensus sequence, compared to an individual circulating viral sequence, may share higher similarity to the different strains within a

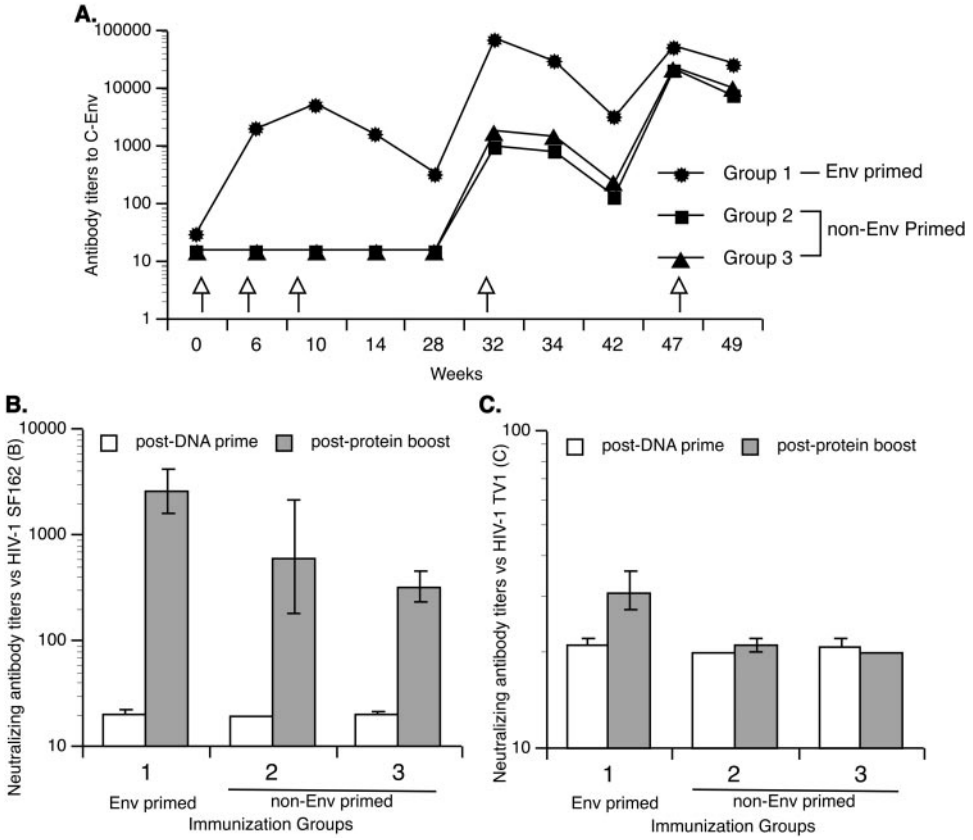

FIG. 7. Humoral immune responses in rhesus macaques immunized with subtype C immunogens. (A) Oligomeric env-binding antibody titers (geometric mean) during the course of immunization. ELISA was performed by using o-gp140ΔV2 TV1 (C-Env) as described in Materials and Methods. (B) Neutralizing antibody titers against HIV-1 subtype B SF162. (C) Neutralizing antibody titers against HIV-1 subtype C TV1. The M7-luc assay was performed as described in Materials and Methods. Titers are the reciprocal of the highest serum dilution at which RLU were reduced 50% compared to virus control wells (no test sample). Two time points were used to evaluate neutralization titers: 2 weeks before the first protein immunization (post-DNA) and 2 weeks after the second protein immunization (post-protein). Group 1 animals were immunized by using a gp140TV1ΔV2 DNA prime/protein boost regimen. Animals in groups 2 and 3 were not primed with gp140TV1ΔV2 DNA but with gag and pol DNA vaccines and given o-gp140TV1ΔV2 protein boosts.

subtype and thus may be more relevant to the common characteristics and epitopes of the different strains. However, our attempt at the selection of a virtual clone, based on published subtype C sequences derived from Botswana, did not result in the expression of functional protein that would bind to CD4 and facilitate fusion (Fig. 2D to F), although this clone exhibited good expression (data not shown). Therefore, it is likely not straightforward that an envelope created from published sequences would necessarily be functional and hence structurally relevant to envelope derived from circulating virus strains. In addition, there is still no clear data on whether consensus or ancestral sequences can offer an advantage over a circulating strain.

