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Polyvalent Envelope Glycoprotein Vaccine Elicits a Broader Neutralizing Antibody Response but Is Unable To Provide Sterilizing Protection against Heterologous Simian/Human Immunodeficiency Virus Infection in Pigtailed Macaques

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The great difficulty in eliciting broadly cross-reactive neutralizing antibodies (NAbs) against human immunodeficiency virus type 1 (HIV-1) isolates has been attributed to several intrinsic properties of their viral envelope glycoprotein, including its complex quaternary structure, extensive glycosylation, and marked genetic variability. Most previously evaluated vaccine candidates have utilized envelope glycoprotein from a single virus isolate. Here we compare the breadth of NAb and protective immune response following vaccination of pigtailed macaques with envelope protein(s) derived from either single or multiple viral isolates. Animals were challenged with Simian/human immunodeficiency virus strain DH12 ($SHIV_{DH12}$) following priming with recombinant vaccinia virus(es) expressing gp160(s) and boosting with gp120 protein(s) from (i) LAI, RF, 89.6, AD8, and Bal (Polyvalent); (ii) LAI, RF, 89.6, AD8, Bal, and DH12 (Polyvalent-DH12); (iii) 89.6 (Monovalent-89.6); and (iv) DH12 (Monovalent-DH12). Animals in the two polyvalent vaccine groups developed NAbs against more HIV-1 isolates than those in the two monovalent vaccine groups ($P = 0.0054$). However, the increased breadth of response was directed almost entirely against the vaccine strains. Resistance to $SHIV_{DH12}$ strongly correlated with the level of NAbs directed against the virus on the day of challenge ($P = 0.0008$). Accordingly, the animals in the Monovalent-DH12 and Polyvalent-DH12 vaccine groups were more resistant to the $SHIV_{DH12}$ challenge than the macaques immunized with preparations lacking a DH12 component (viz. Polyvalent and Monovalent-89.6) ($P = 0.039$). Despite the absence of any detectable NAb, animals in the Polyvalent vaccine group, but not those immunized with Monovalent-89.6, exhibited markedly lower levels of plasma virus than those in the control group, suggesting a superior cell-mediated immune response induced by the polyvalent vaccine.

Neutralizing antibodies (NAbs) have been shown to be critical components of the immune response that controls a variety of viral infections. However, the protective role(s) of NAbs directed against human immunodeficiency virus type 1 (HIV-1) and other primate lentiviruses, which become detectable following acute infections, has been debated over the years and remains unresolved. For example, the clearance of HIV-1 from plasma during the primary infection occurs prior to the appearance of NAbs in newly infected individuals (37). Furthermore, in many studies, vaccinated macaques are able to efficiently control a virus challenge in the absence of detectable NAb, particularly those animals immunized with live, attenuated-virus vaccines (2, 16, 45, 51). Nonetheless, passive immunization experiments have demonstrated the protective effects of NAbs against subsequent challenges with primate lentiviruses (17, 20, 30, 32, 33, 42, 44, 47, 48). In some of these studies, sterilizing immunity could be achieved when high plasma concentrations of NAbs were present prior to virus inoculation. However, the design and/or development of immunogens capable of prospectively eliciting broadly reactive NAbs against multiple virus isolates has been frustratingly unproductive. None of the envelope glycoprotein-based vaccine candidates tested in primates thus far have been able to elicit broadly reactive NAbs, especially against primary isolates.

The HIV-1 envelope glycoprotein contains five highly variable regions, designated V1 through V5, the first four of which form loops through intramolecular disulfide linkage. These variable regions very likely cover significant portions of the exposed surface on the trimeric gp120 complex, as suggested from antigenic probing with monoclonal antibodies (38) and crystallographic data of the envelope core (28). The variable regions of HIV-1 and simian immunodeficiency virus (SIV) gp120 have long been known to be targeted by NAbs, possibly explaining the antigenic variation associated with these regions (9, 19, 22, 23, 27, 34, 43, 46, 55). In contrast, the conserved domains of gp120 are either extensively shielded by carbohydrate moieties, obscured beneath the variable regions, or hid-

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den due to intermolecular protein-protein interactions and do not elicit antibodies that neutralize virions (38, 57).

