Antibodies with Specificity to Native gp120 and Neutralization Activity against Primary Human Immunodeficiency Virus Type 1 Isolates Elicited by Immunization with Oligomeric gp160

THOMAS C. VanCOTT,[1]* J. R. MASCOLA,[2] R. W. KAMINSKI,[1] V. KALYANARAMAN,[3] P. L. HALLBERG,[1]
P. R. BURNETT,[4] J. T. ULRICH,[5] D. J. RECHTMAN,[5] AND D. L. BIRX[4]

*Henry M. Jackson Foundation[1] and Division of Retrovirology,[4] Walter Reed Army Institute of Research, Rockville,
Maryland 20850; Advanced BioScience Laboratories, Kensington, Maryland 20895[3]; Ribi ImmunoChem
Research, Inc., Hamilton, Montana 59840[5]; and Department of Infectious Diseases,
Naval Medical Research Institute, Bethesda, Maryland 20889[2]*

Current human immunodeficiency virus type 1 (HIV-1) envelope vaccine candidates elicit high antibody binding titers with neutralizing activity against T-cell line-adapted but not primary HIV-1 isolates. Serum antibodies from these human vaccine recipients were also found to be preferentially directed to linear epitopes within gp120 that are poorly exposed on native gp120. Systemic immunization of rabbits with an affinity-purified oligomeric gp160 protein formulated with either Alhydrogel or monophosphoryl lipid A-containing adjuvants resulted in the induction of high-titered serum antibodies that preferentially bound epitopes exposed on native forms of gp120 and gp160, recognized a restricted number of linear epitopes, efficiently bound heterologous strains of monomeric gp120 and cell surface-expressed oligomeric gp120/gp41, and neutralized several strains of T-cell line-adapted HIV-1. Additionally, those immune sera with the highest oligomeric gp160 antibody binding titers had neutralizing activity against some primary HIV-1 isolates, using phytohemagglutinin-stimulated peripheral blood mononuclear cell targets. Induction of an antibody response preferentially reactive with natively folded gp120/gp160 was dependent on the tertiary structure of the HIV-1 envelope immunogen as well as its adjuvant formulation, route of administration, and number of immunizations administered. These studies demonstrate the capacity of a soluble HIV-1 envelope glycoprotein vaccine to elicit an antibody response capable of neutralizing primary HIV-1 isolates.

The majority of the vaccines currently being evaluated in clinical trials include forms of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120 or gp160 (2–4, 10, 11, 18, 19, 22, 26–28, 44, 52). These vaccines elicit strong serum binding antibody titers with neutralizing activity against homologous T-cell line-adapted (TCLA) HIV-1 strains and in some cases against heterologous TCLA HIV-1, but not primary HIV-1 isolates (4, 21, 22, 32, 33, 52, 67). In contrast, sera from HIV-1-infected individuals have been shown to neutralize both TCLA and primary HIV-1 strains (31, 32, 50, 67). We have previously demonstrated qualitative differences in antibody recognition of native forms of gp120/gp160 in HIV-1-seropositive sera compared to sera from HIV-1 envelope subunit recipients (61). While HIV-1 sera preferentially bound native forms of gp120/gp160 (36), sera from vaccinees were preferentially reactive against denatured forms of gp120 (61). Furthermore, vaccinee sera had relatively weak recognition of heterologous oligomeric gp120/gp41 expressed on the surface of infected H9 cells, suggesting a lack of antibodies specific for conserved conformational epitopes. These qualitative differences suggest suboptimal formulation of envelope antigens or use of envelope antigens with less than optimal protein conformation. Since the HIV-1 envelope glycoprotein is known to exist as gp120/gp41 oligomers on the virion (14, 45, 51, 57), it is possible that monomeric gp120 and gp160 subunits do not adequately mimic the structure of virion oligomeric gp120/gp41.

The inability of vaccinee sera to neutralize primary HIV-1 may be related to the observations that primary and TCLA strains are differentially susceptible to neutralization by specific monoclonal antibodies (MAbs), soluble CD4, and HIV-1 sera (1, 7, 12, 34, 37, 48, 50, 60). Binding of MAbs to monomeric gp120 was found to be a poor predictor of neutralizing activity against primary HIV-1 isolates, suggesting that the conformation of soluble monomeric gp120 differs substantially from that of virion-associated oligomeric gp120/gp41 (35, 39, 49, 54, 56). Differential exposure of specific epitopes on TCLA and primary HIV-1 isolates has been demonstrated by the lack of recognition by V3 specific antibodies of the V3 loop on monocytropic viruses (6) and the reduced role of V3 antibodies from HIV-1 sera in neutralizing primary compared with TCLA HIV-1$_{MN}$ (64). Alternatively, non-HIV-1-specific factors within HIV-1 sera such as chemokines may contribute to anti-HIV-1 activity (9). However, it is clear that there are immunogenic epitopes on primary HIV-1 isolates that are targets of neutralization by human MAbs (8, 17, 41, 58, 59).

We have recently characterized an oligomeric gp160 protein (o-gp160), affinity purified from chronically HIV-1-infected 6D5 cells and composed of gp160 tetramers, dimers, and monomers (23, 24, 65). This o-gp160 protein binds antibodies produced during acute HIV-1 infection (43) and is reactive with a broad panel of neutralizing MAbs and sera from individuals infected with HIV-1 from clade B and non-clade B HIV-1 (65). o-gp160 is stoichiometrically more reactive with HIV-1 sera than some other recombinant gp120 or gp160 proteins from the same strain, indicating the presence of additional epitopes accessible to HIV-1 serum antibodies (65). We report that o-gp160 formulated in Alhydrogel or monophos-

* Corresponding author. Mailing address: Henry M. Jackson Foundation, 13 Taft Ct., Suite 200, Rockville, MD 20850. Phone: (301) 762-0089. Fax: (301) 762-4177. E-mail: tvancott@hiv.hjf.org.

TABLE 1. Rabbit immune and HIV-1 sera used in this study

| Group | Immunogen[a] | Adjuvant | Route | No. of shots | Endpoint titer (EIA)[b] | | SPR (RU), o-gp160$_{451}$ |
					Mrgp160$_{IIIB}$ (10^3)	o-gp160$_{451}$ (10^3)	
Rabbits							
4A1	gp120$_{IIIB}$	CFA-IFA	Subcutaneous	3	102.4	204.8	836
4A2	o-gp160$_{IIIB}$	CFA-IFA	Subcutaneous	3	204.8	409.6	2,332
5A2	o-gp160$_{IIIB}$	MPL + Ras3c[c]	Mixed[d]	5	102.4	204.8	3,223
5A3	o-gp160$_{IIIB}$	Detox	Mixed	5	51.2	102.4	828
5A4	o-gp160$_{IIIB}$	Ras3c + MPL[e]	Mixed	5	12.8	51.2	794
6B1	o-gp160$_{IIIB}$	Alhydrogel	Mixed	3	25.6	51.2	1,009
6B2	o-gp160$_{IIIB}$	Ras3c	Mixed	3	102.4	204.8	2,914
6B3	o-gp160$_{IIIB}$	MPL-oe	Mixed	3	3.2	6.4	123
6B4	o-gp160$_{IIIB}$	MPL-se	Mixed	3	25.6	51.2	592
6B5	o-gp160$_{IIIB}$	CFA-IFA	Mixed	3	25.6	51.2	517
7A1-1	Mrgp160$_{IIIB}$	Alhydrogel	Intramuscular	3	102.4	204.8	640
7A1-2	Mrgp160$_{IIIB}$	Alhydrogel	Intramuscular	3	51.2	51.2	545
HIV-1 sera							
NA pool ($n = 35$)					204.8	204.8	3,933
HIV-1 (US14)					1,638	1,638	7,500

[a] Immunogens used are described in the text.
[b] Sera from three rabbits per group were pooled, and antibody binding levels were determined by EIA and SPR analysis with the exception of sera from 7A1-1 and 7A1-2, which represent individual rabbit sera.
[c] Rabbits received o-gp160$_{IIIB}$ formulated in MPL-oe for immunizations 1, 3, 4, and 5 and Ras3c for immunization 2.
[d] Mixed route corresponds to combined intramuscular, intradermal, and subcutaneous immunization routes as described in Materials and Methods.
[e] Rabbits received o-gp160$_{IIIB}$ formulated in Ras3c for immunizations 1, 3, 4, and 5 and MPL-oe for immunization 2.

phoryl lipid A (MPL)-containing adjuvant preparations, but not other adjuvants, elicits an antibody response in rabbits which is qualitatively different from that elicited by other HIV-1 envelope subunit vaccines. These antibodies preferentially bind native monomeric gp120, have linear epitope recognition patterns similar to that observed with HIV-1 sera, retain strong reactivity against heterologous strains of monomeric gp120 and cell surface-expressed gp120/gp41, and broadly neutralize TCLA HIV-1 isolates. Additionally, neutralization of several primary HIV-1 isolates by high-titer immune sera was observed. These data represent the first example of a subunit HIV-1 envelope immunogen inducing an antibody response capable of neutralizing primary HIV-1 isolates.

MATERIALS AND METHODS

HIV-1 sera. Serum samples were obtained with informed consent from HIV-1-seropositive individuals enrolled in phase I vaccine therapy trials prior to any immune manipulation at Walter Reed Army Medical Center, using recombinant gp160$_{IIIB}$ (rgp160$_{IIIB}$) from MicroGeneSys (Meriden, Conn.). Sera from 35 early-stage HIV-1-infected patients (47) were pooled (NA pool) and heat inactivated at 56°C for 40 min prior to use. HIV-1 sera US9, US10, US17, and US18 were obtained with informed consent from clinically asymptomatic HIV-1-infected subjects with CD4 lymphocyte counts of >500/μl.