The envelope immunogens described herein were derived from a circulating subtype C HIV strain, TV1. The TV1 isolate was identified from a panel of primary isolates from South Africa by evaluating the levels of expression and function of cloned envelope sequences in vitro. We anticipated that in vitro gene expression levels in transfected cell cultures could give indications of expression and immunogenicity in vaccinated animals. We also reasoned that an envelope with intact cell-cell fusion activity and CD4-binding functions could present epitopes that mimic the native env conformation. Moreover, high-level expression of antigens from DNA and other gene delivery vaccines would be expected to generate higher titers of binding antibodies and potentially improved neutralizing antibody responses. Therefore, we chose to modify the codon usage of the TV1 *env* gene variants to conform to that of highly expressed mammalian genes. All of these sequence-modified TV1 *env* genes showed increased expression compared to their native counterparts. In addition to the analysis of viral sequences, expression profiles, and CD4 binding function, the potency of homologous virus neutralization supported our choice of the TV1 sequence. In preliminary experiments, TV1 was relatively difficult to neutralize by different patient sera, including the TV2 sera; however, the TV1 patient serum could easily neutralize the TV2 strain (data not shown). This result suggested the possibility that the TV1 isolate may produce higher quality neutralization antibodies than the other isolates.

An important requirement of HIV envelope vaccine design is to present or expose conserved epitopes for the induction of potent and consistent antibody responses. The TV1 envelope was therefore modified for potential changes in structure. The

TABLE 2. Neutralizing antibody activity against a panel of six subtype B and seven subtype C HIV strains

Species	Immunization group	Animal no.	% Reduction in RLU[a]												
			6101.10 B	QH0692.42 B	PVO.4 B	3988.25 B	6535.3 B	BG1168.1 B	Du123.6 C	Du151.2 C	Du156.12 C	Du172.17 C	Du422.1 C	ZM18108M.6 C	ZM14654M.7 C
Macaque	gp140ΔV2 TV1 + Gag	1.1	0	2	0	0	44	0	0	0	0	6	0	30	0
		1.2	0	5	0	0	32	1	0	1	8	8	0	10	11
		1.3	32	**55**	45	**52**	**68**	34	**67**	**64**	**64**	**54**	35	**53**	46
		1.4	5	0	0	20	10	1	13	0	0	41	11	18	5
	Gag + Pol	2.1	0	12	0	22	35	0	15	0	27	27	18	30	26
		2.2	30	31	**62**	26	44	13	8	17	38	10	9	25	21
		2.3	0	0	0	0	0	0	0	0	0	0	0	12	0
	Gag − Pol	3.1	15	0	13	31	0	10	18	0	29	12	11	13	5
		3.2	0	0	0	35	35	0	7	0	47	1	21	28	23
		3.3	11	46	7	9	26	0	24	14	41	20	9	31	24
		3.4	0	23	0	26	42	0	26	8	24	23	13	23	23
Rabbit	gp140ΔV2 SF162 (group 1)	1.1	0	0	0	10	**65**	0	6	0	20	10	1	20	38
		1.2	27	**54**	36	41	**60**	31	24	15	36	15	35	24	43
		1.3	0	0	0	0	49	0	0	0	0	0	0	0	0
		1.4	**56**	**62**	**51**	0	**66**	30	0	1	0	0	39	0	41
	gp140 TV1 (group 5)	5.1	0	0	0	0	0	0	0	0	0	0	0	0	0
		5.2	0	0	0	0	2	0	0	0	10	0	1	0	2
		5.3	0	13	0	0	**53**	0	0	0	0	0	0	0	16
		5.4	5	0	0	13	**54**	0	5	0	0	0	0	0	46
	gp140ΔV2 TV1 (group 6)	6.1	0	18	0	16	28	0	5	0	0	0	0	0	13
		6.2	0	0	0	0	15	0	0	0	0	0	0	0	0
		6.3	22	24	0	24	46	6	17	12	45	27	33	14	**50**
	gp140ΔV2 TV1+SF162 (group 8)	8.1	0	0	0	3	42	0	22	12	16	16	8	32	38
		8.2	18	26	0	0	**50**	6	**51**	5	**53**	24	3	27	25
		8.3	0	17	0	0	**57**	0	0	0	0	0	0	13	19
		8.4	0	39	0	0	**71**	0	46	8	6	32	7	39	40

[a] Postprotein samples (i.e., 2 weeks after the fifth immunization) were screened at a 1:15 dilution in triplicate in TZM-bl cells. Values are the percent reduction in RLU relative to the corresponding preimmune sera (in rabbits) or post-DNA sera (in macaques). Neutralizing activity values of ≥50% reduction in RLU are indicated in boldface.