Conserved neutralizing epitopes, present on the unmodified native gp120, have been nearly impossible to identify. To date, only two human anti-gp120 monoclonal NAbs (2G12 and b12), which exhibit relatively broad neutralizing activity, have been isolated (4, 6, 53, 54). Immunization with a variety of envelope glycoprotein preparations (e.g., monomeric gp120, soluble gp160, oligomeric gp140, and virions or virus-like particles) and the use of different vaccine strategies (e.g., whole inactivated virus, subunit, live vector, and DNA vector) usually result in extremely narrow and/or immunogen-specific NAb responses. Theoretically, it might be possible to elicit broadly reactive NAbs by two alternative vaccine strategies: (i) forcing the immune system to preferentially target a conserved gp120 neutralization epitope (assuming its existence) associated with the majority of HIV-1 isolates circulating in a given geographic region, or (ii) immunization with a cocktail of envelope proteins (if feasible) representing the majority of circulating virus isolates, thereby eliciting NAbs against the variable regions of all of the gp120s in the mixture.

Most lentivirus vaccine studies have utilized envelope glycoproteins from either one or, at most, two virus isolates; protective efficacy has usually been assessed using a homologous virus challenge (i.e., the virus isolate used to challenge animals contains the same gp120 as that used for immunization). In reality, however, vaccinated individuals would be expected to encounter an HIV-1 isolate or viral quasispecies containing a gp120 unrelated to the immunogen used for vaccination (heterologous challenge). In this study, we have evaluated a vaccine regimen, based solely on envelope glycoproteins, which utilizes individual or mixtures of both recombinant vaccinia viruses and gp120 boosts to address the following questions pertaining to protection against heterologous virus strains. (i) Is it possible to elicit a broader NAb response by immunization with a mixture (polyvalent) of envelope glycoproteins? (ii) If so, does the breadth of the NAb response extend beyond the virus isolates included in the immunization cocktail? (iii) How does the protective efficacy of the immune response elicited by a polyvalent envelope vaccine compare to that elicited by a monovalent (homologous or heterologous) envelope vaccine? (iv) Will there be antigenic competition between the different envelope glycoprotein components of the polyvalent envelope vaccine cocktail?

MATERIALS AND METHODS

Plasmids and recombinant vaccinia viruses. Recombinant vaccinia viruses that express gp160 of HIV-1 isolates Bal, LAI, RF (vCB43, vCB41, and vCB36, respectively [3]), 89.6 (vBD3 [15]), DH12, and AD8 (vvDHenv and vvADenv, respectively [8]) have been previously described. Virus stocks were propagated in HeLa cells, purified on linear sucrose gradients (29, 31), and resuspended in phosphate-buffered saline (PBS).

To express histidine-tagged gp120 (gp120H), HeLa cells were infected with recombinant vaccinia virus vTF7-3 (18), which expresses T7 RNA polymerase, together with recombinant vaccinia viruses vTM-DHgp120H, vTM-ADgp120H, vTM-LAIgp120H, vTM-RFgp120H, vTM-BALgp120H, or vTM-89.6gp120H, each at a multiplicity of infection of 5 and maintained for 2 to 3 days in the absence of fetal bovine serum. Recombinant vaccinia viruses that express DH12, AD8, and LAI gp120Hs were generated using plasmids (29) that encode the corresponding gp120H as previously described (11). The plasmids for the other three HIV-1 isolates (BAL, RF, and 89.6) were generated using a similar cloning strategy. Briefly, the gp120 coding sequences were amplified by PCR from pCB43, pCB36, and pBD3 (3, 15). The forward primers used for the PCR amplification were 5′-GGGCCCCATGGGAGTGTTGGAGAAATATCAG-3′ (BAL), 5′-GGGCCCCATGGGAGTGATGGAGATGAGGAAG-3′ (RF), and 5′-GGGCCCCATGGGAGTGAAGGAGATCAGGAAG-3′ (89.6). The reverse primers 5′-GGGCCCCTCGAGTTA**ATGGTGATGATGGTGATG**TCTTTTTTCT CTCTGCACCACTC-3′ (BAL and RF) and 5′-GGGCCCCTCGAGTTA**ATGGT GATGATGGTGATG**TCTTTTTTCTCTTTGCACTGTTC-3′ (89.6) encoded six appended histidine residues (in bold print). The amplified PCR fragments were digested with *Nco*I and *Xho*I (introduced into the primers as indicated by underlines) and cloned into pTM-1 (39) for expression under the T7 promoter. The plasmid constructs were sequenced to confirm that no mutations were introduced inadvertently during PCR amplification. All of the recombinant vaccinia viruses employed in this study have been derived from the WR strain of the vaccinia viruses.