Proteins, peptides, and antibodies. Oligomeric gp160 and monomeric gp120 were affinity purified from cell cultures infected with HIV-1$_{IIIB}$ or HIV-1$_{451}$ (o-gp160$_{IIIB}$, o-gp160$_{451}$, and gp120$_{IIIB}$) as described previously (23, 24). Briefly, chronically infected cell lines 6D5$_{451}$ and 6D5$_{IIIB}$ were developed by infection of 6D5 cells (a subclone of the HUT78 cell line) with the primary isolate HIV-1$_{451}$ and the TCLA HIV-1$_{IIIB}$ respectively (16). Radioimmunoprecipitation analysis revealed that this cell line secreted both gp120 and gp160 into the medium (23, 24). The gp120 and gp160 proteins were purified from the serum-free conditioned medium by affinity chromatography using mouse MAbs to HIV-1 gp120 and gp41, respectively (23, 24). Structural analysis showed this gp160 to exist mostly as dimers and tetramers (approximately 75%), with some monomers (65). Baculovirus-expressed recombinant gp160$_{IIIB}$ (Mrgp160$_{IIIB}$) was obtained from MicroGeneSys. rgp120$_{IIIB}$ and rgp120$_{MN}$ from CHO cells and reduced, carboxymethylated gp120$_{IIIB}$ (rcmgp120$_{IIIB}$ and rcmgp120$_{MN}$) were provided by Genentech Inc., South San Francisco, Calif. (29) for use in serologic assays. Peptides LAI-74, -99, -132, -246, -348, -420, -454, and -480 (25 to 40 amino acids in length) were synthesized to >90% purity by Synthecell Corporation (Gaithersburg, Md.) as assessed by reverse-phase high-pressure liquid chromatography,

amino acid analysis, and sequencing; peptides LAI-31, -53, -112, -191, -211, -283, -319, and -373 were synthesized by standard solid-phase methods as described previously (46). The peptide nomenclature used refers to the strain of virus the sequence is based on (LAI) and the position of the starting amino acid within gp120 (42). MAb 1C1, specific for a linear epitope within C5 of gp120, was purchased from RepliGen Corp., MAb P43110, specific for a discontinuous epitope within gp120 (62, 66), was purchased from Advanced BioScience Laboratories (Kensington, Md.), and MAb 9305 (13), specific for V3 of HIV-1$_{IIIB}$, was purchased from Dupont (Wilmington, Del.). Immunoglobulin (Ig) was purified from rabbit serum, using protein A as instructed by the manufacturer (Bio-Rad, Hercules, Calif.).

Adjuvants. Aluminum hydroxide gel adjuvant (Alhydrogel) was purchased from Superfos Biosector A/S (Vedbaek, Denmark). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Sigma Immunochemicals (St. Louis, Mo.). The following MPL-containing adjuvant preparations were provided by Ribi ImmunoChem Research, Inc. (Hamilton, Mont.): MPL-oe, a 1.0% (vol/vol) squalene oil-in-water emulsion containing 50 μg of MPL per ml; MPL-se, a 1.0% (vol/vol) squalene oil-in-water emulsion containing 250 μg of MPL per ml; Detox, a 1.0% squalene oil-in-water emulsion containing 25 μg of MPL per ml and 250 μg of cell wall skeleton from *Mycobacterium phlei* per ml; and Ribi adjuvant system—three components (Ras3c), a 2.0% (vol/vol) squalene oil-in-water emulsion containing 250 μg of MPL, cell wall skeleton, and synthetic dicorynomycolate (S-TDCM) per ml.

Immunization of rabbits. New Zealand white (NZW) female rabbits (14 to 16 weeks of age, 2.0 to 2.5 kg) were purchased from HRP, Inc. (Denver, Pa.). Animals were randomized to the various vaccine groups and individually housed in wire rabbit cages at Biocon Laboratories, Rockville, Md. The various rabbit immunogenicity studies conducted were named 4A, 5A, 6B, and 7A (Table 1). In study 4A, NZW female rabbits (three rabbits per group) were primed with 200 μg of gp120$_{IIIB}$ (4A1) or o-gp160$_{IIIB}$ (4A2) in CFA and boosted with 100 μg of gp120$_{IIIB}$ (4A1) or o-gp160$_{IIIB}$ (4A2) in IFA subcutaneously at weeks 4 and 8. In study 5A, NZW female rabbits (three rabbits per group) were immunized at 0, 3, 6, 10, and 18 weeks with 70 μg of o-gp160$_{IIIB}$ formulated with MPL-oe (5A2), Detox (5A3), or Ras3c (5A4). For the second immunization only, groups 5A2 and 5A4 received o-gp160$_{IIIB}$ formulated with Ras3c and MPL-oe adjuvants, respectively. Immunizations were given in 1.0-ml volumes, using a mixed route (0.3 ml intramuscularly into each hind leg, 0.05 ml intradermally in six sites, and 0.1 ml subcutaneously in the neck region). Rabbits were bled prior to the initial immunization and 1 and 2 weeks after each immunization. In study 6B, NZW rabbits (three per group) were immunized at 0, 3, and 18 weeks with 70 μg of o-gp160$_{IIIB}$ formulated in Alhydrogel (6B1), Ras3c (6B2), MPL-oe (6B3), MPL-se (6B4), or CFA-IFA (6B5). Immunizations were given by using the mixed route described above. Rabbits were bled several times prior to the initial immunization and 1 and 2 weeks after each immunization. The study 7A, rabbits (three per group) were immunized intramuscularly (0.5 ml in each rear hind leg) with 85 μg of Mrgp160$_{IIIB}$ in Alhydrogel at 0, 1, and 4 weeks.

Enzyme-linked immunosorbent assay (ELISA) measurement of serum IgG binding. Affinity-purified o-gp160$_{IIIB}$ or o-gp160$_{451}$ (1.25 µg/ml) or various synthetic peptides (1 µg/ml) corresponding to HIV-1$_{LAI}$ gp160 in phosphate-buffered saline (PBS; pH 7.4) containing 0.01% thimerosal were coated overnight at 4°C onto Immulon 2 microtiter plates (Dynatech, Chantilly, Va.) and assayed as described previously (62, 65). Briefly, plates were washed twice with wash buffer (PBS with 0.1% Tween 20 [pH 7.4]) prior to the incubation with twofold dilutions of serum diluted in serum diluent (wash buffer with 5% skim milk, pH 7.4) for 1 h at 37°C. Plates were washed three times with wash buffer and incubated with horseradish peroxidase-conjugated goat anti-rabbit (Boehringer Mannheim, Indianapolis, Ind.) IgG (diluted 1:2,000 in serum diluent) or goat anti-human (Kirkgaard & Perry, Gaithersburg, Md.) IgG (diluted 1:8,000 in serum diluent). After a 1-h incubation at 37°C, plates were washed three times, after which substrate (ABTS; Kirkgaard & Perry) was added. The reaction was stopped with 0.5% sodium dodecyl sulfate after 30 min at 37°C.

Determination of antibody binding by using SPR. Real-time binding interactions between ligand (gp120 or gp160 covalently linked to a biosensor matrix) and ligate (antibodies in solution) were measured by using surface plasmon resonance (SPR) (BIAcore; Pharmacia Biosensor, Piscataway, N.J.) as described in detail elsewhere (15, 25, 55, 63). Briefly, the system utilizes the optical properties of SPR to detect alterations in protein concentration within a dextran biosensor matrix. HIV-1 envelope glycoproteins were covalently linked via free amine groups to the biosensor dextran matrix as described previously (62). The amount of protein immobilized can be determined directly from the immobilization process (55). Appropriate matrices were prepared by injecting across the activated matrix 30 µl of the following: rgp120$_{IIIB}$ (20 µg/ml in sodium acetate buffer [NaAc], pH 4.5); rgp120$_{MN}$ (20 µg/ml in NaAc, pH 5.0); rcmgp120$_{IIIB}$ (70 µg/ml in NaAc, pH 4.0); rcmgp120$_{MN}$ (35 µg/ml in NaAc, pH 4.0), or o-gp160$_{451}$ (20 µg/ml in NaAc, pH 5.0). This was followed by deactivation with an injection of 1 M ethanolamine.

Antibody binding to native and denatured monomeric gp120. Native/denatured gp120$_{IIIB}$ and gp120$_{MN}$ antibody binding ratios were determined as previously described (62). MAbs and sera were diluted in HBS running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% BIAcore surfactant P20 [pH 7.4]), and 30-µl aliquots were injected through the gp120 matrices at a flow rate of 5 µl/min. Prior to injection of antibody, and immediately afterward, HBS buffer alone flowed through each flow cell. The net difference in signal between baseline and approximately 20 s after completion of antibody injection was taken to represent the binding value of that particular sample. The native/denatured gp120 antibody binding ratios were determined by dividing the amount of serum binding to rgp120 (in resonance units) by the amount of serum binding to rcmgp120 (in resonance units) after normalizing for any differences in the rgp120 and rcmgp120 matrix concentrations. Control MAbs which were previously shown to bind specifically to denatured gp120 (1C1), native gp120 (P43110), or similarly to both native and denatured gp120 (V3) were used as controls (62). Matrices were regenerated by using 60 mM H$_3$PO$_4$ (rgp120 and rcmgp120) or 10% formic acid (o-gp160) prior to injection of the next sample.

