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Comparable Antigenicity and Immunogenicity of Oligomeric Forms of a Novel, Acute HIV-1 Subtype C gp145 Envelope for Use in Preclinical and Clinical Vaccine Research

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ABSTRACT

Eliciting broadly reactive functional antibodies remains a challenge in human immunodeficiency virus type 1 (HIV-1) vaccine development that is complicated by variations in envelope (Env) subtype and structure. The majority of new global HIV-1 infections are subtype C, and novel antigenic properties have been described for subtype C Env proteins. Thus, an HIV-1 subtype C Env protein (CO6980v0c22) from an infected person in the acute phase (Fiebig stage I/II) was developed as a research reagent and candidate immunogen. The gp145 envelope is a novel immunogen with a fully intact membrane-proximal external region (MPER), extended by a polylysine tail. Soluble gp145 was enriched for trimers that yielded the expected "fan blade" motifs when visualized by cryoelectron microscopy. CO6980v0c22 gp145 reacts with the 4E10, PG9, PG16, and VRC01 HIV-1 neutralizing monoclonal antibodies (MAbs), as well as the V1/V2-specific PGT121, 697, 2158, and 2297 MAbs. Different gp145 oligomers were tested for immunogenicity in rabbits, and purified dimers, trimers, and larger multimers elicited similar levels of cross-subtype binding and neutralizing antibodies to tier 1 and some tier 2 viruses. Immunized rabbit sera did not neutralize the highly resistant CO6980v0c22 pseudovirus but did inhibit the homologous infectious molecular clone in a peripheral blood mononuclear cell (PBMC) assay. This Env is currently in good manufacturing practice (GMP) production to be made available for use as a clinical research tool and further evaluation as a candidate vaccine.

IMPORTANCE

At present, the product pipeline for HIV vaccines is insufficient and is limited by inadequate capacity to produce large quantities of vaccine to standards required for human clinical trials. Such products are required to evaluate critical questions of vaccine formulation, route, dosing, and schedule, as well as to establish vaccine efficacy. The gp145 Env protein presented in this study forms physical trimers, binds to many of the well-characterized broad neutralizing MAbs that target conserved Env epitopes, and induce cross-subtype neutralizing antibodies as measured in both cell line and primary cell assays. This subtype C Env gp145 protein is currently undergoing good manufacturing practice production for use as a reagent for preclinical studies and for human clinical research. This product will serve as a reagent for comparative studies and may represent a next-generation candidate HIV immunogen.

Human immunodeficiency virus (HIV) vaccine candidates capable of eliciting broad, protective humoral immune responses would significantly advance prevention strategies to control the AIDS pandemic. The diversity of circulating HIV type 1 (HIV-1) envelope (Env) sequences and structures has complicated HIV-1 immunogen design and has impaired the development of a globally efficacious vaccine. Env diversity of up to 20% within a subtype and up to 35% between subtypes has been reported (1), leading vaccine developers to focus on common antigenic features most frequently represented on infectious viral particles. The role of the subtype in cross-protective responses has long been a subject of debate, but numerous studies have indicated that the HIV-1 subtype can play a role in the nature of the functional antibody responses elicited by both vaccination and infection (2–4). Subtype C accounts for >50% of the current global infections (5, 6) and for the majority of incident cases in southern Africa. Subtype C has also been shown to exhibit greater kinetics, magnitude, and breadth of neutralizing antibody (NAb) development than other subtypes during the course of natural infection (3, 7, 8). Early NAb responses in humans to subtype C infection are predominantly strain specific, targeting the V1V2 and C3 regions of gp120, and can be quite potent (8–11). Subtype C Env proteins from the acute infection phase also appear to have certain genetic features in common, such as shorter gp120 variable regions and fewer potential N-linked glycosylation sites, indicating selective pressure at transmission, where infection may be established by a single infectious particle (3, 5, 6, 12, 13). Subtype C

envelopes derived from the acute infection phase are therefore of considerable interest for vaccine design.

The development of broadly reactive NAbs occurs after 2 to 3 years in only 10 to 30% of infected individuals (14–18) and can be mediated by a single or a small number of antibody specificities (19). Some of these broadly reactive antibodies target quaternary neutralizing epitopes (QNEs) that exist in the context of trimeric Env (10). Monoclonal antibodies (MAbs) that show preferential binding to the Env quaternary structure, such as the PG9, PG16, PGT141-145, and CH01-04 MAbs, recognize epitopes and glycosylation motifs in V1, V2, and V3 (20–22) and have particularly potent antiviral activity. Animal studies have demonstrated that protection is achieved with relatively low titers of HIV-specific antibodies if the antibodies are targeted to the native Env trimer on the virion surface (14, 23). NAbs targeting QNEs, if elicited via vaccination, may prevent infection in humans if present at sufficient titers prior to exposure to HIV. The development of HIV Env trimers to elicit these functional antibodies has thus become a critical priority for HIV vaccine development.

The RV144 human clinical trial was the first to achieve HIV prevention, with an estimated 31.2% vaccine efficacy at 42 months following initial immunization. Monomeric gp120, in combination with an ALVAC canarypox prime, provided protective immunity; however, broadly NAbs were not found to be a correlate of efficacy (24, 25). In the posttrial analysis of correlates of protective immunity, V1V2 IgG binding titers were found to correlate inversely with HIV-1 acquisition (24, 26, 27), with vaccine-induced responses influencing amino acid composition of V2 at positions 169 and 181 (28). Additionally, nonneutralizing V1V2-specific IgG3 antibodies were found to mediate polyfunctional antiviral activity through FcR-mediated effector function, indicating that both the specificity and the quality of V1V2-specific responses may have contributed to protection. Given this encouraging but limited success with gp120 monomers, it is currently thought that the use of trimeric Env vaccines will improve functional antibody responses, as they potentially harbor QNEs not found on gp120 monomers. Kovacs et al. demonstrated that trimeric gp140 Env proteins of both subtypes A and C elicited NAbs capable of more potent neutralization of tier 1 viruses than the matched monomeric gp120 proteins (29). These Env proteins have been engineered to form trimers through the addition of trimerization motifs such as the T4 bacteriophage fibritin "foldon" (Fd) domain (29). Recent studies using stable, cleaved subtype A trimers have indicated that cleavage can influence whether or not soluble Env trimers adopt more native conformations that appear to mimic the trimer on the virion surface (30). Negative-stain electron microscopy (EM) at 5.8 Å resolution suggested that cleaved trimers form homogeneous structures. In particular, antigenicity data indicate that these structures may resemble native Env spikes on virus particles. These aggregate-free, MPER-truncated SOSIP Env trimers have thus been extensively characterized for antigenicity and structure (30–34). However, a direct comparison of cleaved, stabilized, and uncleaved trimeric Env proteins, in terms of immunogenicity in nonhuman primates or other relevant animal systems, has not yet been published. Thus, it remains to be determined which form of Env will be the most immunogenic and, most importantly, which will demonstrate protective antibody response levels in humans.

Here we describe the research grade production and the antigenic and immunogenic features of a mammalian-cell-produced, acute subtype C HIV-1 envelope immunogen. This protein, which was down-selected from four East African HIV-1 subtype C strains, was derived from an individual in the acute phase of HIV-1 infection. The gp145 protein assembles into physical trimers with no engineered trimerization motifs and binds many of the well-described broadly neutralizing MAbs. CO6980v0c22 gp145 Env is currently undergoing good manufacturing practice (GMP) production for use in research, preclinical studies, and human clinical trials and may represent a next-generation HIV vaccine immunogen.

MATERIALS AND METHODS

Design and cloning of gp145 *env* gene for protein expression. Envelope sequences were derived from two acute-phase HIV-1 subtype C infected individuals and one early seroconverting HIV-1 subtype C-infected individual. Four *env* genes from subtype C R5 HIV-1 strains (CO6838v1c48, CO6980v1c3, CO6980v0c22, and CO3728v2c6) were codon optimized and synthesized by GeneArt, AG (Regensburg, Germany). To maximize expression in Chinese hamster ovary (CHO) cells, the *env* genes were synthesized incorporating *Cricetulus griseus* (Chinese hamster) codon bias. *Cis*-acting motifs that may reduce translational efficiency (i.e., internal TATA boxes, chi sites, ribosomal entry sites, RNA secondary structure, and repeat sequences) were eliminated from the sequences. Two versions of each *env* gene were synthesized, each without the native signal peptide, (i) full-length gp160 and (ii) delta cleavage (DC) full-length gp160 with substitutions in the gp120/gp41 cleavage sites (R503S, R508S, and R511S for CO6980v0c22 and CO6980v1c3 and R508S and R511S for CO6838v1c48 and CO3728v2c6) to prevent protease cleavage. For production of the selected gp145 protein, the CO6980v0c22 gp160DC gene was selected and modified to terminate at position 648, after the YIK motif within the MPER domain, and to include a three-lysine C-terminal extension. The synthesized, codon-optimized *env* genes were cloned into mammalian expression plasmid pSWTIPK3 in frame with the tissue plasminogen activator signal peptide.

Comparison of *env* gene expression for downselection. CHO-K1 cells and human embryonic kidney (HEK293H) cells were transiently transfected with the gp160 and gp160DC *env* genes by lipofection (Lipofectamine 2000; Invitrogen, Carlsbad, CA). Protein expression was evaluated in both culture medium and cell lysates by antigen capture enzyme-linked immunosorbent assay (ELISA) with human anti-gp41 MAb 4E10

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and immunoblot analysis of 4E10-immunoprecipitated Env, followed by detection with pooled gp160 (subtype B)- and gp120 (subtype C)-immunized rabbit serum.