Protein purification. Recombinant gp120H was purified from the culture supernatant using a one-step metal-chelate affinity purification procedure (Ni-NTA; Qiagen). The culture supernatant was prepared by removing the cells by centrifugation at $1,000 \times g$ for 10 min at 4°C. Then 1 M Na_2HPO_4 was added to the supernatant (50 mM final concentration) to raise the pH (to >8.0), and vaccinia virus was inactiviated with NP-40 (0.5%). The mixture was stored overnight at 4°C, the resulting $CaPO_4$ precipitate was removed by centrifugation ($10,000 \times g$ for 30 min), and the supernatant was further clarified by filtration through a 0.2-μ ZapCap bottletop filter unit (Schleicher and Shuell). The Ni-NTA resin (10-ml bed volume per liter of supernatant) was equilibrated using three wash cycles of 50 mM sodium phosphate buffer (pH 8.0; 10 bed volumes per cycle) and was added to the filtrate while being continuously stirred for 4 to 16 h at 4°C. The resin was collected by pouring the mixture into a 25-ml EconoColumn (Bio-Rad) and washed using 10 bed volumes of the equilibration buffer containing 500 mM NaCl. The column was connected to an UV detector (Pharmacia), and the absorption at 280 nm was monitored during the purification procedure. Nonspecifically bound material was removed using approximately 10 to 20 bed volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 500 mM NaCl and 20 mM imidazole. Recombinant gp120H was eluted using 50 mM sodium phosphate buffer (pH 8.0) containing 200 mM imidazole. The peak fractions were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and dialyzed against PBS (100 volumes) at 4°C for 16 to 24 h. The purified gp120H was concentrated to approximately 1 mg/ml using a 10,000-dalton cutoff ultrafilter unit (Millipore). Typically, 5 mg of purified gp120H was produced from 1 liter of culture supernatant.

Animal experiments. Pigtailed macaques (*Macaca nemestrina*) were maintained in accordance with American Association for Accreditation of Laboratory Animal Care standards and were housed in a biosafety level 2 facility; biosafety level 3 practices were followed. Animal anesthetization and bleeding were done as previously described (48). The macaques were immunized (see Fig. 1 for schedule) intradermally with the indicated recombinant vaccinia viruses on four separate sites on their backs, about 4 cm from each other. A total of 5×10^7 PFU in 0.5 ml (0.125 ml on each location) was injected per animal per immunization. Thus, in the two polyvalent vaccination groups, in which the macaques were immunized with mixtures containing either five or six different envelope glycoproteins, individual animals received either 10^7 or 0.83×10^7 PFU of each recombinant vaccinia virus, respectively. The monkeys were boosted intramuscularly with a total of 100 μg of recombinant gp120H combined with 100 μg of QS-21 adjuvant (Aquila Biopharmaceuticals) in 0.5 ml. The animals vaccinated with mixtures of gp120 were immunized with either 20 or 16.7 μg of each gp120H. The macaques were subsequently challenged intravenously with 100 50% tissue culture infectious doses ($TCID_{50}$) of simian/human immunodeficiency virus strain DH12 ($SHIV_{DH12}$) stock prepared in macaque peripheral blood mononuclear cells (PBMC) (49) (previously referred to as $SHIV_{DH12}MD14YE$).