Monomeric gp120 capture assay. Antibody binding to monomeric gp120 captured from detergent-treated HIV-1 was measured by using an assay first described elsewhere (38). H9 cells (15 × 10^6) were acutely infected with 500 µl of stock of either HIV-1$_{IIIB}$, HIV-1$_{MN}$, HIV-1$_{RF}$, or HIV-1$_{451}$ at 15 × 10^6 cells/ml for 60 min at 37°C. Cells were washed twice with PBS and once with cRPMI (RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin solution, and 1% L-glutamine solution), resuspended in 30 ml of cRPMI, and transferred to a T-75 tissue culture flask. Cultures were fed at day 3 and every subsequent 2 days with 10 ml of cRPMI. At peak p24 antigen production, supernatant was harvested and lysed with Empigen BB (1.0%) for 1 h at room temperature. Lysates were diluted in PBS (with 5% skim milk and 0.1% Tween 20) and captured for 1 h at room temperature by sheep antibody (D7324) which had been adsorbed overnight at 4°C to wells of Immulon 2 microtiter plates (2.5 µg/ml in sodium bicarbonate buffer [pH 9.6]). Test sera were diluted in PBS (with 5% skim milk, 0.1% Tween 20, and 5% normal goat serum), titrated down the plate (twofold), and detected with appropriate horseradish peroxidase-labeled secondary antibody as described above. Binding activity of the various rabbit immune sera was compared to that for an HIV-1 serum pool (NA pool).

Flow cytometric assessment of antibody binding to cell surface-expressed gp120/gp41 complexes. H9 cells were acutely infected with TCLA HIV-1 as described above. At times ranging between 3 and 7 days, depending on the time course of the individual infection, 5 × 10^6 H9 cells infected with HIV-1$_{IIIB}$, HIV-1$_{MN}$, or HIV-1$_{RF}$ were removed and washed three times in wash medium (PBS [pH 7.4], 0.1% bovine serum albumin, 0.01% thimerosal) and resuspended at 10 × 10^6 cells/ml (61). At these time points, CD4 expression on these HIV-1-infected H9 cells was downregulated as measured by staining by antibodies L3a and OKT4, suggesting that minimal binding against free gp120 complexed to cell surface CD4 may be measured. Twenty microliters of cells was mixed with 20 µl of serum (1:50, 1:250, and 1:1,250) for 1 h at 4°C while mixing. The cells were washed two times with wash medium, and 2 µl of goat anti-human IgG conjugated to phycoerythrin (PE) or goat anti-rabbit IgG-PE (Southern Biotechnologies, Birmingham, Ala.) was added for 30 min at 4°C. After an additional three washes, the cells were fixed overnight with 1% paraformaldehyde at 4°C and analyzed on a FACscan (Becton Dickinson). Fifteen

thousand cells in total were acquired, and cells falling within a defined forward and side scatter profile were analyzed for mean PE fluorescence. Binding to uninfected H9 cells was subtracted for each of the rabbit sera to control for nonspecific cell surface binding. Binding by each of the rabbit immune sera was normalized to the binding of the NA pool to account for differences in concentration of cell surface-expressed HIV-1 envelope. This was accomplished by dividing the mean fluorescence of each of the rabbit sera by the mean fluorescence of the NA pool. Additionally, a strong HIV-1 serum was also included and normalized to the NA pool. Data are presented as the means of three to four separate assays.

Virus neutralization assay. Virus isolates BZ167, CM237, BK132, US1, US2, and US3 were obtained from investigators of the U.S. Military HIV Research Program. Isolates TH014, IIIB, MN, and RF were obtained from the NIH AIDS Research and Reagent Repository. All primary isolates were initially cultured on phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) and were low passage. None were passaged through neoplastic cell lines. All viruses described above have been previously shown to be genetic subtype B (30, 31). The one clade E isolate used (9461) was a gift from Jay Levy (University of California, San Francisco). For expansion to high-titered virus stocks, 2 × 10^7 fresh PHA-stimulated donor PBMC were infected overnight and monitored for production of HIV-1 p24 antigen (Coulter [Hialeah, Fla.] enzyme immunoassay [EIA]). Supernatant collected at a single time point (between days 6 and 10) was stored in 1-ml aliquots in the vapor phase of liquid nitrogen. To prepare laboratory-adapted HIV strains (MN, IIIB, and RF), virus supernatant from chronically infected H9 cells was used to infect PHA-stimulated human PBMC as described above. Unless otherwise specified, assessment of neutralizing antibody activity of serum or purified Ig was performed as previously described (31, 32), with minor modifications. PHA-stimulated PBMC were used as target cells for both primary and TCLA strains, and virus growth kinetics and median tissue culture infective dose (TCID$_{50}$) were determined within the assay format. The same donor cryopreserved PBMC were used in the titration and neutralization assays. All sera and purified Ig were heat inactivated at 56°C for 40 min prior to use.

Dilutions of test sera or purified Ig were aliquoted in quadruplicate wells of a 96-well microtiter plate (25 µl per well). Culture media without antibody, pooled normal human serum (Sigma), and preimmune rabbit sera served as controls for baseline virus growth. An equal volume of virus stock (25 µl), representing 25 to 50 TCID$_{50}$, was added to each well. After 30 min at 37°C, 2 × 10^5 PHA-stimulated PBMC were added and incubated overnight at 37°C. Cells were then washed extensively to remove p24 antigen and plasma anti-p24 antibody and transferred to a 96-well microtiter plate with culture medium containing 20 U of human recombinant interleukin-2 per ml. Inhibition of PBMC infection was assessed by quantitative p24 measurement of cell supernatants during the early virus growth phase (days 4 to 6 for the viruses in this study). Average p24 antigen output in control (normal human serum) wells was usually between 50 and 250 ng/ml. Serum dilutions causing 50 and 90% reduction in p24 antigen were calculated by linear regression analysis. For rabbit sera tested at a single dilution, results were reported as percent neutralization (decrease in p24 antigen level in immune sera wells compared to preimmune sera). In all studies, the serum dilution was defined as the dilution of serum in the presence of virus and before addition of PBMC.

RESULTS

Immunogenicity of o-gp160 in rabbits. The primary objective of this study was to evaluate the qualitative and quantitative immune responses elicited by an o-gp160 immunogen formulated in several different adjuvants. In particular, studies were conducted to determine whether a soluble HIV-1 envelope protein could elicit antibodies preferentially reactive with native forms of HIV-1 gp120, in contrast to that observed previously with other subunit vaccines in phase I clinical trials (61). A summary of the vaccine groups analyzed is given in Table 1. Group 4A rabbits received either affinity-purified monomeric gp120$_{IIIB}$ or o-gp160$_{IIIB}$ in Freund's adjuvant, while group 5A rabbits received o-gp160$_{IIIB}$ formulated with Alhydrogel or MPL-containing adjuvants (MPL-oe, Detox, or Ras3C). Study 6B was designed to confirm the results obtained in study 5A as well as to test immunogenicity when the interval between the second and third immunizations was increased from 3 to 15 weeks. Study 6B also included groups which received o-gp160$_{IIIB}$ formulated in either Ras3C alone (6B2) or MPL-oe alone (6B4), in contrast to groups 5A2 and 5A4, which received alternating formulation of o-gp160$_{IIIB}$ in Ras3C and MPL-oe. Group 7A received CD4 binding-incompetent, baculovirus-expressed Mrgp160$_{IIIB}$ in Alhydrogel.
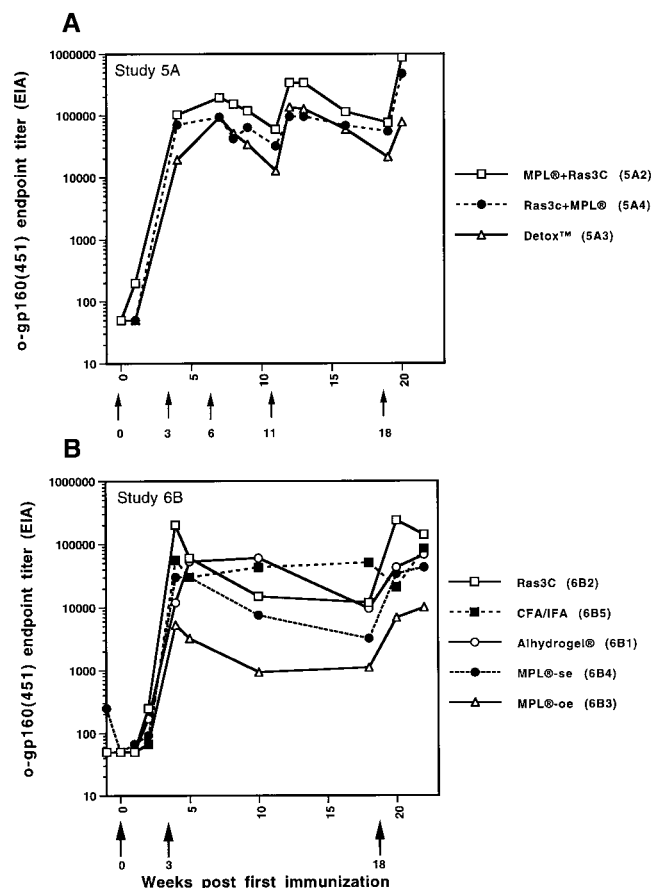
FIG. 1. Immunogenicity of o-gp160$_{IIIB}$ formulated with different adjuvants in rabbits. Serum IgG endpoint titers were measured against o-gp160$_{451}$ at multiple time points over the course of immunizations. The initial serum dilution tested was 1:100; any sera negative at 1:100 were graphed as 1:50. The arrows indicate the timing of immunizations. Rabbits were immunized by using a mixed route of administration at 0, 3, 6, 11, and 18 weeks with o-gp160$_{IIIB}$ in MPL-oe–Ras3c, Ras3c–MPL-oe, or Detox adjuvant (A) or 0, 3, and 18 weeks with o-gp160$_{IIIB}$ in Ras3c, CFA-IFA, Alhydrogel, MPL-se, or MPL-oe (B).

gp120 and Mrgp160 formulated with MPL-containing adjuvants were not included in this study.