Expression and purification of the CO6980v0c22 gp145 protein. CHO-K1 cells were electroporated with NruI-linearized pSWCO6980v0c22 gp145 DNA, and stably transfected cultures were selected with puromycin. Stable cell lines were cloned and established by limiting dilution, and HIV Env expression was assessed as described above. The selected CHO cell line expressing CO6980v0c22 gp145 was then adapted for growth in protein-free medium. Expressing cells were expanded to volumes of 3 to 30 liters, and conditioned medium was clarified by centrifugation and concentrated by tangential-flow filtration. Lectin affinity chromatography with *Galanthus nivalis* lectin agarose (Vector Laboratories, Burlingame, CA), followed by anion exchange on Q-Sepharose fast flow (GE Healthcare, Little Chalfont, United Kingdom), was used to purify gp145 from the supernatant. The protein was then further concentrated, buffer exchanged into phosphate-buffered saline (PBS), and sterile filtered. Protein purity and final concentration were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the Bradford assay, respectively. Gel filtration column chromatography (with tandem Superose 6 and Superdex 200 26/60 columns [GE Healthcare, Pittsburgh, PA]) was used to separate the different oligomeric forms of gp145 on the basis of previously described procedures (35). Each lot of pooled fractions was then buffer exchanged into PBS and filtered with a 0.22-μm filter. To determine the purity and the oligomeric forms present, each pooled fraction was analyzed by blue native PAGE (BN-PAGE). Five micrograms of each gp145 fraction was resolved on a 4 to 16% Novex Bis-Tris gel (Invitrogen, Grand Island, NY) and visualized via Coomassie blue staining. To further clarify the identities of the oligomeric forms, 5 μg of purified unfractionated or fractionated gp145 was cross linked with 5 mM ethylene glycol bis(succinimidylsuccinate) (EGS) and resolved by SDS-PAGE (36). Cross-linked phosphorylase *b* (Sigma Chemical Co., St. Louis, MO) was used as a protein molecular mass marker. Proteins were resolved on a 3 to 8% NuPAGE Tris acetate polyacrylamide gel (Invitrogen, Grand Island, NY) under reducing and nonreducing conditions and stained with Coomassie blue. Densitometric analysis was used to estimate the molecular mass of each gp145 species. Endotoxin contamination in all fractions was also assessed by colorimetric *Limulus* amebocyte lysate assay (Lonza, Basel, Switzerland), and levels were <3.3 EU/mg in all of the lots.

Negative-stain cryo-EM and determination of oligomeric species ratios. To determine the oligomeric species ratios by negative-stain EM, 3 μl of the gp145 trimeric fraction in PBS was placed on 300-mesh copper grids coated with continuous carbon film, stained with 2% uranyl acetate, and imaged with a JEOL 1230 120-kV electron microscope at ×40,000 magnification. A total of 31 micrographs were taken. To determine percentages of gp145 monomers, dimers, trimers, and higher-order oligomers, particles were indiscriminately boxed from micrographs by reference-free automated techniques and then reference-free class averaging was performed (37). Particles belonging to class averages resembling each multimer, based on area of intensity, were grouped into respective multimeric classes. Particles displaying a small point in the center of the box were determined to be monomers, while particles displaying two lobes were classified as dimers. Trimeric particles had three clear lobes, and higher-order oligomers appeared as large aggregated particles.

To visualize gp145 in the enriched trimeric fractions by cryo-EM, 3 μl of the gp145 trimer fraction in PBS was placed on 300-mesh copper grids coated with a holey carbon film, plunged into liquid ethane, kept submerged in liquid nitrogen, loaded onto a Gatan cryoholder, and imaged with a JEOL 2100FEG 200-kV electron microscope at ×80,000 magnification. A total of 98 micrographs were taken at an electron dose of 15 $e^{-}/Å^2$ and a sampling step of 1.25 Å/pixel. Reference-free classification was performed (37), and representative class averages were generated in preparation for single-particle reconstruction work, which is the subject of an additional ongoing structural study (Moscoso et al., unpublished data).

BLI and SPR for antigenicity assessment. Env-binding dissociation constants (K_d values) and rate constants were measured on the FortéBio Octet (biolayer interferometry [BLI]) and Biacore 3000 (surface plasmon resonance [SPR]) instruments according to the following procedures and as described previously (38–40). To perform BLI analysis, unfractionated CO69080v0c22 gp145 and trimer and dimer fractions, as well as the CO6980v0c22 gp120 monomer, were biotinylated at a 1:1 molar ratio by mixing with the NHS-PEG4 biotinylation reagents for 2 h on ice according to the manufacturer's instructions (Thermo Fisher, Rockford, IL). Zeba desalt spin columns were then used to remove free biotin, and buffer exchange into PBS was performed. The binding affinities of CO6980v0c22 Env fractions and Env-specific MAbs or sCD4 were measured on a FortéBio Octet Red system (FortéBio, Menlo Park, CA) (41). This system monitors the interference of light reflected from the surface of the sensor and bound biomolecules (41). Binding of IgG molecules to the tethered target results in thickening of the surface, which is monitored in real time. The biotinylated CO6980v0c22 Env fractions were immobilized on streptavidin biosensors at a single concentration of 10 μg/ml. After the baseline was reached, sensors were dipped into six 2-fold dilutions of the MAbs or sCD4 at various concentrations for association. Sensors with analyte-ligand complexes were then moved into kinetics buffer (FortéBio, Menlo Park, CA) to measure dissociation rates. Binding of MAb 17b to the CO6980v0c22 fractions in the presence of sCD4 was due to the exposure of this epitope after Env binding to CD4. The CO6980v0c22 gp145 fractions or gp120 monomer were immobilized on the surface of the biosensors, washed, and then dipped into kinetics buffer containing 75 nM sCD4. The second association phase was performed in the presence or absence of MAb 17b, and dissociation was subsequently performed in kinetics buffer for 1,800 s. Association and dissociation rates were measured in real time and calculated with the Octet Molecular Interaction System software, which fit the observed global binding curves to a 1:1 binding model. Nonspecific binding of the MAb to the sensor and/or a buffer-only reference was subtracted from all curves. Rate constants were calculated by using at least three different concentrations of analyte to achieve a χ^2 value of <1 and an R^2 value of >0.90.

To perform SPR analysis, anti-human IgG Fc antibody (Sigma Chemical Co., St. Louis, MO) was immobilized on a CM5 sensor chip to about 15,000 response units (RU) and each antibody was captured to about 50 to 100 RU on three individual flow cells for replicate analysis, in addition to one flow cell with the control Synagis MAb on the same sensor chip. Nonspecific binding of Env gp145 to the control surface and/or blank buffer flow was subtracted for each MAb-Env binding interaction. The antibody capture level on the sensor surface was optimized for each MAb to minimize rebinding and any associated secondary effects. All curve-fitting analyses were performed by using a global fit of multiple titrations to the 1:1 Langmuir model. Mean rate constants and K_d values were calculated from at least three measurements on individual sensor surfaces with equivalent amounts of captured antibody. All data analysis was performed with the BIAevaluation 4.1 analysis software (GE Healthcare, Pittsburgh, PA).

ELISA measurement of V2 MAb binding. A panel of conformation-dependent V2 MAbs developed from subtype B HIV-infected individuals was used to characterize V1V2 exposure of gp145, including 697 (42), 2158 (43), 2297 (44), 1357 (45), 1361 (45), 1393 (46), and 830A (43), and the irrelevant anti-parvovirus B19 MAb 1418. gp145 protein was used to coat ELISA plates directly, blocked with 2% bovine serum albumin (BSA) in PBS, and incubated with MAbs, starting at 10 μg/ml. The plates were washed, and the bound MAbs were detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG (Fc) (Southern Biotech, Birmingham, AL). After washing, the substrate *p*-nitrophenylphosphate was added and the plates were read at 410 nm.

Flow cytometric assessment of cell-expressed α4β7 binding. The binding of CO6980v0c22 gp145 to α4β7 was assessed by flow cytometry based on a method described by Nawaz et al. (47). Briefly, the α4β7-expressing cell line RPMI8866 was incubated in staining buffer (PBS with

10% normal human IgG and 10% mouse IgG) in the presence or absence of 2 μg of α4 MAb HP2/1 (Beckman Coulter, Indianapolis IN) for 15 min on ice in a 96-well plate. Biotin-labeled gp145 (0.5 μg) was added, and the mixture was incubated for 30 min on ice. Cells were washed twice with staining buffer and then stained with NeutrAvidin-phycoerythrin (PE; Thermo Fisher, Rockford, IL) and β7-fluorescein isothiocyanate (BioLegend, San Diego, CA). Following staining, cells were washed twice and fixed with 4% paraformaldehyde. Data were acquired on an LSR-II flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Animal immunization protocols. Two independent rabbit studies were performed; the first was to assess the effect of various formulations with unfractionated gp145, and the second was to assess differences in the gp145 multimeric fractions in immunogenicity. For study 1, female New Zealand White rabbits were immunized under a protocol approved by the Walter Reed Army Institute of Research Institutional Animal Care and Use Committee (IACUC). Five groups of four rabbits each were immunized intramuscularly in alternating caudal thigh muscles three times at 4-week intervals with 25 μg (groups 1 to 4) or 0 μg (group 5, control) of unfractionated CO6980v0c22 gp145 protein. The three adjuvants tested were (i) aluminum hydroxide (Alhydrogel; Invitrogen, San Diego, CA) at 0.6 mg Al^{3+}/dose, (ii) L(DMPG/MPLA) (dimyristoyl phosphatidylcholine [DMPC]:cholesterol:dimyristoyl phosphatidylglycerol [DMPG]) at a ratio of 9:7.5:1 (50 mM phospholipids containing 100 μg of monophosphoryl lipid A [MPLA]/dose) (48), and (iii) L(PIP/MPLA) (DMPC:cholesterol:phosphatidylinositol-4-phosphate [PIP] at a ratio of 1:1.5:1 (50 mM phospholipids containing 100 μg of MPLA/dose) (49). Unfractionated gp145 was formulated as follows: group 1, aluminum hydroxide plus gp145 (aluminum hydroxide mixed with gp145); group 2, L(DMPG/MPLA) plus gp145 [L(DMPG/MPLA) mixed with gp145]; group 3, L(DMPG/MPLA plus gp145) [L(DMPG/MPLA)-encapsulated gp145] (50); group 4, L(PIP/MPLA plus gp145) [L(PIP/MPLA)-encapsulated gp145]; group 5, L(DMPG/MPLA) [L(DMPG/MPLA) without gp145]. Blood was collected at 2-week intervals starting 2 weeks before the first immunization and ending 2 weeks after the last immunization. Serum was stored at −80°C until analysis. For study 2, five groups of four female New Zealand White rabbits were immunized intramuscularly with different purified multimeric forms of gp145 protein, all formulated by mixing aluminum hydroxide with gp145 by using the same method, schedule, and dosing as described for study 1 (see Table 3). The following groups were analyzed: group 1, unfractionated gp145; group 2, dimeric gp145; group 3, trimeric gp145; group 4, higher-order oligomeric gp145; group 5, aluminum hydroxide alone. The study 2 immunization protocol was approved by the ABL, Inc., IACUC.