TABLE 3. Comparison of HIV-specific and non-HIV-specific neutralizing antibody activities in macaque sera[a]

Animal	ID50 in TZM-bl cells		ID80 in TZM-bl cells	
	MuLV	Du123.6	MuLV	Du123.6
1.1	17	<15	<15	<15
1.2	53	52	17	<15
1.3	33	46	<15	<15
1.4	<15	<15	<15	<15
Positive control[b]	>50	0.25	>50	2.70

[a] Values are the reciprocal of the highest serum dilution or lowest monoclonal antibody concentration at which RLU were reduced 50% (ID50) or 80% (ID80) compared to virus control wells (no test sample).

[b] Threefold dilutions of monoclonal antibody 4E10, ranging from 50 to 0.02 μg/ml, were tested. The indicated concentrations were required to reduce RLU by 50 or 80%.

TV1 strain has a relatively longer V1 loop than the subtype B SF162 strain and contains six more N-linked glycosylation sites. As proposed in the "glycan shield" model suggested by Wei et al., additional glycosylation sites may help HIV escape neutralization by masking the exposure of conserved neutralizing antibody epitopes (27). Thus, we reasoned that removal on N-linked glycosylation sites by deletion of the V2 loop or deletion of both V1 and V2 loops would result in a better env immunogen, one with better exposed neutralizing and/or non-neutralizing epitopes. Studies of homologous TV1 neutralization showed that, at 2 weeks after the fourth immunization, the V1 and V2-deleted gp140 structures appeared to elicit higher titers of homologous neutralizing antibody responses than the intact gp140 (Fig. 5B; group 5 versus group 6, $P = 0.0548$; group 5 versus group 7, $P = 0.0598$). At 2 weeks after the fifth immunization, although all groups showed reduced TV1 neutralization, gp140 ΔV2TV1 was significantly better than intact gp140TV1 in inducing homologous neutralization ($P = 0.0491$). Even before the protein immunizations, DNA priming with ΔV2TV1 gp140 resulted in neutralization activity against the heterologous subtype C TV2 strain in all four immunized rabbits compared to fewer animals in the intact TV1 gp140 group (Fig. 6B). Also, gp140ΔV2 elicited the highest titer of oligomeric env binding antibody titers among all of the TV1 gp140 forms at 2wp3 (Fig. 4B, group 6 versus group 5 and 7 combined values, $P = 0.0183$). These results suggest that V2 deletions may expose epitopes that can enhance the potency of neutralizing and non-neutralizing antibody activity. The antibody binding results and the neutralizing antibody activities measured against TV1, SF162, and TV2 indicated that the gp140 with V2 deleted was the most immunogenic of all genes tested. In addition, immunization of rabbits with V2-deleted gp140s from both the subtype C TV1 and subtype B SF162 yielded high titers of env-binding antibodies and neutralizing antibodies against both the subtype B and C vaccine strains, HIV-1 SF162 and TV1, respectively, indicating that these two immunogens can be effectively combined in a multivalent vaccine approach.

Based on these results in rabbits, the gp140ΔV2TV1 immunogen was further evaluated into a pilot study in nonhuman primates. Rhesus macaques primed with gp140 ΔV2TV1 envelope DNA and boosted with the corresponding protein developed high titers of env-specific binding antibodies and neutralizing antibodies against the neutralization-sensitive, subtype B SF162 strain, as well as modest titers of neutralizing activity against the subtype C TV1 vaccine strain. These responses were higher than those observed in two other unprimed groups that received two protein immunizations alone. Breadth assessments of the immune sera were performed with a panel of HIV pseudoviruses derived from early seroconverters. One of the four macaques from the first group exhibited broadly neutralizing activity against three sexually transmitted R5-tropic clade B strains from Trinidad and the United States and four sexually transmitted R5-tropic clade C HIV strains from Durban and Zimbabwe (Table 2). However, this activity appeared to be non-HIV specific, since it was observed against MuLV pseudotypes, underscoring the importance of including a non-HIV control in screening assays. Elicitation of HIV-specific, consistent, and broad neutralizing antibody responses continues to be a major challenge that we hope to overcome, at least in part, with improved vaccine adjuvants and more efficient delivery systems.

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