The antibody responses to o-gp160$_{IIIB}$ formulated in several different adjuvants (study 5A) are summarized in Fig. 1A. All three rabbits in each adjuvant group (5A2, 5A3, and 5A4) seroconverted to o-gp160 after two immunizations, with one of the groups (5A2) reaching a titer of $>10^5$. Additional boosting of antibody titers above that obtained after two shots was not obtained until after the fourth and fifth shots. Two of the three groups (5A2 and 5A4) reached titers of $>10^5$ after four immunizations. Maximum titers were obtained after the fifth shot in all three groups, and these bleeds were used for all subsequent binding and functional studies. A summary of group 6B immunogenicity is given in Fig. 1B. Responses were seen in all o-gp160$_{IIIB}$-immunized groups (Alhydrogel, Ras3C, MPL-oe, MPL-se, and CFA-IFA) 2 weeks after a single shot and were boosted substantially after the second. The strongest antibody response was obtained with the Ras3C group, which reached titers of $>10^5$ after the second shot. Lower titers obtained in 6B3 than in 5A2 or 5A4 suggested that MPL-oe on its own was a less potent adjuvant than when combined with Ras3C. Maximum titers in all groups were obtained after the third shot, and these sera were used for all subsequent studies.

Sera from all groups were screened against Mrgp160$_{IIIB}$ and o-gp160$_{451}$ by EIA and o-gp160$_{451}$ by SPR to compare total HIV-1 envelope-specific binding levels to each other and to those for sera from HIV-1-infected individuals (Table 1). Screening against Mrgp160$_{IIIB}$ and o-gp160$_{451}$ was included to compare reactivities against linearized and oligomeric forms of gp160. Binding titers against the two proteins as measured by EIA were comparable within each group; sera strongly reactive against Mrgp160$_{IIIB}$ were also strongly reactive against o-gp160$_{451}$. Rabbit sera with the strongest reactivity by EIA included 4A1, 4A2, 5A2, 6B2, and 7A1-1, and all had titers comparable to that obtained with NA pool. HIV-1 serum US14 was included as an example of HIV-1 sera with exceptionally high antienvelope binding activity. It is important to note that direct comparisons cannot be made between the reactivities of the rabbit and human sera in the EIA format because different conjugates are used for antibody detection. Table 1 also shows binding against o-gp160$_{451}$ determined by SPR. This assay has been demonstrated previously to be more sensitive to conformation-specific antibodies and allows direct comparison of antibody binding levels in different species (62). Differential binding to o-gp160$_{451}$ was obtained when measured by EIA or SPR for several of the groups. For example, groups 5A2, 6B2, and NA pool retained relatively strong binding to o-gp160$_{451}$ by SPR, while the reactivity of 7A1-1, 4A2, and 4A1 was diminished compared to EIA reactivity. The enhanced binding of sera 7A1-1, 4A1, and 4A2 measured in the EIA format relative to SPR is most likely attributable to binding to epitopes accessible due to partial o-gp160$_{451}$ denaturation upon adsorption to the plastic surface of the EIA plate (62). Sera from groups 4A1, 4A2, 5A2, 6B2, and 7A1, all with comparable EIA antibody titers, were selected for further analysis.

Antibodies bind preferentially to native, monomeric gp120. Serum binding to native and denatured forms of monomeric gp120 is shown in Fig. 2. The native/denatured gp120 binding ratio of NA pool (native/denatured gp120 binding ratio of >1) is in agreement with previous results showing that sera from HIV-1-infected individuals bind preferentially to native gp120 (36, 61). In contrast to HIV-1 sera, sera from rabbits immunized with Mrgp160$_{IIIB}$ (7A1-1 and 7A1-2) or immunized subcutaneously with monomeric gp120$_{IIIB}$ (4A1) or o-gp160$_{IIIB}$ (4A2) formulated with CFA-IFA preferentially bound to denatured forms of both gp120$_{IIIB}$ and gp120$_{MN}$ (native/denatured gp120 binding ratio of <1). Binding profiles from these groups (7A1, 4A1, and 4A2) are similar to those obtained in a previous study involving sera from human vaccinees receiving recombinant CHO gp120$_{IIIB}$ (Genentech) or rgp160$_{IIIB}$ (MicroGeneSys) in alum or rgp160$_{IIIB}$ (ImmunoAG) in alum and deoxycholate (61).

To determine whether adjuvant formulation or route of administration of immunogen affected specificities of antibodies elicited during immunization, sera from rabbits immunized with o-gp160$_{IIIB}$, formulated with Alhydrogel (6B1), MPL-containing (6B2 and 6B4), or Freund's (6B5) adjuvants and administered by the mixed route were similarly evaluated. Sera from groups 6B1, 6B2, and 6B4 preferentially bound native gp120 (native/denatured gp120 binding ratio of >1), more consistent with the binding profiles of antibodies generated during natural HIV-1 infection. Rabbits receiving o-gp160$_{IIIB}$ formulated in CFA-IFA (6B5) and administered by the mixed immunization route had ratios somewhat lower than those in the other groups (6B1, 6B2, and 6B4), suggesting that CFA-IFA formulation itself may have some impact on the conformational structure of o-gp160 and alter recognition of native gp120 epitopes. Interestingly, native/denatured gp120 binding ratios for group 6B5 were higher than those for group 4A2,
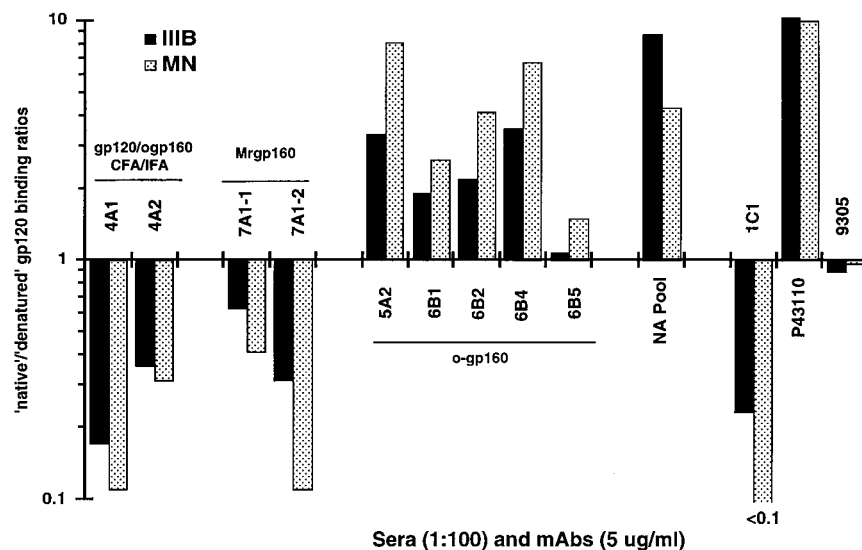
FIG. 2. Native/denatured gp120 binding ratios of sera from immunized rabbits and HIV-1-infected individuals. Groups are described in Table 1. Sera from groups 5A2, 6B1, 6B2, and 6B4 preferentially bound native gp120 similarly to HIV-1 sera. Control antibodies include MAb 1C1 specific for denatured gp120 (native/denatured gp120 binding ratio of <1.0), MAb P43110 specific for properly folded gp120 (ratio of >1), MAb 9305 specifically for a linear epitope within the V3 loop of gp120 exposed on both native and reduced forms of gp120 (ratio of ~1), and NA pool, sera pooled from 35 HIV-1-seropositive individuals.

suggesting that the inclusion of intradermal and intramuscular routes of administration may be more favorable for the induction of antibodies with specificities to native gp120. More recent data have demonstrated that sera from rabbits given o-gp160$_{IIIB}$ formulated in Ras3c by the intramuscular route alone also preferentially bound native gp120 (data not shown).

Recognition of native versus denatured forms of gp120 was further examined as a function of the number of immunizations. As shown in Table 2, groups 6B1, 6B2, and 6B4, but not 6B5, had increases in relative recognition of native gp120 after a third immunization, suggesting a maturation of the immune response toward the recognition of epitopes preferentially accessible on native gp120.