ELISA assessment of binding antibodies from immunized rabbit sera. Binding antibody responses of rabbits to the following HIV Env antigens were measured by ELISA as described previously (51): gp120 subtype C (C.ZA.1197MB; Immune Technology Corp., New York, NY), 77.01% sequence identity to CO6980v0c22; gp140 subtype C (CN54; Polymun, Klosterneuburg, Austria), 79.74% sequence identity to CO6980v0c22; gp41 subtype B (IIIB; Abcam, Cambridge, MA), 81.65% sequence identity to CO6980v0c22; gp145 subtype C immunogen, 100% sequence identity to CO6980v0c22; cyclic V2 peptides from the immunizing CO6980v0c22 strain; V2 Con C, 74.36% sequence identity to CO6980v0c22. Briefly, 96-well Immulon 2 U-bottom microtiter plates were coated with proteins or peptides overnight, blocked with 5% milk in PBS containing 0.1% Tween 20, washed, and incubated for 1 h at room temperature with serum from individual rabbits in triplicate. The plates were then washed, and bound antibody was detected with horseradish peroxidase-labeled, affinity-purified goat anti-rabbit IgG and 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate. Plates were read at 405 nm on a SpectraMax 250 plate reader (Molecular Devices, Sunnyvale, CA). Matched prebleed sera were used as the negative control for each animal. Endpoint titers were calculated as the highest dilution that yielded an optical density reading greater than or equal to twice the background value after subtraction of the mean absorbance of triplicate wells lacking antigen from wells containing antigen.

ELISA binding to scaffolded HIV-1 envelope V1V2. Binding ELISAs were performed as previously described (24). Briefly, Immulon 4 plates were coated with 1 μg/ml gp70-V1V2 overnight at 4°C and then washed six times with PBS containing 0.05% Tween 20, pH 7.4. Plates were incubated for 1.5 h at 37°C with sera diluted in blocking medium (2% BSA, 5% fetal bovine serum, 5% sheep serum in PBS). The plates were washed six times, and then horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA) diluted 1:6,000 in PBS containing 0.05% Tween 20 was added and the mixture was incubated for 1.5 h at 37°C. After washing, 3,3′,5,5′-tetramethylbenzidine substrate (Bio-Rad Laboratories) was added for 2 min of incubation at room temperature to develop color and the reaction was stopped with 50 μl of H_2SO_4. The plate A_{450}s were read with respect to an A_{570} reference. All reagents were added in a volume of 100 μl/well; at least two experiments were performed.

Binding by BLI to V3 and MPER peptide. Autologous biotinylated gp145 V3 (TRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAY) and MPER (RNEKDLLALDSWNNLWNWFDISNWLWYIKKKK) peptides (GenScript) were immobilized on SAX sensors (FortéBio) at concentrations of 6.25 and 12.5 μg/ml, respectively, for 250 s. After the baseline was established, the loaded sensors were dipped into duplicate wells of rabbit serum diluted 1:8 in sample diluent buffer (FortéBio) until saturation, for approximately 800 s. MAbs 447-52D and 4E10 were used as positive controls for each peptide, which yielded significant binding to V3 and MPER, respectively. Binding responses (nanometer) were monitored in real time and quantified at the end of the association step with the Octet Molecular Interaction System software.

TZM-bl neutralization assay. Pseudovirus (PV) neutralization assays were performed as previously described (7, 52). Briefly, PVs were produced with a pSG3ΔEnv DNA plasmid encoding the HIV backbone and a plasmid encoding either the homologous CO6980 *env* or heterologous *env* genes, including GS015 (subtype C), MW965 (subtype C), SF162 (subtype B), BZ167 (subtype B), CM235 (subtype CRF01_AE), NI1046 (subtype CRF01_AE), DU422 clone1 SVPC5 (subtype C), ZM197M.PB7 SVPC6 (subtype C), ZM214M.PL15 SVPC7 (subtype C), TZA246 (subtype C), PBL288 (subtype C), TZBD9/11 (subtype C), and murine leukemia virus (MuLV) (nonspecific control). The following plasmids were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: MW965 (catalog no. 3094), SF162 (catalog no. 10463), DU422 clone1 SVPC5 (catalog no. 11308), ZM197M.PB7 SVPC6 (catalog no. 11309), and ZM214M.PL15 SVPC7 (catalog no. 11310). Rabbit serum or MAbs were tested for the ability to neutralize the HIV Env-containing PVs in the single-cycle TZM-bl neutralization assay. The neutralization activity of postimmunization serum was determined on the basis of the reduction of *luc* reporter gene expression and compared to that obtained in wells containing virus and preimmunization serum. Relative light units (RLU) were detected with the EnVision luminometer (PerkinElmer Life Sciences, Shelton, CT), and percent neutralization (percent reduction of RLU) was calculated for each serum dilution or MAb concentration. Neutralization dose-response curves were fitted by nonlinear regression, and the final titer is reported as the reciprocal of the dilution of serum necessary to achieve 50% neutralization (50% inhibitory dose [ID_{50}]). As a negative control, a PV containing MuLV *env* was used to evaluate nonspecific viral inhibition of serum. For MAbs, the concentration of antibody required to obtain 50% neutralization (the 50% inhibitory concentration [IC_{50}]) is reported.

PBMC neutralization assay. Peripheral blood mononuclear cell (PBMC) neutralization assays were performed as previously described, with replication-competent, *Renilla reniformis* luciferase (LucR)-expressing HIV-1 reporters with an NL4-3 backbone (49, 53) or with full-length *env* in a native backbone (54). Briefly, each infectious molecular clone (IMC) was produced with a DNA plasmid encoding the *env* expressed in either the native backbone (CO6980 [subtype C], ETH2220 [subtype C], or CM235 [subtype CRF01_AE]) or an NL4-3 HIV-1 backbone express-

ing the ectodomain of either SF162 or BaL *env*. IMC stocks were diluted to a concentration that yielded $>1 \times 10^5$ RLU at 4 days postinfection. Serum and virus were incubated together for 1 h prior to the addition of 1×10^5 phytohemagglutinin- and interleukin-2-stimulated PBMCs. Following overnight incubation at 37°C, additional medium was added and the culture was again incubated at 37°C for 72 h. Cultures were then lysed, and substrate was added in accordance with the manufacturer's instructions (*Renilla* luciferase assay system; Promega, Corp., Madison, WI). RLU were measured with the EnVision luminometer. Neutralization dose-response curves were fitted by nonlinear regression, and the final titer is reported as the reciprocal of the dilution of serum necessary to achieve 50% neutralization.

Nucleotide sequence accession number. The CO6980v0c22 gp160 HIV-1 Env sequence has been deposited in GenBank under accession number KR261061.

RESULTS

Acute subtype C Env selection. Four full-length *env* gene sequences derived from plasma from one early-phase and two acute-phase subtype C HIV-1-infected individuals were codon optimized for expression in mammalian cells and synthesized to produce two constructs, gp160 (full-length Env without the native signal peptide) and gp160DC (gp160 with mutations in the gp120/gp41 cleavage sites to disrupt processing). CHO-K1 (data not shown) and HEK293H (see Fig. S1 in the supplemental material) cells were transiently transfected with the two constructs for each of the four strains, and Env expression was assessed after 48 h. The gp160 and gp160DC proteins were detected by antigen capture ELISA at low levels in the medium (presumably associated with microvesicles) and cell lysates of CHO-K1 cells for two of the four strains (CO6980v0c22 and CO3728v2c6) (data not shown). Expression by HEK293H cells was detected in the medium for all Env proteins except CO6838v1c48, and similar amounts of Env protein were observed in the cell lysates for all Env proteins by ELISA-based antigen capture with MAb 4E10, which recognizes an epitope within MPER (see Fig. S1A in the supplemental material). MPER accessibility on HEK293H-expressed Env proteins was compared by antigen capture (see Fig. S1A in the supplemental material) and immunoprecipitation (IP) with MAb 4E10, followed by Western blotting with a subtype C polyclonal serum (see Fig. S1B in the supplemental material). Expression of HIV-1 BaL Env was detected in both cell lines and served as the positive control; no protein was detected in nontransfected cells (negative control), as expected. CO6980v0c22 gp160 and gp160DC demonstrated the highest expression, both by antigen capture of protein in the medium (presumably associated with microvesicles) (see Fig. S1A in the supplemental material) and by IP and Western blot analysis (see Fig. S1B in the supplemental material). Processing of gp41 was observed only for the CO6980v0c22 isolate, with gp41 evident in cells transfected with the gp160 construct (see Fig. S1B, red arrow). CO6980v0c22 Env was selected for further development on the basis of the apparent higher Env protein expression levels and superior MAb 4E10 detection of gp160 and gp41 species. In addition to higher expression levels, the selection of the CO6980v0c22 Env clone was obtained from a person at Fiebig stage I in the acute phase of HIV-1 infection.