Enzyme-linked immunosorbent assay (ELISA). Nunc Maxisorp 96-well plates were coated with purified $HIV\text{-}1_{DH12}$ gp120H (20 ng per well) in 50 μl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) for 1 h at 37°C. The coating mixture was replaced with 200 μl of blocking buffer (2.5% skim milk, 25% fetal bovine serum, in PBS) and incubated for 1 h at 37°C. Plates were washed twice with PBS-T (PBS containing 0.1% Tween 20). Serially diluted antiserum in 200 μl of blocking buffer was added to each well and incubated for 1 h at 37°C. The plates were washed three times with PBS-T and 100 μl of horseradish peroxidase-conjugated goat anti-human antibodies were added (1:5,000 dilution; Pierce, Rockford, Ill.) for 1 h at 37°C. The plates were washed again three times with PBS-T, and 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added. The reaction was stopped after 10 min with 50 μl of 2 N H_2SO_4, and absorbency was measured at 450 nm using a 96-well plate spectrophotometer (Bio-Tek Instruments).

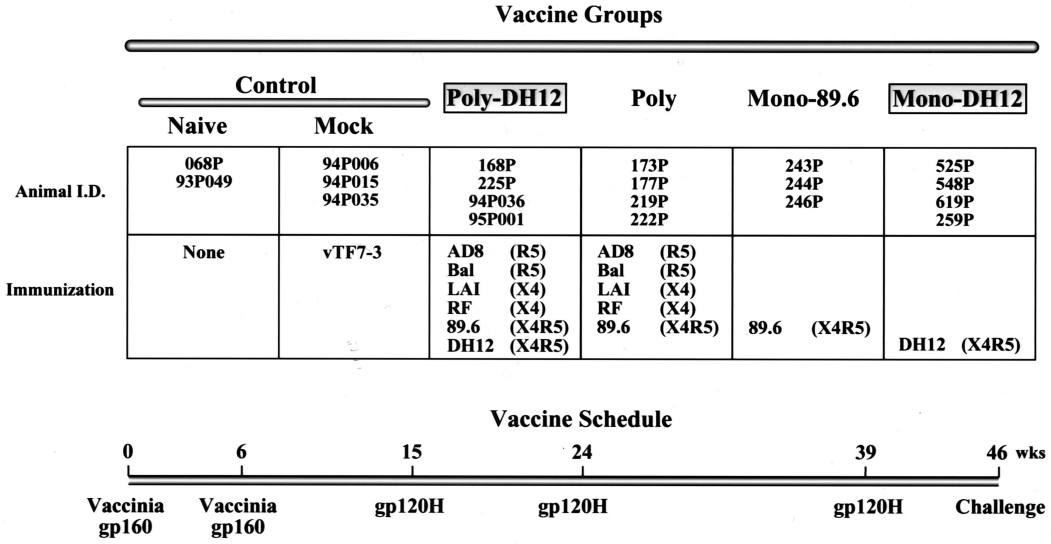

FIG. 1. Vaccine strategy and immunization schedule. Twenty animals were divided into five vaccine groups: control (naïve and mock), Polyvalent-DH12, Polyvalent, Monovalent-89.6, and Monovalent-DH12. Animals were immunized twice (weeks 0 and 6) with recombinant vaccinia viruses expressing gp160 of the HIV-1 isolates indicated, followed by three immunizations (weeks 15, 24, and 39) with gp120s from the same isolates. The coreceptors used by the HIV-1 isolates are indicated. The animals in the mock immunization group were infected with recombinant vaccinia virus vTF7-3, which expresses T7 RNA polymerase. The animals were challenged with $SHIV_{DH12}$ on week 46. I.D., identity.