Linear epitope recognition. The various rabbit immune and HIV-1 sera were screened against a panel of linear peptides corresponding to gp120$_{IIIB}$, and the results are summarized in Table 3. Regions of gp120 covered by these peptides (25 to 40 amino acids in length) are indicated as Cn and Vn, where C and V indicate conserved and variable regions of gp120 and n equals 1 to 5, depending on the region within gp120 (29); the numbering is based on the HIV-1$_{LAI}$ strain (42). Individual sera from two to three rabbits per group were screened by EIA, and endpoint titers were determined against each of the peptides. NA pool was included as a comparison. The two predominant epitopes recognized by NA pool are epitopes at the N terminus (C1) and the C terminus (C5) of gp120. Sera from groups 4A1, 4A2, and 7A1 recognized the greatest number of linear epitopes, 13, 12.5, and 10, respectively, and were the same groups that preferentially bound denatured gp120 (native/denatured gp120 binding ratios of <1.0) (Fig. 2). These sera were particularly reactive with peptides within conserved regions (C1, C2, C3, and C4). Recognition of epitopes within conserved regions is consistent with native/denatured binding ratios of <1 measured against homologous rgp120$_{IIIB}$ as well as the heterologous gp120$_{MN}$. Groups whose sera recognized fewer peptides, 5A2 (2.0), 6B1 (5.3), and 6B2 (5.3), preferentially bound native gp120 (Fig. 2). Increased linear epitope recognition and preferential reactivity with denatured gp120 are consistent observations since many of the linear, contigu-

ous epitopes within gp120 are thought to be relatively inaccessible on natively folded gp120 (39).

Monomeric gp120 capture ELISA. To determine whether the rabbit antisera were capable of recognizing monomeric gp120 from HIV-1$_{IIIB}$ as well as from heterologous HIV-1 strains, the sera were screened against gp120 captured from detergent-treated HIV-1 culture supernatants. These included gp120 from HIV-1$_{IIIB}$ (Fig. 3A), HIV-1$_{MN}$ (Fig. 3B), HIV-1$_{RF}$ (Fig. 3C), and HIV-1$_{451}$ (Fig. 3D). Sera from groups 6B2-pre, 6B2, 6B1, 7A1-1, and 7A1-2 were selected for this analysis. Pooled preimmune serum from group 6B (6B2-pre) served as a negative control for each assay. NA pool gave the strongest binding signal against each of the different gp120 proteins studied, but as mentioned previously, direct comparison cannot be made since different conjugates are used to detect binding rabbit and human Ig in the EIA format. Therefore, assuming the HIV-1 serum pool to be comprised of antibodies with broad specificity and capable of binding to many clade B gp120 proteins, comparisons were made between the breadth of rabbit antisera and HIV-1 sera gp120 binding.

Each of the evaluated rabbit sera bound to gp120 from HIV-1$_{IIIB}$ in the rank order NA pool >6B2>6B1>7A1-1> 7A1-2≫6B2-pre (Fig. 3A). This reactivity pattern is consistent with the SPR binding against o-gp160$_{451}$, but not Mrgp160$_{IIIB}$

TABLE 2. Maturation of the rabbit anti-gp120 immune response

Group	Adjuvant	Native/denatured gp120 binding ratio[a]	
		Bleed 2a	Bleed 3a
6B1	Alhydrogel	1.02 ± 0.22	1.95 ± 0.59
6B2	Ras3c	1.86 ± 0.09	2.20 ± 0.42
6B4	MPL-se	1.59 ± 0.05	3.45 ± 1.98
6B5	CFA-IFA	1.10 ± 0.06	0.98 ± 0.08

[a] Rabbits were immunized with o-gp160$_{IIIB}$ by the mixed immunization route. Ratios (determined by SPR) are means and standard deviations of three rabbits per group. Bleeds 2a and 3a were obtained 1 week after the second and third immunizations, respectively.

TABLE 3. Linear gp120 peptide recognition of sera from rabbits immunized with various HIV-1 Env subunit vaccines

Treatment[a]	Endpoint serum dilution as determined by ELISA[b]																No. with titer of >1:800[c]
	C1, 31[d]	C1, 53	C1, 74	C1, 99	C1/V1, 112	V1, 132	V2/C2, 191	C2, 211	C2, 246	C2/V3, 283	V3/C3, 319	C3, 348	C3/V4, 373	C4, 420	V4/C5, 454	C5, 480	
4A1-1	−	++	+++	++	++	+	+++	+++	+++	+++	+	+++	+++	++	+++	+++	13.0
4A1-2	+	++	+++	+++	++	−	++	++++	++++	−	++	++	++	++	+++	+++	
4A2-1	+++	+++	+++	+++	++	++	++	++	+++	+++	+++	++	−	++	+++	++	12.5
4A2-2	−	++++	++++	++	−	−	+	++	−	++	++	+++	+	++	++	+++	
7A1-1	+	+++	+++	+	−	++	+	+	++	−	++	++	−	++	++	++	10.0
7A1-2	−	+++	+++	+++	−	−	++	++	+++	++	++	+	−	++	+++	++	
6B5-1	++	++	++	+	+	++	+	+	++	−	++	+	−	++	+	++	
6B5-2	++	++	++	+	−	+	−	+	+	−	++	−	−	+	+	++	7.0
6B5-3	−	+++	++	+	+	+++	+	+	++	−	−	−	−	++	++	++	
6B1-1	−	−	−	−	−	−	−	−	−	−	++	−	−	−	−	++	
6B1-2	−	+	++	++	++	++	+	+	++	−	+	−	−	+	+++	++	5.3
6B1-3	−	+	++	+++	++	+++	−	+	++	−	++	−	−	−	+	++	
6B2-1	−	+	+	+	−	+	−	+	−	−	+++	−	−	++	++	+++	
6B2-2	−	++	++	++	++	+	−	++	++	−	+	−	−	+	++	+++	5.3
6B2-3	−	++	+	+	−	++	+	+	−	−	++	−	−	+	++	+++	
5A2-1	−	+	+	+	−	−	+	+	−	−	+	−	−	+	−	++	
5A2-2	−	−	+	++	−	+	+	+	−	−	++	−	−	−	−	++	2.0
5A2-3	−	−	+	++	−	+	+	+	−	−	+	−	−	−	−	++	
NA pool	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	2.0

[a] Immunogen, adjuvant, and route of administration for each of the rabbit vaccine groups (given in parentheses) are as follows: 4A1 (gp120$_{IIIB}$, CFA-IFA, subcutaneous); 4A2 (o-gp160$_{IIIB}$, CFA-IFA, subcutaneous); 7A1 (Mrgp160$_{IIIB}$, Alhydrogel, intramuscular); 5A2 (o-gp160$_{IIIB}$, MPL plus Ras3C, mixed route); 6B5 (o-gp160$_{IIIB}$, CFA-IFA, mixed route); 6B1 (o-gp160$_{IIIB}$, Alhydrogel, mixed route); 6B2 (o-gp160$_{IIIB}$, Ras3C, mixed route).
[b] ++++, >1:51.2k; +++, >1:6.4k; ++, >1:800; +, >1:100; −, <1:100.
[c] Average number of peptides recognized with a serum endpoint titer of >1:800 for each serum group.
[d] Starting amino acid in gp120.

or o-gp160$_{451}$ by EIA (Table 1). Similar binding profiles against HIV-1$_{MN}$ were obtained for NA pool, 6B2, and 6B1, with relatively weaker binding by 7A1-1 and 7A1-2 (Fig. 3B). A similar trend was observed against gp120 from HIV-1$_{RF}$ (Fig.

3C). There was some drop in reactivity of 6B2 and 6B1 compared to NA pool (2-fold) but a more pronounced decrease in 7A1-1 and 7A1-2 (100-fold). Binding by these two sera was detectable only at the lowest dilution tested (1:100). Binding to
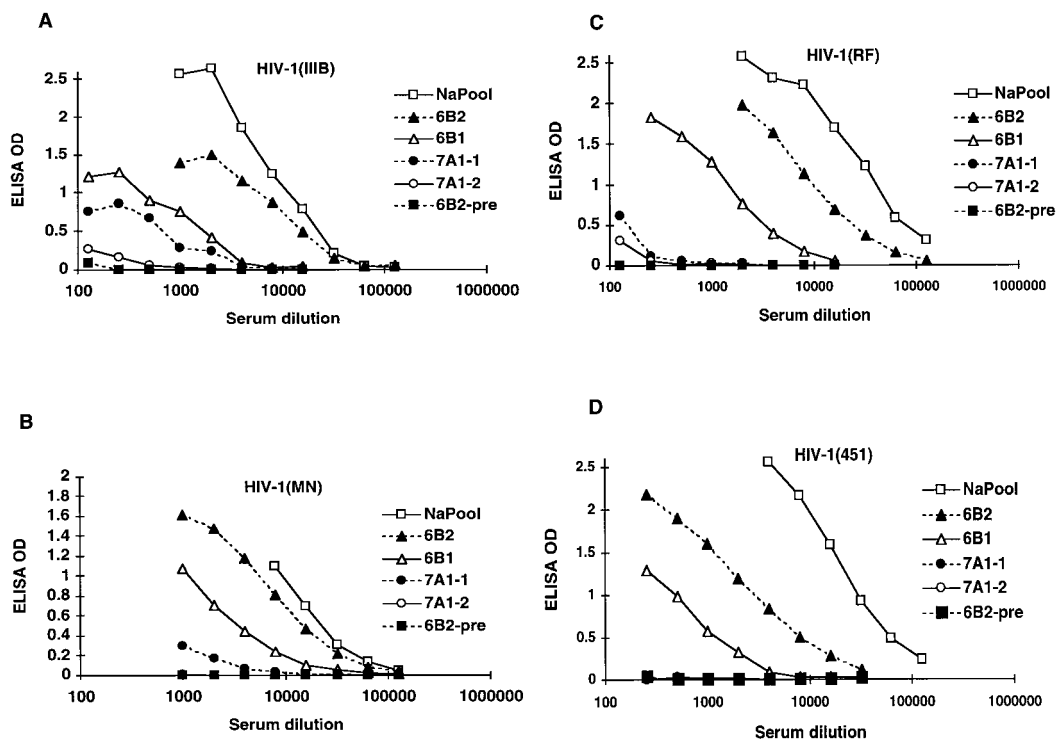