Separation and characterization of multimeric forms of CO6980v0c22 gp145. CO6980v0c22 gp145 Env, here referred to gp145, was produced by truncating CO6980v0c22 gp160DC Env prior to the transmembrane domain, at position 648, and then extending the C terminus by three additional lysine (K) residues. The three lysine residues distinguish gp145 from other gp140 Env proteins and were added to the C terminus of gp145 to increase hydrophilicity and to attempt to enhance the exposure of the MPER domain (55, 56). Alignment of a subtype C consensus sequence (Con C) produced from the Los Alamos database for HIV-1 Env sequences with the CO6980v0c22 gp145 Env sequence (see Fig. S2 in the supplemental material) reveals similarities. The gp145 protein was expressed and purified to >90% homogeneity from CHO cell culture supernatant as described in Materials and Methods. The unfractionated preparation was resolved by BN-PAGE, and the various multimers present under native conditions are shown in Fig. 1A. This analysis demonstrated trimers and dimers to be the major multimeric forms of gp145 (Fig. 1A, lane 1). By tandem gel filtration chromatography, the gp145 higher-order oligomers (Fig. 1A, lane 2), trimers (Fig. 1A, lane 3), and dimers (Fig. 1A, lane 4) were further purified and electrophoresed by nondenaturing BN-PAGE. SDS-PAGE analysis of cross-linked or untreated gp145 mixtures or purified species was utilized to confirm the nature of the different enriched or purified multimers. The fully reduced, monomeric protein migrates on SDS-PAGE at an approximate molecular mass of 145 kDa (Fig. 1B). The different oligomeric fractions were cross linked with 5 mM EGS and then separated with a Tris-acetate NuPAGE gel under nonreducing conditions. In Fig. 1B, the specified oligomeric forms are demonstrated for the unfractionated and reduced (lane 1), unfractionated non-cross-linked (lane 2), unfractionated cross-linked (lane 3), enriched higher-order oligomeric (lane 4), cross-linked purified trimeric (lane 5), and cross-linked purified dimeric (lane 6) forms. The migrations of the gp145 multimers on BN-PAGE are greater than those predicted by the standards used and greater than those observed on SDS-PAGE. This is a phenomenon that has been observed by others (31, 57) and might be explained by the large amount of glycan present on Env and/or physical or chemical properties of the different gel systems.

EM with fresh preparations in vitreous ice was then used to generate a class-averaged, top-down view of each of the oligomeric species found in both unfractionated or enriched trimeric fractions of gp145, as shown in Fig. 1C (1, monomer; 2, dimer; 3, trimer; 4, higher-order oligomer). By this negative-stain analysis, class average-derived percentages of species visualized in the enriched trimeric fraction of the CO6980v0c22 gp145 protein were determined (see Fig. S3A in the supplemental material for a representative micrograph); 59.7% of the enriched trimeric fraction was estimated to exist as true physical trimers (see Fig. S3B). These different species appear to exist in a state of dynamic equilibrium. Thus, while a substantial amount of trimers remains, since this protein was not cleaved and was not physically stabilized by an Fd motif or engineered disulfide bonds, approximately 40% of the protein in this preparation existed as nonnative structures.

Antigenicity of CO6980v0c22 gp145. The dissociation constants for interactions between HIV-specific MAbs and HIV Env proteins were assessed via BLI with the FortéBio Octet or by SPR with a Biacore 3000 (Table 1). Specific MAbs targeting immunogenic regions of Env were selected for analysis. The high-throughput Octet platform (left side of Table 1) was used to analyze the binding of HIV-specific MAbs to specific forms of the CO6980v0c22 proteins, including gp120 monomers, gp145 dimers, gp145 trimers, and unfractionated gp145, and that of a limited number of MAbs to the BG505 SOSIP.664 trimer (Table 1). Strong binding to the gp145 species was detected with soluble

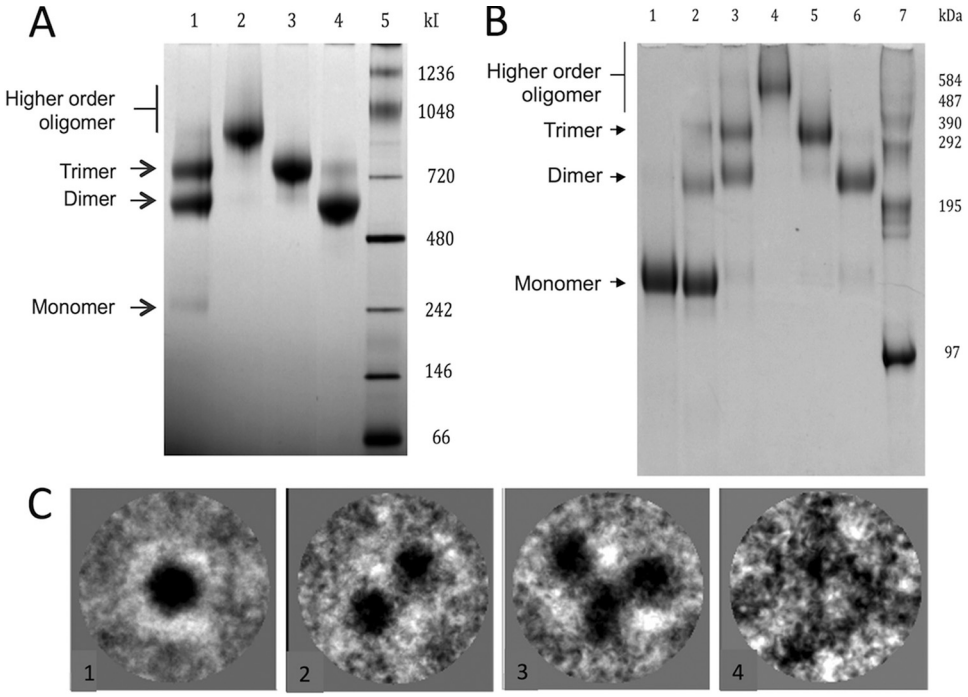

FIG 1 Purified gp145 was fractionated and analyzed by BN-PAGE (A; lane 1, unfractionated gp145; lane 2, higher-order oligomer; lane 3, trimer; lane 4 dimer; lane 5, molecular mass marker) and SDS-PAGE under nonreducing conditions (B; lane 1, reduced unfractionated gp145; lane 2, unfractionated gp145; lane 3, cross-linked unfractionated gp145; lane 4, cross-linked higher-order oligomers; lane 5, cross-linked trimer; lane 6, cross-linked dimer). EGS-cross-linked phosphorylase *b* was run in lane 7 as a protein molecular mass marker. The positions and natures of the deduced oligomeric species are indicated by the arrows at the left. (C) Averaged, top-down views of gp145 monomer (part 1), dimer (part 2), trimer (part 3), and oligomer (part 4), as visualized by cryo-EM.

CD4 (sCD4) and CD4 binding site MAbs variable-loop-specific PG9 (QNE specific) and 2158 (V2 specific). The gp41 cluster I MAb, F240, also bound well to the gp145 trimer fraction, indicating disulfide loop exposure in the expressed gp41 domains. V3-specific MAbs 447-52D, PGT121, and PGT126 demonstrated various degrees of binding to the trimeric fraction; PGT121 bound well, while PGT126 binding was weak, with a very quick off rate (see Table S1 in the supplemental material). MAb 447-52D demonstrated a K_d of 9.90 nM; however, the interaction demonstrated a rapid on-and-off rate (see Table S1). Similar kinetics were also observed for N-glycan-specific MAb 2G12 (see Table S1). A binding affinity of 27.1 nM was detected when using the trimeric fraction and MPER-specific MAb 4E10; however, MPER-specific MAb 10E8 and gp120-gp41 interface-specific MAb PGT151 demonstrated only weak binding to the purified trimer fraction at a very high concentration. No binding of trimeric gp145 by MAb PGT145 was detected. No apparent difference in antigenicity was detected when the various gp145 fractions were assessed against a smaller panel of MAbs (Table 1). The antigenicity of unfractionated gp145 was also evaluated for comparison to other HIV Env proteins, including subtype C gp140 1086c (58) and subtype B gp120 proteins 6240Δ11 and 63521Δ11 (each containing an N-terminal 11-amino-acid deletion) (59) by SPR with the Biacore 3000. CO6980v0c22 gp145 and gp140 1086c bound MAb 4E10; however, no binding to the MPER-specific 2F5 MAb was detected, as expected, since the 2F5 epitope is mutated in the CO6980v0c22 strain, as it is in many subtype C strains (60). The binding of sCD4 and MAb VRC01 to the CD4 binding site was comparable for all four of the proteins tested by SPR. While PG9 and PG16 did not bind to gp140 1086c, they bound to CO6980v0c22 gp145 with affinities comparable to or greater than those observed for gp120 proteins 624Δ11 and 63521Δ11 (Table 1). These data indicate the exposure of several important epitopes on the gp145 trimer but poor binding by quaternary-structure-dependent MAbs PGT145 and PGT151. However, neither of these MAbs neutralized the CO6980v0c22 PV (discussed below for Table 2), indicating that the sequences necessary for these antibodies to be effective may not be present. Furthermore, the binding of nonneutralizing MAbs such as b6, 17b, and F240 verified the presence of nonnative Env structures in the preparations (Table 1).

In addition to utilizing BLI and SPR to explore the exposure of the V2 region, a panel of seven conformation-dependent V2-specific MAbs was used to evaluate gp145 binding by ELISA. As shown in Fig. 2A, binding of gp145 was detected by MAbs 2158 and 697, which bind to the midloop region of V2 in an area that includes the α4β7 tripeptide binding motif. Binding by MAb 2297, which binds to a different but overlapping V2 epitope (61, 62), was also detected. V2-specific MAbs 1357, 1361,1393, and 830A, which bind to epitopes that are similar to or overlap that of 697, did not bind to gp145. Differential binding of these V2-specific MAbs to subtype C gp120 Env proteins has previously been observed (44). Nonspecific anti-parvovirus MAb 1418 did not bind and served as the negative control in the ELISA (Fig. 2A). In addition, the V2-mediated binding of gp145 to the α4β7 integrin receptor at the cell surface was determined by a flow cytometric assay. As shown in Fig. 2B, biotinylated gp145 bound to RPMI 8866 cells, as detected with PE-labeled streptavidin. The addition of α4 MAb HP2/1 inhibited gp145 binding, yielding a signal com-

TABLE 1 Antigenicity of CO6980v0c22 gp145[a]

Epitope and analyte	Octet CO6980v0c22 analysis					Biacore 3000 analysis			
	gp120 monomer	gp145 dimer	gp145 trimer	Unfractionated gp145	BG505 SOSIP.664, gp140	CO6980v0c22, unfractionated gp145	1086c, gp140	624Δ11, gp120	63521Δ11, gp120
CD4bs									
CD4	15.3	9.32	5.53	11.9	ND′[b]	36.1	20.1	17.0	23.2
VRC01	0.058	0.11	0.149	0.318	<0.001[c]	2.47	3.60	21.2	1.10
b6	<0.08[c]	<0.022[c]	<0.016[c]	<0.032[c]	ND′	ND[d]	ND	ND	ND
b12	1.56	1.08	1.09	1.38	ND′	ND	ND	ND	ND
CD4i C1									
17b (CD4i)	48.4	31.1	41.8	47.4	ND′	ND	ND	ND	ND
17b + CD4	<0.006[c]	<0.015[c]	<0.064[c]	<0.085[c]	ND	ND	ND	ND	ND
V1/V2 QNE									
PG9	5.20	8.26	5.60	8.26	ND′	55.7	—[e]	22.7	240
PG16	252	334	142	131	1.23	199	—	597	664
PGT145	ND	ND	—	ND	0.053	ND	ND	ND	ND
V2									
697D	ND	ND	ND	ND	ND	+/−[f]	174	7.40	21.0
2158	ND	ND	0.2	ND	ND	4.80	42.6	2.70	1.60
V3									
PGT121	ND	ND	93.3	ND	ND′	ND	ND	ND	ND
PGT126	ND	ND	+/−	ND	ND′	ND	ND	ND	ND
447-52D	9.97	41.9	9.90	10.6	ND′	ND	ND	ND	ND
gp120-CHO, 2G12	116	375	527	506	ND′	50.1	ND	0.70	2.70
gp120-gp41 interface, PGT 151	ND	ND	+/−	ND	0.13	ND	ND	ND	ND
gp41 cluster I, F240	ND	ND	0.024	ND	ND′	ND	ND	ND	ND
MPER									
2F5	—	—	—	—	ND	—	—	—	—
4E10	—	54.7	27.1	39.9	ND	426	+/−	—	—
10E8	—	—	+/−	—	ND	ND	ND	ND	ND

[a] The values shown are antibody binding dissociation constants (K_d [nanomolar]) for CO6980v0c22 gp145 determined with the FortéBio Octet or Biacore 3000. sCD4 and HIV-specific antibodies that target critical regions of Env, including CD4bs, V1V2, and MPER, were used to probe the epitope display of CO6980v0c22 gp145. Three additional Env antigens, 1086c gp140, 624Δ11 gp120, and 63521Δ11 gp120, were tested with the Biacore 3000 for comparison.
[b] ND′, the antigenicity of BG505 SOSIP.665 gp140 has previously been evaluated and reported (34, 92).
[c] Accurate KD values could not be assessed beyond this value; less than 5% dissociation was achieved.
[d] ND, not determined.
[e] —, no binding detected at 1,000 nM.
[f] +/−, weak binding detected at 1,000 nM.

parable to that of the control without gp145 protein and verifying the specificity of the gp145-α4β7 interaction.

Neutralization sensitivity of the CO6980v0c22 PV. The exposure of neutralizing epitopes on CO6980v0c22 Env was further evaluated by testing the neutralization sensitivity of this Env expressed on a PV, with a panel of neutralizing MAbs for assessment within the TZM-bl neutralization assay. The neutralization profile of CO6980v0c22 was compared to six additional subtype C PVs, three isolated from acute-phase infections and three from chronic-phase infections, as indicated in Table 2. The CO6980v0c22 PV was sensitive to neutralization by MAbs VRC01, PG9, and PG16 (Table 2), similar to most of the other subtype C PVs and in accordance with the binding data (Table 1). Interestingly, neither MAb PGT145 nor PGT151 (which also did not bind trimeric gp145) neutralized the CO6980v0c22 PV. Also of note, although sCD4 and IgG1 MAb b12 bound to CO6980v0c22 gp145, neither sCD4 nor IgG1 MAb b12 neutralized the CO6980v0c22 PV at concentrations of <25 μg/ml. V1V2/V3 MAbs that demonstrated weak binding or rapid on-and-off rates (447-52D, PGT121, PGT126), were also unable to neutralized the CO6980v0c22 PV at concentrations of <25 μg/ml. MAb 4E10, which bound to CO6980v0c22 gp145, demonstrated modest neutralization of CO6980v0c22 PV. In contrast, MPER-specific MAb 10E8, which demonstrated poor binding to the gp145 trimer, neutralized the PV, similar to the other subtype C PVs tested. These data, taken together with the antigenicity data determined by BLI and SPR, revealed profiles of epitope exposure on the gp145 protein subunits different from those on PV Env during a neutralization assay.

Immunogenicity of CO6980v0c22 gp145. Two immunogenicity studies were carried out with New Zealand White female rabbits to determine the performance of this protein as a candi-

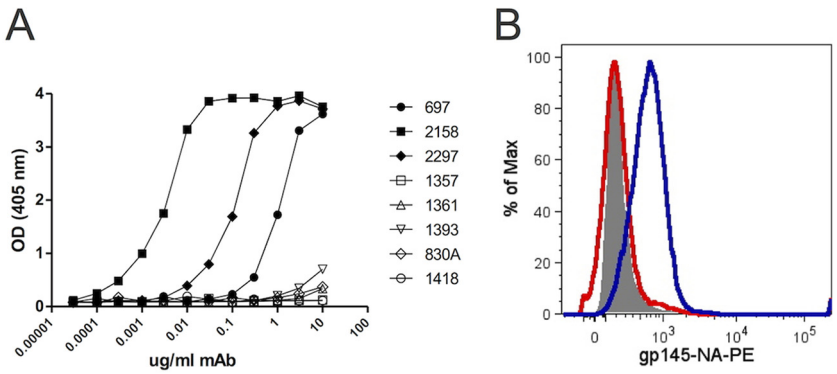

FIG 2 CO6980v0c22 gp145 binding to V2-specific antibodies and α4β7. (A) HIV V2-specific antibodies were tested by ELISA for binding to CO6980v0c22 gp145. MAbs 697, 2158, and 2297 bound (black symbols), while MAbs 1357, 1361, 1393, and 830A and nonspecific MAb 1418 did not bind (open symbols). (B) CO6980v0c22 gp145 binding to α4β7 was determined by using flow cytometry to measure the binding of biotinylated gp145 to RPMI 8866 cells. gp145 binding (blue peak) was observed as a shift from the cell control without gp145 (shaded area) and was inhibited by the incubation of RPMI 8866 cells with α4 antibody HP2/1 before the addition of gp145 (red peak). A single representative experiment is shown. OD, optical density.

date HIV vaccine immunogen. In the first study, unfractionated CO6980v0c22 gp145 was formulated by mixing or encapsulating the protein with three different adjuvants (Table 3, study 1), and immunogenicity was tested with four rabbits per group at a gp145 dose of 25 μg/ml given at weeks 0, 4, and 8; sera were collected at 2-week intervals, ending with the terminal bleed at week 12. Titers of antibody binding to week 10 sera were determined by ELISA of the following HIV Env antigens: subtype B gp41, homologous CO6980v0c22 gp145 immunogen (unfractionated), subtype C gp120, and subtype C gp140 (Fig. 3A). The adjuvant formulation of gp145 did not significantly influence binding antibody titers, and no statistically significant differences between groups immunized with aluminum hydroxide and liposomal groups were observed. The highest titers observed were those of antibodies to the unfractionated gp145 immunogen (overall mean = 65,870), but this was not statistically significant. Week 0 and 10 sera from nine rabbits in study 1 from groups 1, 3, and 5 were tested (at 1:10, 1:50, 1:100, and 1:1,000) for binding to the subtype B V1V2-gp70 fusion protein used in the RV144 immune-correlate assay (24); binding at a 1:50 serum dilution is demonstrated in Fig. 3B. Robust binding was observed at serum dilutions of up to 1:100, with no notable differences observed between adjuvant groups. The magnitude of binding to this gp70V1V2 protein detected in gp145-immunized rabbit sera was comparable to, and in many cases higher than, the levels measured in humans in the RV144 phase III human trial (27). The same sera were used to test binding to V3 and MPER peptide by BLI (Fig. 3C). Robust and comparable binding to V3 peptide was observed for groups 1 and 3; no binding to the MPER peptide was observed (data not shown).

Study 2 was carried out to compare the immunogenicities of the different multimeric fractions of gp145, all formulated in aluminum hydroxide, as described in Table 3, study 2. Study 2 week 10 sera were tested for binding to heterologous subtype C gp120 and gp140 Env proteins, as well as to the cyclic V2 peptides derived from CO6980v0c22 and Con C. Immunization of rabbits with the different multimers elicited comparable titers of antibodies binding to all of the antigens tested by ELISA, with no significant differences detected between the groups (Fig. 3D). Preimmunization and placebo (studies 1 and 2, group 5) sera did not bind to any of the Env antigens tested by ELISA, as expected (data not shown).