FIG. 3. Binding of rabbit immune and HIV-1 sera to monomeric gp120 captured from detergent-treated HIV-1$_{IIIB}$ (A), HIV-1$_{MN}$ (B), HIV-1$_{RF}$ (C), and HIV-1$_{451}$ (D). Sera were titrated by using serial twofold dilutions against each captured gp120. Groups are as follows: 6B1, o-gp160$_{IIIB}$ in Alhydrogel, mixed route; 6B2, o-gp160$_{IIIB}$ in Ras3c, mixed route; 7A1-1,2, baculovirus rgp160$_{IIIB}$ in Alhydrogel, intramuscular. OD, optical density; 6B2-pre, pooled preimmune serum from group 6B (negative control).
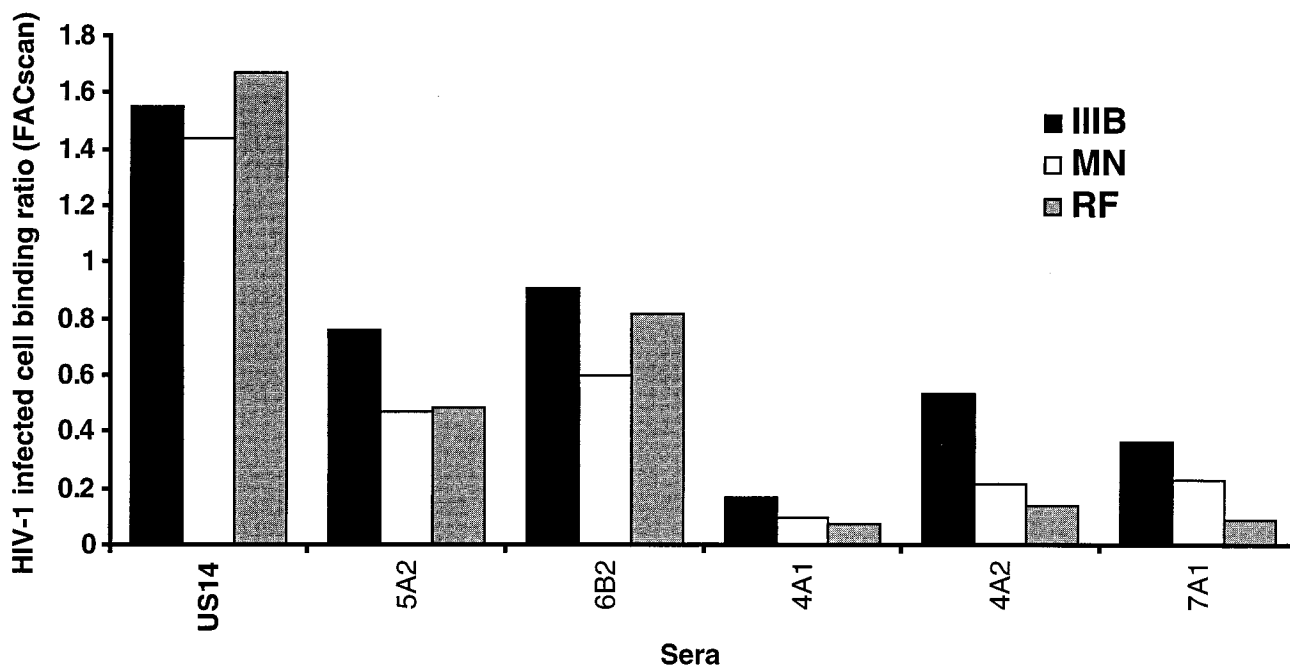
FIG. 4. Binding of rabbit immune and HIV-1 sera to H9 cells acutely infected with either HIV-1$_{IIIB}$, HIV-1$_{MN}$, or HIV-1$_{RF}$ as measured by flow cytometry. Data are plotted as the test serum/HIV-1 serum pool (NA pool) binding ratio, with binding measured in mean PE fluorescence. Values of >1 indicate serum binding levels greater than those obtained with NA pool. HIV-1 serum US14 and rabbit sera 5A2 and 6B2 retain consistent binding against all three TCLA HIV-1 isolates, while sera 4A1, 4A2, and 7A1 bind with reduced efficiency to HIV-1$_{RF}$. Groups are described in Table 1.

HIV-1$_{451}$ by both 6B2 and 6B1 dropped an additional twofold but was still relatively strong, while no detectable binding was measured by 7A1-1 or 7A1-2 at the lowest dilution tested (Fig. 3D). The ability of 7A1-1 and 7A1-2 sera to bind o-gp160$_{451}$ (Table 1) but not monomeric gp120$_{451}$ (Fig. 3D) indicates that the epitopes recognized in the former are within gp41 or exposed only after adsorption to the plastic surface of the EIA plates. These data indicated that sera from rabbit groups 6B2 and 6B1 (o-gp160$_{IIIB}$ formulated with Alhydrogel or Ras3c) and with native/denatured gp120 binding ratios of >1, but not groups 7A (Mrgp160$_{IIIB}$ in Alhydrogel, native/denatured gp120 binding ratios of <1), retained comparable antibody binding levels to divergent strains of monomeric gp120.

Binding to the surface of cells acutely infected with HIV-1. Several groups have reported that the conformation of cell surface-expressed oligomeric gp120/gp41 may differ from that of monomeric forms of gp120 and that binding to the former may better correlate with the functional activity of any given antibody (49, 56). Sera from rabbit groups 5A2, 6B2, 4A1, 4A2, and 7A1 were assayed for binding to oligomeric gp120/gp41 expressed on the surface of H9 cells acutely infected with HIV-1$_{IIIB}$, HIV-1$_{MN}$, or HIV-1$_{RF}$ by using flow cytometry (Fig. 4). The data are expressed as a ratio of rabbit serum binding to NA pool serum binding. NA pool was reactive with all HIV-1-infected H9 cells and is representative of a broadly reactive antibody population. Values greater than 1 indicate stronger binding relative to NA pool, while values less than 1 indicate weaker binding relative to NA pool. Expression of data in this manner allowed for normalization for the amount of cell surface HIV-1 envelope expression, which varied temporally during acute infection. HIV-1 serum US14 was included as an example of sera with high levels of HIV-1 envelope-specific antibody, as demonstrated by ratios of >1 against cells infected with each of the isolates (IIIB, MN, and RF). Binding ratios

were determined at three separate serum dilutions (1:50, 1:250, and 1:1,250) to ensure that binding values below saturation levels were used to calculate ratios. Serum pools from each of the rabbit groups had their strongest binding ratios against H9 cells infected with the homologous HIV-1$_{IIIB}$. Those serum pools which were most reactive by SPR analysis (Table 1) were also most reactive against HIV-1$_{IIIB}$-infected H9 cells. Of note, binding differences among the rabbit groups were observed against the heterologous HIV-1 isolates. While sera from HIV-1 (US14) and rabbit groups 5A2 and 6B2 bound with similar efficiencies to IIIB, MN, and RF, sera from

TABLE 4. Neutralization of TCLA HIV-1 isolates by immune sera

Group[a]	Neutralization titer[b]					
	HIV-1$_{IIIB}$		HIV-1$_{MN}$		HIV-1$_{RF}$	
	50%	90%	50%	90%	50%	90%
4A1	98	<8	81	<8	<8	<8
4A2	83	<8	<8	<8	<8	<8
5A2	587	24	1,086	137	430	24
6B1	126	<8	302	16	8	<8
6B2	1,220	13	1,846	284	429	10
6B3	<8	<8	<8	<8	<8	<8
6B4	121	<8	50	<8	<8	<8
6B5	52	<8	259	<8	<8	<8
7A1	39	<8	559	19	<8	<8
NA pool	175	9	11,468	2,162	1,543	159
US18	888	165	3,046	811	960	139
US9	698	70	2,263	398	968	72
US10	10	<8	866	70	155	<8

[a] US18, US9, and US10 are sera from HIV-1-seropositive individuals with strong, moderate, and weak neutralization capacity, respectively.
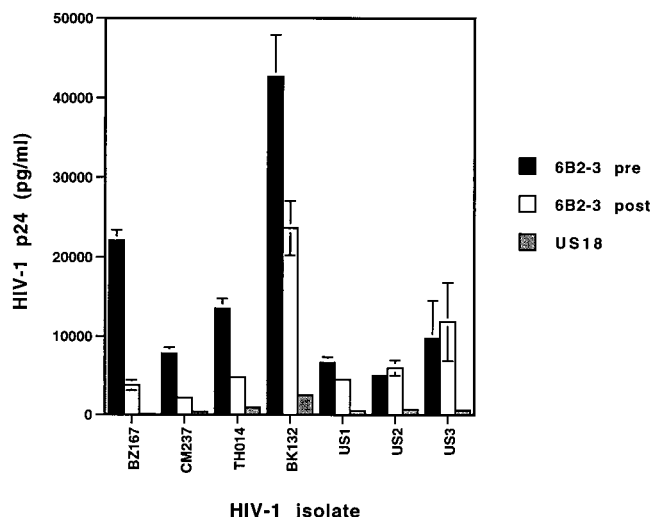[b] Serum dilution capable of inhibiting viral growth by 50 and 90%.