NAbs elicited by vaccination with CO6980v0c22 gp145. NAbs elicited in rabbits were detected with both the TZM-bl and PBMC neutralization assay platforms. To compare the abilities of the different adjuvants with gp145 to elicit NAbs, study 1 week 10 sera were screened at a 1:40 (TZM-bl assay) or 1:50 (PBMC assay) dilution. Neutralization, defined as a >50% reduction of infection, was not observed in preimmunization and placebo sera (data not shown). With the TZM-bl assay, all of the rabbit groups neutralized the tier 1 sensitive viruses GS015 (subtype C) and SF162 (subtype B), and rabbits immunized with gp145 in aluminum hydroxide neutralized BZ167 (subtype B) (Fig. 4A). However, minimal neutralization of the autologous CO6980v0c22 PV and two tier 2 CRF01_AE PVs was detected (Fig. 4A). All of the sera were also screened against MuLV PV as a nonspecific viral control,

TABLE 2 Neutralization sensitivity of the CO6980v0c22 PV[a]

				Epitope/mAb:	CD4bs					V2			V3		V1V2/V3	V1V2		gp120-CHO	gp120-gp41	MPER				IC50
Virus Isolate	Subtype	Tier	Origin	Stage	b6	sCD4	VRC01	b12	3BNC117	697	PGT145	447	PGT126	PGT121	PG9	PG16	2G12	PGT151	F240	4E10	10E8	2F5	(ug/ml)
CO6980v0_C22	C	2	Tanzania	Fiebig I/II	>25	>25	0.5	>25	0.3	>25	>25	>25	>25	>25	0.1	4.1	>25	>25	>25	22.1	8.8	>25	>25
SF162	B	1A	USA	Fiebig VI	0.5	0.1	0.1	0.03	0.03	>25	>25	0.4	0.001	5.4	>25	>25	1.2	0.02	>25	9.8	0.1	2.1	10 - 25
ZM197M.PB7	C	1B	Zambia	Fiebig ≤VI	>25	3.4	0.7	16.9	0.6	>25	0.8	>25	>25	>25	0.6	0.8	>25	0.02	>25	0.7	0.2	21.8	2 - 10
DU422, clone 1	C	2	S. Africa	Fiebig V	>25	5.1	>25	0.5	>25	>25	>25	>25	0.2	0.1	1.0	1.2	>25	0.4	>25	1.8	0.8	>25	<2
ZM214M.PL15	C	2	Zambia	Fiebig ≤VI	>25	5.0	0.6	1.4	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	4.8	1.4	>25	
TZA246	C	2	Tanzania	Fiebig ≤VI	>25	1.6	1.3	0.1	>25	>25	>25	>25	>25	>25	>25	>25	>25	0.05	>25	3.5	1.0	>25	
PBL288	C	2	Ethiopia	Fiebig ≤VI	>25	1.3	1.3	>25	0.2	>25	>25	>25	0.03	0.02	>25	6.1	>25	>25	>25	4.5	1.3	>25	
TZBD9/11	C	2	Tanzania	Fiebig ≤VI	>25	6.9	0.1	>25	>25	>25	0.3	>25	>25	0.03	0.4	0.04	>25	0.01	>25	3.6	1.4	>25	

[a] A panel of NAbs was used to assess the neutralization sensitivity of CO6980v0c22 in comparison with that of SF162 and other subtype C PVs. Each IC_{50} is the mean value of two independent experiments.

TABLE 3 Immunization schemes used in studies 1 and 2[a]

Group	Study 1		Study 2	
	Test article	Adjuvant and formulation	Test article	Adjuvant and formulation
1	Unfractionated gp145	Aluminum hydroxide + gp145	Unfractionated gp145	Aluminum hydroxide + gp145
2	Unfractionated gp145	L(DMPG/MPLA) + gp145	gp145 dimer	Aluminum hydroxide + gp145
3	Unfractionated gp145	L(DMPG/MPLA + gp145)	gp145 trimer	Aluminum hydroxide + gp145
4	Unfractionated gp145	L(PIP/MPLA + gp145)	gp145 multimer	Aluminum hydroxide + gp145
5	None	L(DMPG/MPLA)	None	Aluminum hydroxide

[a] For study 1, unfractionated gp145 with aluminum hydroxide and liposomal adjuvants were prepared to encapsulate (groups 3 and 4) or mix with (group 2) the test article. For study 2, unfractionated and different fractionated gp145 oligomers were formulated with aluminum hydroxide adjuvant. In both studies, New Zealand White female rabbits were immunized at weeks 0, 4, and 8 with 25 μg of gp145 per rabbit per dose via the intramuscular route and blood was collected at weeks −2, 0, 4, 8, 10, and 12.

and neutralization was not detected (Fig. 4A), indicating that nonspecific neutralization was not observed. Greater breadth was detected in the PBMC assay, in which all five of the viruses tested were neutralized by serum from one or more groups (Fig. 4B). The highest neutralizing activity was observed in animals immunized with L(DMPG/MPLA plus gp145) (group 3, hatched bars); all four rabbits had serum NAbs to all five IMCs, representing subtypes B, C, and CRF01_AE. In addition, group 3 [L(DMPG/MPLA plus gp145), encapsulated] showed significantly higher overall neutralization than group 2 [L(DMPG/MPLA) plus gp145,

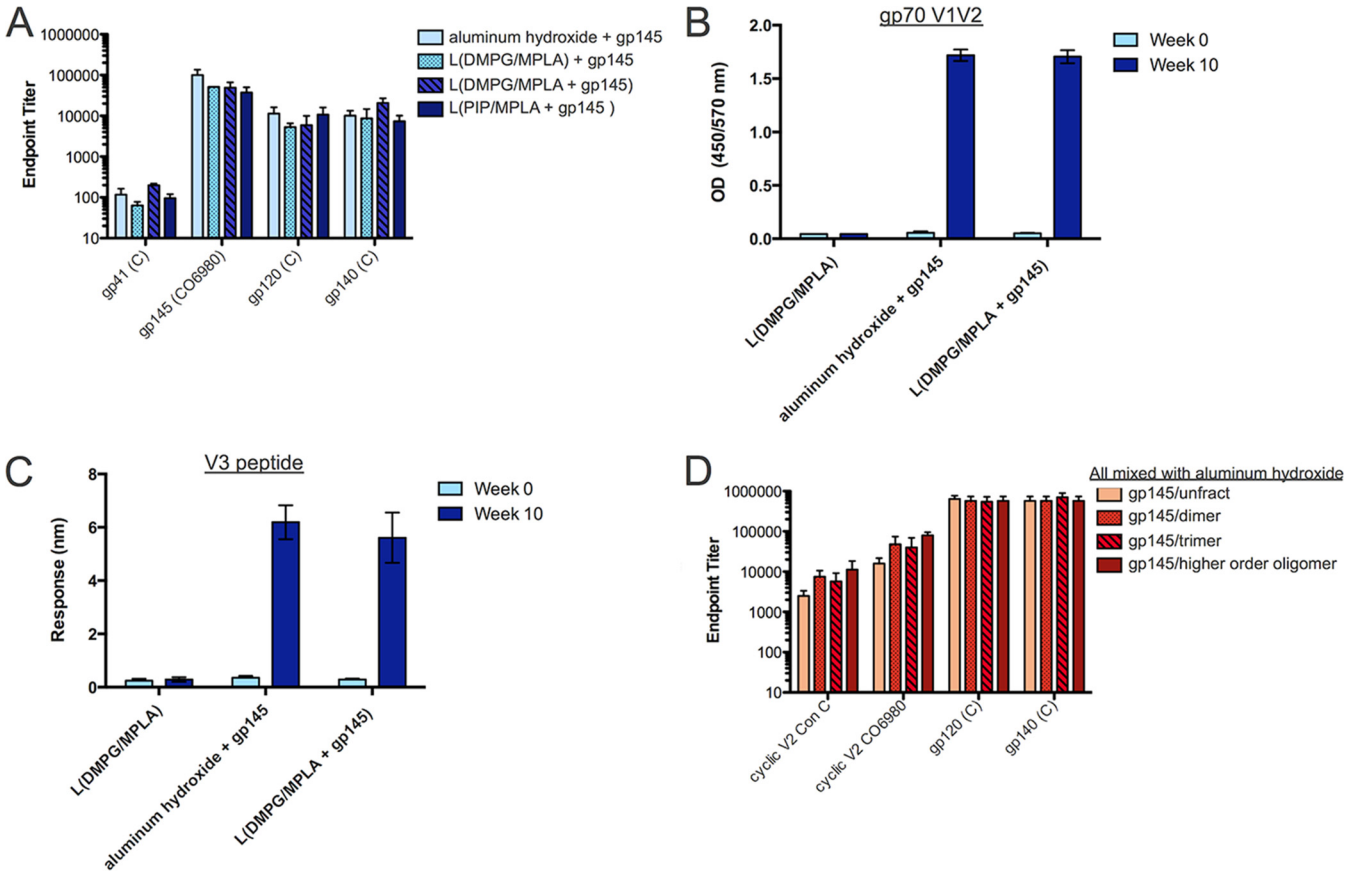

FIG 3 Binding antibody titers elicited by vaccination with CO6980v0c22 gp145. (A) Geometric mean binding antibody titers were determined with week 10 sera collected 2 weeks after the final immunization in study 1. Immunogenicity of unfractionated gp145 was evaluated in four different adjuvant formulations: aluminum hydroxide plus gp145 (solid light blue), L(DMPG/MPLA) plus gp145 (blue bars with black dots), L(DMPG/MPLA plus gp145) (hatched bars), and L(PIP/MPLA plus gp145) (solid dark blue bars). Binding antibody responses to the following four Env antigens were tested: IIIB gp41 (subtype B), CO6980v0c22 gp145 (subtype C), C.ZA.1197MB gp120 (subtype C), and CN54 gp140 (subtype C). The data are the geometric mean endpoint titers of individual serum samples from four rabbits in each group assayed in triplicate and the standard error of the mean (SEM). (B and C) Week 0 and 10 sera from nine rabbits. One rabbit in group 5, L(DMPG/MPLA) without gp145; four rabbits in group 1; and four rabbits in group 3 were tested for antibodies binding to the gp70 V1V2 scaffolded antigen by ELISA (B) and a V3 peptide by BLI (C). (D) Study 2 binding antibody responses to the following four Env antigens: cyclic V2 from Con C (subtype C) and CO6980v0c22 (subtype C), C.ZA.1197MB gp120 (subtype C), and CN54 gp140 (subtype C). The bars indicate the responses of animals immunized with unfractionated (solid pink bar) or fractionated gp145 (dimer, red bar with black dots; trimer, hatched bar; higher-order oligomer, solid dark red bar). Antigen for all groups was formulated by mixing gp145 with aluminum hydroxide. The data are the mean endpoint titers of individual serum samples from four rabbits in each group assayed in triplicate and the SEM.

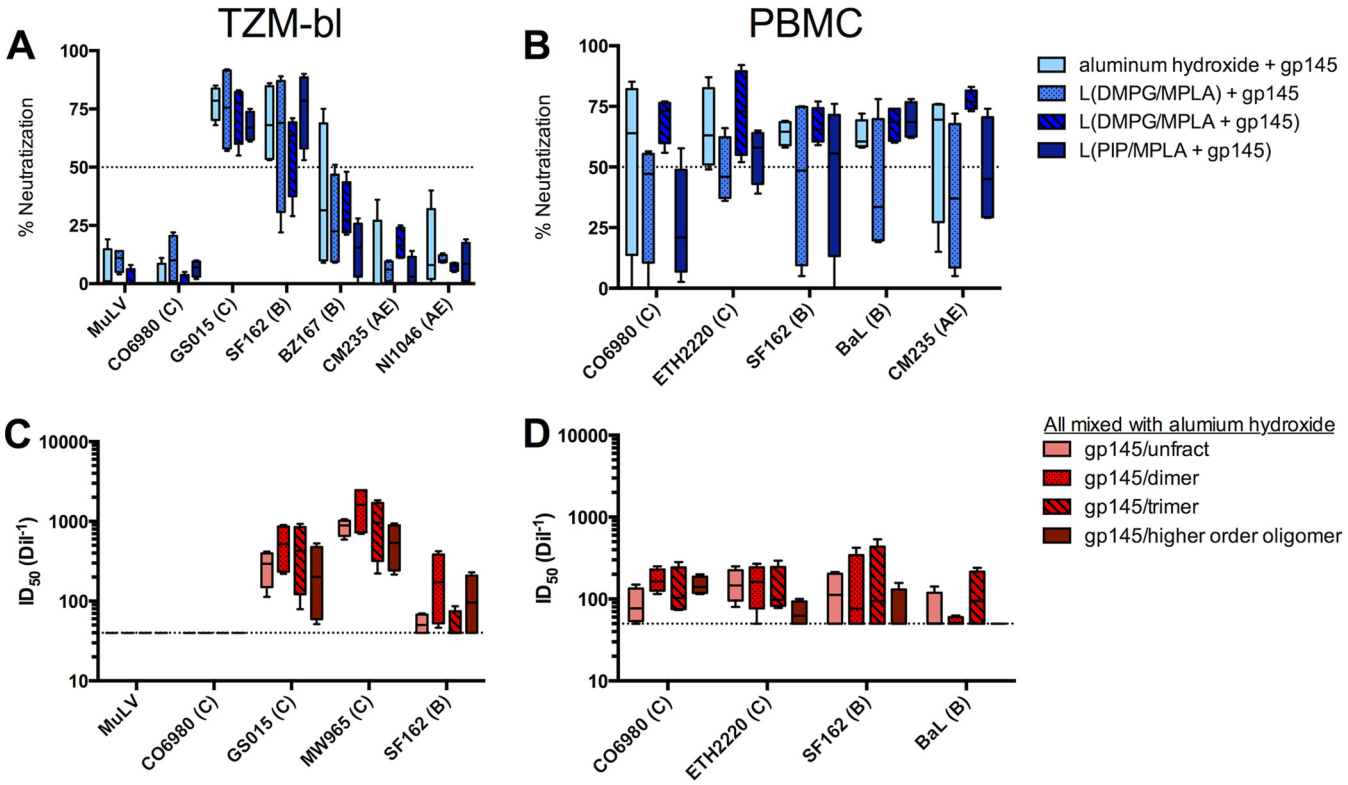

FIG 4 Detection of NAbs elicited by vaccination with CO6980v0c22 gp145. NAbs were detected by both the TZM-bl (A and C) and PBMC (B and D) neutralization assay platforms with viruses from subtypes B (SF162, tier 1A; BZ167, tier 1B; BaL, tier 1B), C (CO6980, tier 2; GS015, tier 1A; ETH2220, tier 1B; MW965, tier 1A), and CRF01_AE (CM235, tier 2; NI1046, tier 2). For panels A and B, week 10 sera were screened at a dilution of 1:40 (TZM-bl) or 1:50 (PBMC); percent neutralization was calculated as the reduction of infection observed in the postimmunization sera compared to the preimmunization sera. The dashed line at 50% represents the negative cutoff for neutralization. The box plots represent the mean percent neutralization and SEM of the four individual rabbits in each group. Individual values were calculated as the mean of two independent experiments. In the graphs in panels C and D, the group mean ID_{50}s from study 2 are presented and the box plots are as described above.

mixed; $P = 0.0002$] and group 4 [L(PIP/MPLA plus gp145); $P = 0.0015$], but it was not significantly higher than that of group 1 (aluminum hydroxide plus gp145) (Fig. 4B).

To further assess the temporal development and magnitude of the NAbs in study 1, sera from all animals were assayed at all time points to obtain 50% inhibitory dilutions in the TZM-bl assay with the sensitive tier 1 subtype C GS015 PV (which was neutralized 100% by the week 10 sera in screening assays (Fig. 4A). As shown in Fig. 4A, neutralizing activity was detectable after the second immunization at week 8, peaked 2 weeks after the third immunization at week 10, and began to wane by week 12. The highest GS015 NAb titers were observed in animals immunized with gp145 mixed with aluminum hydroxide (group 1). Sera from these rabbits in group 1 were also tested by the PBMC neutralization assay using subtype B BaL LucR IMC. Similar kinetics were observed in the PBMC assay, where NAbs to BaL were detected after two immunizations (see Fig. S4B in the supplemental material). Preimmunization and week 10 sera from rabbits 1 and 3 in group 1 were IgG depleted with protein G beads, and the resulting depleted-serum titers were determined by the TZM-bl assay with GS015 PV and by the PBMC neutralization assay with BaL LucR IMC. Depletion of IgG from the sera removed all virus-inhibitory activity (data not shown), indicating that the rabbit serum neutralization observed was IgG mediated.

To compare the NAb titers elicited by different gp145 fractions, sera from study 2 were assessed in the TZM-bl neutralization assay as shown in Fig. 4C. For this study, titers of antibodies against four PVs and MuLV in week 10 serum were determined, and the ID_{50} was determined. MuLV was used as a negative control, and no nonspecific neutralization was observed (Fig. 4C). As observed in study 1, MuLV and the homologous tier 2 CO6980v0c22 PV were not neutralized in the TZM-bl assay (Fig. 4A). Tier 1 PVs of subtypes B and C were neutralized, and the highest peak titers observed were those of antibodies to the sensitive subtype C MW965 PV (Fig. 4C). In addition, week 10 sera from the four different groups in study 2 were tested against four different LucR IMCs in the PBMC neutralization assay. Modest titers of antibodies against all four IMCs were achieved, including the two tier 2, subtype C LucR IMCs (Fig. 4D). While autologous neutralization was not detected in the TZM-bl assay, NAb titers were positive in 100% of the rabbits immunized with the dimer, trimer, or higher-order oligomers when using the vaccine-homologous CO6980v0c22 LucR IMC. The overall frequency of response to the vaccine-homologous IMC (100%) was higher than that to any of the other three IMCs tested (ETH2220, 81%; SF162, 44%; BaL, 38%). There were no statistically significant differences observed in the neutralization of any of the viruses with sera from the different groups immunized with specific purified multimers (Fig. 4C and D).

The kinetics and magnitude of the responses to immunization

with the different gp145 multimers from study 2 were also assessed. Neutralization of GS015 PV in the TZM-bl assay was determined over time as shown in Fig. 4C. Similar to the responses in study 1, NAb titers peaked 2 weeks after the third immunization (week 10). The purified multimer preparations elicited similar NAb titers to GS015, while animals immunized with aluminum hydroxide alone as a placebo (group 5) demonstrated no NAb activity, as expected.

DISCUSSION

HIV vaccines capable of conferring sterilizing immunity have been under development since HIV was identified in 1983. Human clinical trials utilizing recombinant gp120 subunits alone have been unsuccessful in eliciting protective NAbs, including the phase III efficacy trials VAX003 (utilizing recombinant gp120 proteins from subtype B MN and CRF01_AE A244) and VAX004 (utilizing recombinant gp120 proteins from subtypes B MN and GNE8) (63–67). In these trials, the neutralizing activity in vaccinee sera was restricted to highly sensitive (tier 1) or vaccine-homologous strains and was unable to inhibit infection of primary isolates *in vitro* (67, 68). In the RV144 phase III trial, while NAb activity did not correlate with protection, binding of IgG to the V1V2 region of Env correlated inversely with HIV acquisition, indicating a role for antibody in protection (24). A subsequent study in which macaques were immunized with a regimen designed to mimic the RV144 trial identified high-avidity antibodies to gp120, specifically to the V1V2 epitope, as critical for protection against infection (69).

These vaccine results, along with the recent identification of broadly neutralizing QNE MAbs whose epitopes include regions in V1 and V2 (20–22), have underscored the need for improved Env proteins that represent a sufficient mimic of the conformational forms of the virion spike (70). Additionally, combinations of NAbs that bind distinct epitopes have been shown to mediate additive virus-neutralizing activity (71, 72). The goal of this study was to design an HIV-1 subtype C Env from the acute phase of infection that would sufficiently mimic the antigenic properties of Env spikes on virions and elicit improved titers of functional antibodies. We chose a subtype C Env protein from an infected patient in the acute phase to represent an acute-phase virus from the most globally prevalent subtype, with the hope that the neutralizing cross-reactivity observed in natural infection with East African subtype C (7) could be recapitulated via vaccination.