FIG. 5. Neutralization of several primary HIV-1 isolates by an HIV-1 serum and a serum from one of the rabbits in group 6B2. Sera from rabbit 6B2-3, preimmunization (pre) and after the third immunization (post), at a 1:10 dilution were incubated with approximately 25 to 50 $TCID_{50}$ of primary isolate BZ167, CM237, TH014, BK132, US1, US2, or US3. Data indicate the means and standard errors of the percent reduction of HIV-1 p24 in the presence of the 6B2-3 pre- and postimmunization sera.

groups 4A1, 4A2, and 7A1 bound 60, 74, and 76%, respectively, less efficiently to RF. These data indicate that sera from o-gp160-immunized rabbits efficiently bind cell surface-expressed oligomeric gp120/gp41 from multiple HIV-1 isolates.

HIV-1 neutralization capacity of rabbit immune sera. Pooled sera from several rabbit vaccine groups were selected for analysis of functional neutralizing antibody against TCLA HIV-1_{IIIB}, HIV-1_{MN}, and HIV-1_{RF}. The 50 and 90% neutral-

ization titers for each of the sera are summarized in Table 4. NA pool was included for comparison, as were US18, US9, and US10, which were selected based on strong, moderate, and weak neutralization of primary isolates, respectively (reference 32 and unpublished data). Each HIV-1 serum except US10 had detectable 90% neutralization titers against HIV-1_{IIIB}, HIV-1_{MN}, and HIV-1_{RF}, demonstrating broad neutralization capacity against TCLA HIV-1. Two groups of rabbit sera (5A2 and 6B2) also exhibited 90% neutralization activity against all three HIV-1 isolates. The 90% titers were weaker than that obtained with the NA pool or strong HIV-1 sera but comparable with the titers for weak and moderate sera. This broad neutralizing activity against TCLA HIV-1 correlated with the strong binding against infected cells (Fig. 4). Sera from rabbit groups 4A1, 6B1, 6B4, 6B5, and 7A1 had neutralizing activity against homologous HIV-1_{IIIB} and HIV-1_{MN} but not HIV-1_{RF}. This lack of neutralization activity of 4A1, 4A2, and 7A1 against HIV-1_{RF} is consistent with the loss in binding to H9 cells infected with HIV-1_{RF} (Fig. 4). Despite similar EIA antibody binding titers for groups 4A2 (o-gp160$_{IIIB}$ in CFA-IFA) and 5A2 and 6B2 (o-gp160$_{IIIB}$ in MPL-containing adjuvants), group 4A2 bound less efficiently to HIV-1-infected cells and had substantially less neutralization against MN and RF. Sera from 6B3 had no activity against any HIV-1 tested, consistent with its relatively low antibody binding titers (Table 1).

Sera with the broadest neutralization activity against TCLA HIV-1 were selected to assay against seven primary HIV-1 isolates. These viruses were chosen to represent a spectrum of primary isolate neutralization susceptibilities. Isolates CM237, TH014, US1, US2, and US3 were phenotyped as non-syncytium inducing by MT2 assay, while BZ167 and BK132 were syncytium inducing (data not shown). Of the seven viruses, BZ167 was the most susceptible to neutralization by a panel of HIV-1-positive sera (data not shown). Neutralization data from a representative assay for one of the sera (6B2-3) are
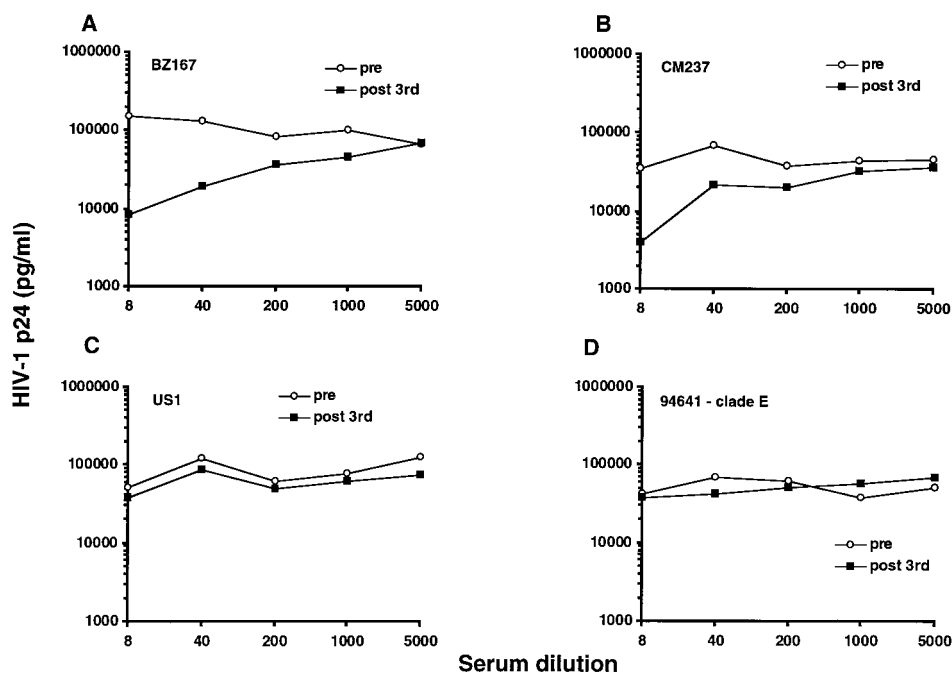

FIG. 6. Neutralization of primary HIV-1 isolates by purified Ig from rabbit serum. Five fivefold dilutions of rabbit Ig purified from rabbit serum before and after three immunizations with o-gp160$_{IIIB}$ formulated in Ras3c adjuvant were tested for neutralization against HIV-1_{BZ167} (A), HIV-1_{CM237} (B), HIV-1_{US1} (C), and HIV-1_{9461} (clade E) (D).

TABLE 5. Neutralization of primary HIV-1 isolates by rabbit immune and HIV-1 sera

Serum	Mean HIV-1 neutralization (% reduction in p24)[a] $\pm$ SD ($n = 2$–4)							Total
	BZ167	CM237	TH014	BK132	US1	US2	US3	
Rabbits								
5A2	95 ± 6	90 ± 6	83 ± 1	ND	<50	75	ND	3/5
6B2-1	84 ± 10	92 ± 2	<50	<50	<50	<50	<50	2/7
6B2-2	91 ± 7	<50	<50	<50	<50	<50	<50	1/7
6B2-3	88 ± 6	82 ± 11	61 ± 10	<50	<50	<50	<50	2/7
4A1	<50	<50	ND	ND	ND	ND	ND	0/2
4A2	<50	<50	ND	ND	ND	ND	ND	0/2
7A1	<50	<50	ND	ND	ND	ND	ND	0/2
HIV-1 sera								
US7	96 ± 2	59 ± 7	ND	<50	62	<50	ND	1/5
US9	91 ± 11	80 ± 13	86 ± 5	ND	76	60 ± 8	ND	3/5
US10	83 ± 14	96 ± 4	97 ± 1	65	<50	<50	ND	3/6
US18	97 ± 2	97 ± 3	94 ± 5	94 ± 5	93 ± 10	95 ± 1	94 ± 7	7/7

[a] Percent reduction in p24 antigen from the corresponding rabbit preimmune serum or normal human serum control. ND, not determined.

shown in Fig. 5. Serum 6B2 inhibited growth of BZ167, CM237, TH014, BK132, US1, and US2 by 85, 82, 65, 45, 32, −5, and −12%, respectively. The absence of US1, US2, or US3 neutralization indicated the effect was virus specific and not attributable to the presence of some nonspecific inhibitor of viral or cell growth. To further test neutralization specificity, pre- and postimmunization sera from rabbit 6B2-3 were Ig purified and assessed for neutralization activity against HIV-1 strains BZ167, CM237, US1, and 9461 (clade E) (Fig. 6). Twofold dilutions of Ig were incubated with each of the primary viruses. Concentrations of Ig used were normalized to serum o-gp160$_{451}$ binding activity prior to purification (as measured by SPR) to correct for some nonspecific loss in Ig during the purification procedure. Rabbit Ig neutralized both BZ167 and CM237 (>80%), with a corresponding <10% loss in growth of 9461. These data demonstrate that the Ig-purified fraction of the rabbit polyclonal sera retained comparable neutralizing activities against BZ167 and CM237, with minimal nonspecific effects against a non-clade B HIV-1 primary isolate.

Neutralization data for individual sera from group 6B2 and pooled sera from groups 4A1, 4A2, 5A2, and 7A1 are summarized in Table 5 along with data for several HIV-1 sera with weak (US7), moderate (US9 and US10), and strong (US18) neutralizing activity. HIV-1 sera US7, US9, US10, and US18 had >80% neutralizing activity against one of five, three of five, three of six, and seven of seven primary isolates, respectively. Individual sera from group 6B2 had >80 neutralizing activity against two of seven, one of seven, and two of seven isolates tested, while pooled sera from group 5A2 neutralized three of five isolates tested. Neutralization profiles of the rabbit sera were similar to those of HIV-1 sera with weak to moderate neutralizing activity. Sera from groups 4A1, 4A2, and 7A1 had no detectable activity against the two more susceptible isolates, HIV-1$_{BZ167}$ or HIV-1$_{CM237}$, and were therefore not tested against the other primary isolates.

DISCUSSION

Designing an HIV-1 immunogen capable of eliciting an immune response with functional neutralizing activity against primary HIV-1 isolates is considered an important objective in HIV-1 vaccine design. In this study, we have demonstrated that neutralizing activity against some, but not all, primary HIV-1 isolates can be elicited in rabbits by immunizing with an affin-

ity-purified oligomeric gp160 formulated in Alhydrogel or MPL adjuvant preparations. Neutralizing activity did not extend to all primary HIV-1 isolates tested but rather included only those isolates previously found to be relatively susceptible to HIV-1 serum-mediated neutralization and was comparable to that observed with weak to moderate neutralizing HIV-1 seropositive sera. Importantly, both the quantity and the quality of the antibody response were critical. The quality of the immune response was influenced by several parameters, including immunogen conformation, adjuvant formulation, and route of vaccine administration. Additionally, there appears to be a threshold antibody titer required to detect neutralization of primary isolates.

A goal of our current preclinical HIV-1 vaccine development plan was to elicit an antibody response comparable to that observed in sera from HIV-1-infected individuals both in quality and in quantity and thus be sufficient to achieve broad HIV-1 neutralization. As described previously, our evaluation of antibody responses elicited by candidate vaccines included assays which measured antibody binding to HIV-1 envelope possessing proper tertiary and quaternary structure (61). Analysis of antibody binding dependence on intact monomeric gp120 tertiary structure was evaluated by measuring reactivity against native and reduced forms of monomeric gp120 by using SPR. Using this assay, we previously showed that antibodies elicited by some of the current IIIB envelope glycoprotein vaccine products (CHO gp120, baculovirus gp160, and vaccinia virus gp160) in human volunteers bound preferentially to linear epitopes accessible on denatured gp120 and independent of intact tertiary gp120 structure (61). This is in contrast to the profile observed in sera from HIV-1-infected individuals (36), perhaps partially explaining the inability of these vaccine products to elicit broadly neutralizing antibody.

The antibody response elicited by o-gp160$_{IIIB}$ formulated with Alhydrogel, MPL, and Ras3c preferentially bound native gp120 similar to that observed with HIV-1 serum. This profile was not observed, however, in rabbits immunized with a monomeric gp120$_{IIIB}$ in CFA-IFA or with baculovirus-expressed gp160$_{IIIB}$ in Alhydrogel. It is important to note that this study did not include a vaccine arm with monomeric gp120$_{IIIB}$ in Alhydrogel or MPL-containing adjuvants, although in a previous study (61), CHO rgp120$_{IIIB}$ formulated in Alhydrogel elicited an antibody profile similar to that observed in the present study with monomeric gp120 in CFA-IFA. It remains possible that a monomeric gp120 protein formulated in MPL-contain-

ing adjuvants or other adjuvants would elicit an antibody profile preferentially reactive with native gp120, and further studies are needed to address this issue. The antibody response to o-gp160$_{IIIB}$ in CFA-IFA yielded different results depending on the route of administration: subcutaneous yielded antibodies preferentially directed to denatured gp120 (group 4A2), while a combination of intramuscular, intradermal, and subcutaneous yielded antibodies directed similarly to both forms of gp120 (group 6B5). These data suggest an impact of both adjuvant formulation and route of administration on the quality of the immune response to gp120 by altering the recognition of epitopes accessible on linearized forms of gp120. Freund's adjuvant disruption of protein conformation structure has been suggested in some studies (53) but disputed by others (5). More direct studies to assess the impact of immunization route on the quality of the immune response are required and are ongoing.

Preferential serum recognition of denatured gp120 was reflected both by an increase in the number of linear gp120 epitopes recognized by peptide EIA and by reduced binding to heterologous strains of gp120. Sera from naturally infected individuals recognize a limited number of linear peptides, predominantly epitopes within the V3 and C5 regions of gp120. This was confirmed in the present study, as the dominant linear gp120 responses were directed at peptides located within the C terminus of gp120 (LAI-480) and within V3, with some weaker reactivity against a peptide located at the N terminus (C1) of gp120 (LAI-31). Reactivity against V3, however, was not with the LAI strain but against an MN V3 peptide (data not shown). Sera from groups 4A1, 4A2, and 7A strongly recognized multiple peptides (13, 12.5, and 10, respectively) compared to HIV-1 sera, which recognized only 2 peptides. Rabbits receiving o-gp160$_{IIIB}$ in Alhydrogel, Ras3c, or the Ras3c-MPL-oe combination recognized considerably fewer linear peptides, 5.3, 5.3, and 2.0, respectively. Formulation in CFA-IFA resulted in recognition of an intermediate number of epitopes ($n = 7$), again consistent with its lower native/denatured gp120 binding ratio. All groups had comparably strong reactivities against the C terminus of gp120. Since many of these linear epitopes are not predicted to be exposed on the native gp120 (39, 40), antibodies recognizing such epitopes would contribute to binding against only the denatured gp120, not native gp120, resulting in lower native/denatured gp120 binding ratios. Therefore, a higher native/denatured gp120 binding ratio is consistent with decreased recognition of unexposed linear gp120 and a greater concentration of binding antibody directed against exposed epitopes.

To assess the functional capacity of immune sera, HIV-1 neutralization studies were performed against both TCLA and primary HIV-1 isolates. As a comparison, several sera from HIV-1-infected individuals with strong, moderate, and weak overall neutralizing activity were included. Neutralizing activity against the homologous HIV-1$_{IIIB}$ was present in the majority of the rabbit immune sera and all HIV-1 sera tested and was therefore not a useful indicator of differences among the various vaccine groups. However, substantial differences in the ability to neutralize heterologous TCLA HIV-1 were observed. Sera with high o-gp160 antibody binding titers ($>102.4 \times 10^3$) and with preferential binding to native gp120 (native/denatured gp120 binding ratios of >1) (5A2, 6B2, and HIV-1 sera) neutralized HIV-1$_{RF}$, while sera from groups with lower serum o-gp160 binding titers and/or with preferential binding to denatured gp120 had no HIV-1$_{RF}$ neutralizing activity. The neutralization profiles against IIIB, MN, and RF were consistent with binding to both monomeric gp120 captured from heterologous TCLA isolates and H9 cells infected with heterologous

TCLA isolates. Neutralizing activity against HIV-1$_{IIIB}$, HIV-1$_{MN}$, and HIV-1$_{RF}$ for sera 5A2 and 6B2 was comparable to that for weak to moderate HIV-1 sera.

Those sera with the strongest and broadest neutralizing activity against TCLA HIV-1 were tested against several primary HIV-1 isolates. The primary isolates included all belong to HIV-1 clade B and have been found to be differentially susceptible to neutralization by HIV-1 sera (reference 32 and unpublished results). Isolates CM237, TH014, US1, and US2 are non-syncytium inducing, and BZ167 and BK132 are syncytium inducing. Postimmunization sera from groups receiving o-gp160 in MPL-containing adjuvants (5A2 and 6B2) neutralized primary HIV-1 isolates CM237 and BZ167 ($>80\%$ at 1:10 serum dilution). Sera from rabbits with high o-gp160 EIA binding titers, but with preferential antibody binding to denatured gp120 (4A1, 4A2, and 7A1), failed to neutralize these two primary isolates. Group 5A2 receiving five o-gp160$_{IIIB}$ immunizations had additional neutralizing activity against TH014, with borderline activity against US2, suggesting that repeated immunizations may increase the breadth of neutralizing antibody response. Previous studies have demonstrated increased neutralization of laboratory-adapted HIV-1 after repeated immunizations (20). Immune sera were not capable of neutralizing several of the primary isolates examined (BK132, US1, and US3), a neutralization profile similar to that observed in assays using HIV-1 sera characterized as having weak to moderate neutralizing activity (US7, US9, and US10). This result suggests that some primary isolates may be resistant to neutralization by antibodies elicited by o-gp160, the immune sera may lack the necessary type-specific neutralizing antibodies for these specific primary isolates, or a quantitative threshold of appropriate antibody was not achieved. Studies using o-gp160 proteins from other HIV-1 strains to compare neutralizing activities against other primary HIV-1 isolates are ongoing.

We have demonstrated the feasibility of using a soluble HIV-1 envelope glycoprotein preparation to elicit, in small animals, an antibody response capable of neutralizing primary HIV-1 isolates, using PHA-stimulated PBMC as targets. The specificities of immune serum antibodies responsible for the observed neutralizing activity have not been identified. Since monomeric gp120 is known to be the target for several potent neutralizing MAbs (8, 17, 58, 59), it is possible that the use of an oligomeric form of gp160 protects and enhances presentation of these critical epitopes for immune recognition. Several correlates between neutralization activity and antibody binding properties can be drawn: the quality of antibody as demonstrated by preferential recognition of native gp120, minimal recognition of unexposed linear epitopes, strong cross-reactive binding against divergent clade B monomeric gp120 proteins and cell surface-expressed oligomeric gp120/gp41 complexes, and neutralization of multiple TCLA HIV-1 isolates. Sera with these binding properties were more likely to have neutralizing activity against primary isolates, suggesting that antibodies specific for conserved epitopes requiring proper tertiary and possibly quaternary structure may play a greater role in mediating neutralization of primary isolates.

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