Trimeric gp140 molecules are relatively labile and require modifications to enhance stability and promote trimerization (73, 74). In addition to the elimination of the furin cleavage site, other studies have introduced stabilizing disulfide bonds between cleaved domains (75, 76) or used C-terminal trimerization motifs (77, 78) to stabilize gp140 Env. In this study, we chose to alter the CO6980v0c22 Env gp140 sequence minimally; however, specific modifications were introduced as follows. (i) The cleavage site between gp120 and gp41 was mutated to prevent cleavage, (ii) a full-length MPER was maintained, and (iii) the C terminus (at amino acid 648) was extended by three lysine residues to increase the hydrophilicity of this region and promote MPER display (55). Trimers, dimers, and monomers existed in the enriched trimeric preparation in dynamic equilibrium, with approximately 60% actual trimers exhibiting the fan blade motif, as visualized by cryo-EM analyses. The structure of gp145 is currently being evaluated in greater detail by EM techniques (Moscoso et al., unpublished data).

We assessed this novel, acute-phase gp145 Env protein for the exposure of neutralization epitopes and of epitopes in the V1V2 and V3 domains, as well as epitopes in the MPER on gp41. CO6980v0c22 gp145 maintains exposure of some of these critical domains, as shown by the antigenicity and immunogenicity data reported here. Neither cleavage-dependent, trimer-specific MAb, PGT145 nor PGT151 (21, 79, 80), binds this subtype C gp145 trimer, as previously observed with other uncleaved trimers (30). In addition, the results obtained with CD4i MAb 17b, with or without sCD4, are perplexing. Another group (81) has reported that CD4i antibodies typically do not bind the structurally intact envelope protein in the absence of CD4; however, MAb 17b has been reported to bind to some trimeric gp140 Env proteins (82, 83). MAb 17b did bind all of the CO6980v0c22 species, and this binding was substantially increased in the presence of sCD4 (Table 1). Tran et al. also demonstrated the binding of MAb 17b to a portion of trimers on virions of AT-2-inactivated HIV-1 BaL, a CD4-dependent strain (84). The possible reasons for this observation are under study and may be revealed by ongoing gp145 structural studies (Moscoso et al., unpublished data).

Negative-stain EM has indicated that stabilized, cleaved trimers form homogeneous structures resembling native Env spikes on virus particles (30). In contrast, uncleaved trimers are highly heterogeneous, adopting a mixture of conformations and forming different multimers, as is described here for unfractionated and trimeric CO6980v0c22 gp145. Antigenicity studies with a subtype A strain (BG505) and neutralizing versus nonneutralizing MAbs revealed the expression of quaternary-structure-dependent epitopes on cleaved-stabilized trimers and poor exposure of nonneutralizing gp120 and gp41 epitopes that are occluded on more native structures (34, 85). The Fd-stabilized trimers described by Kovacs et al. (29) were also not bound by nonneutralizing MAb b6, while the matched strain gp120 proteins were bound. Comparisons of the antigenicity of the CO6980v0c22 gp145 trimers and the gp120 monomer from the matched strain revealed comparable binding of MAb b6 to gp120 monomers and all multimers. The significance of this binding and the impact that exposure of the b6 epitope has on the immunogenicity of gp145 remain to be determined. These data, combined with the binding of nonneutralizing MAbs 17b and F240 and the cryo-EM data, also verify the presence of nonnative Env species. Further studies, including additional antigenic characterization of gp145, identification of NAb and non-NAb specificities elicited by gp145 and the immunogenicity of gp145, and simian-human immunodeficiency virus (SHIV) challenge studies with nonhuman primates, are in progress. A phase I safety trial with humans is also under review, in preparation for possible future clinical trials.

Given that V1V2-specific binding antibodies were a correlate of protection in the RV144 trial (24), we investigated V2 exposure on the gp145 immunogen. Binding ELISAs, BLI, and SPR with V2-specific MAbs confirmed the exposure of the V2 loop, and V2-specific antibodies were elicited in gp145-immunized rabbits. These elicited antibodies bound to subtype C V2 and also to the subtype B gp70-V1V2 scaffold protein utilized in the study of RV144 immune correlates. Additionally, CO6980v0c22 gp145 maintains a structure that allows it to bind to conformational V2-specific MAbs, as well as to cell-expressed $\alpha4\beta7$, providing further evidence that the V2 region of gp145 is accessible. It has

recently been demonstrated that binding to α4β7 is not a common property of many currently available HIV-1 gp120 and gp140 proteins (86). The binding of gp145 to α4β7 integrin was validated by blocking the binding to the α4 subunit with an α4-specific MAb (Fig. 2B). The significance of binding to α4β7 is still a subject of considerable study requiring further characterization. However, two recent nonhuman primate studies highlight the role of α4β7 in HIV infection, demonstrating that an increase in the percentage of cells expressing high levels of α4β7 at the mucosa correlated with increased susceptibility to HIV infection (87) and that an anti-α4β7 antibody can be protective against SHIV infection when administered prior to and during repeated low-dose intravaginal challenges (88). These results suggest that the α4β7-binding site on HIV has biological relevance for vaccine design. It remains to be determined whether or not the V2-specific antibodies elicited by gp145 Env are functional *in vivo* in antibody-dependent cell-mediated cytotoxicity (ADCC) or in the inhibition of viral binding to α4β7 integrin. In ongoing nonhuman primate studies with gp145 as an immunogen, it will be critical to assess the potential for this immunogen to elicit ADCC and other functional responses, which is suggested by the exposure of the V2 and F240 (89, 90) epitopes, as well as the elicitation of V2-specific antibodies. Because of these properties, this gp145 Env could represent an incremental improvement over gp120 or gp140 structures that are incapable of binding α4β7 or that have truncations within the MPER.

Our results also indicate that structural differences exist between the CHO-expressed CO6980v0c22 gp145 protein and the 293T-produced CO6980v0c22 PV. Antigenic exposure of some epitopes on the gp145 protein did not always correlate with neutralization of the PV. When expressed as the functional Env protein on a PV, CO6980v0c22 was neutralized by the VRC01, 3BNC117, PG9, PG16, and 10E8 MAbs, indicating accessibility of the CD4 binding site, QNE, and MPERs on the pseudovirions. However, at ≤25 μg/ml, MAb b12 and sCD4 did not inhibit the CO6980v0c22 PV in the TZM-bl assay. Conversely, while MAb 10E8 demonstrated potent neutralization of the PV, it bound poorly in a BLI analysis. Additionally, antibodies elicited by CO6980v0c22 gp145 did not neutralize the homologous PV in the TZM-bl assay; however, titers of antibody to the CO6980 IMC were observed in a PBMC assay. We have observed this discrepancy between assay platforms previously when evaluating human vaccinee sera (91, 92, 97). The differences could be attributed to the detection of additional types of antibody-mediated viral inhibition in the PBMC assay (93), differences in cell surface HIV receptor expression (94), differences in the cell production systems, or differences in the Env cleavage state of the CO6980v0c22 gp145 protein versus the CO6980v0c22 PV. Importantly, it has been shown that antigenic exposure of desired epitopes does not always predict the elicitation of epitope-specific NAbs upon immunization (58, 81).

Using T4 bacteriophage fibritin Fd domain-stabilized trimers, Kovacs et al. have demonstrated that subtype A and C gp140 trimers elicited a NAb response with incremental improvement over the response elicited by gp120 monomers when comparing matched-strain proteins in immunized guinea pigs (29). They suggested that these stable gp140 trimers more accurately mimic the antigenic properties of the native Env spike than do gp120 monomers and are thus better immunogens. Taking these observations into account and in the interest of resource conservation, we expressed only the uncleaved gp145 protein at the time that the two immunogenicity studies described here were conducted and did not have available the matched-strain gp120 protein for immunization. At a later time, gp120 was expressed to allow for comparative antigenicity studies. Upon assessment of the immunogenicity of purified dimers, trimers, and higher-order oligomers, we found that preparations of these species exhibited comparable immunogenicity in rabbits. The trimeric fraction prepared by size exclusion chromatography was enriched to approximately 60% trimer, with nonnative monomers and dimers still present in what is presumed to be a state of dynamic equilibrium, as the trimer is not fully stabilized. The heterogeneity of the enriched fractions could potentially mask differences in antigenicity and immunogenicity between oligomeric species. However, these data demonstrate certain immunogenic features of this acute-phase clade C Env protein that is now undergoing production under GMP conditions and suggest that the additional purification efforts required to enrich for gp145 trimers may not result in improved immunogenicity in macaques or humans.

To date, there remain a relatively limited number of HIV envelope proteins that have been successfully scaled up for production under GMP conditions at a quantity sufficient for testing in multiple different clinical research protocols. Recent efforts at the Division of AIDS, National Institutes of Health, have been directed at improving the access to antigens that are produced in sufficient quantities such that critical studies of formulation, route, dosing, and schedule, with different regimens and vectors, can be conducted with proteins vetted for safety (95). Recent preclinical studies suggest that a mixture of multiple soluble Env proteins may boost NAb production, compared with the use of a single Env trimer alone (96), highlighting the potential of combining Env subunits. The efforts to increase the availability of good laboratory practice- or GMP-produced Env proteins, which include investigators from several institutes, companies, and universities, should provide better access to several HIV-1 envelope subunits (including gp120 proteins, cleaved/stabilized trimers, Fd trimers, gp140 trimers, and CO6980v0c22 gp145) that are currently in the pipeline. Furthermore, the sharing of these reagents will allow the conduct of preclinical and clinical studies to address pressing questions pertaining to the formulation, selection, and use of Env proteins, such that optimal immunogenicity and protective immunity may be achieved.

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Francine McCutchan is an independent consultant.

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