Western immunoblot. $SHIV_{DH12}$ particles were concentrated 100-fold from virus-infected MT-4 cell culture medium by ultracentrifugation as previously described (26). Concentrated virus particles (40 μl), supplemented with 300 ng of purified DH12 gp120H (see above), were resuspended in SDS-PAGE sample buffer and subjected to SDS-PAGE on a preparatory gel. Following electrotransfer onto a nitrocellulose membrane, immunoblotting was performed in a multichamber immunoblot apparatus (Bio-Rad) as previously described (29). Briefly, blots were incubated with macaque plasma samples (1:50 dilution) followed by goat anti-human immunoglobulin G conjugated with horseradish peroxidase (Pierce). Protein bands were visualized with SuperSignal chemiluminescent substrates (Pierce) using the manufacturer's protocol.

Neutralization assay. Three different neutralization assays were employed in this study: complete virus neutralization in MT-4 cells, MT-2 cell killing reduction assay, and *gag* antigen reduction assay using PBMC. $SHIV_{DH12}$, propagated in either chimpanzee or macaque PBMC, was used in the complete virus neutralization assay as previously described (48). In some cases a chimeric HIV-1, AD8-DHenv ($HIV\text{-}1_{AD8}$ containing the gp160 coding region of $HIV\text{-}1_{DH12}$ [10]), was also used. Serially diluted plasma samples, collected at the indicated times postimmunization, were incubated with 100 $TCID_{50}$ for 1 h at room temperature. Triplicate- or quadruplicate-infected MT-4 cell cultures were maintained for 2 weeks. Virus replication was determined by measuring reverse transcriptase activity in culture supernatants as previously described (56). The titer represents the inverse of the serum dilution (before adding cells) that resulted in no detectable virus replication in all of the replicate wells.

Neutralization of SHIV-HXB2, SHIV-89.6, and HIV-1 strains RF and MN was determined in MT-2 cells by a reduction in virus-induced cell killing, measured by neutral red uptake as previously described (36). All of the virus stocks were produced in H9 cells, except for SHIV-89.6, which was produced in human PBMC. Virus (500 $TCID_{50}$) was incubated in triplicate with dilutions of serum for 1 h at 37°C. Cells were added and the incubation continued until approximately 80% of cells in virus control wells (cells plus virus but no serum sample) exhibited syncytium formation (usually 4 to 6 days). Neutralization titers are defined as the dilution of serum in the presence of virus (before the addition of cells) at which 50% of cells were protected from virus-induced killing. A 50% reduction in cell killing corresponds to an 85 to 90% reduction in viral *gag* antigen synthesis in this assay (5, 41).

Neutralization of $SHIV_{DH12}$ and HIV-1 strains AD8 and BAL, all produced in human PBMC, was determined by a reduction in *gag* antigen synthesis, as previously described (14, 35). Serum samples were diluted in interleukin 2 (IL-2)-containing (4%) growth medium and mixed with virus (500 $TCID_{50}$) in triplicate for 1 h at 37°C. Phytohemagglutinin-stimulated PBMC were subsequently added to each well. The virus inoculum and antibodies were removed 24 h later by 3 washes with 200 μl of growth medium, and the washed cells were maintained in 200 μl of IL-2-containing growth medium. Culture supernatants (25 μl) were collected on a daily basis thereafter and mixed with 225 μl of 0.5% Triton X-100 for quantification of Gag antigen. SHIV p27 and HIV-1 p24 were quantified by antigen ELISA as described by the supplier (SHIV p27 was from Organon-Teknika/Akzo, Durham, N.C.; HIV-1 p24 was from DuPont/NEN Life Sciences, Boston, Mass.). The 25-μl volume of culture fluids removed each day was replaced with 25 μl of fresh IL-2-containing growth medium. The percent reduction in Gag antigen synthesis is reported relative to the amount of the protein synthesized in the presence of preimmunization serum.

Virus load measurements. Plasma samples were prepared from blood collected with Acid Citrate Dextrose (ACD)-A solution (Becton Dickinson) as the anticoagulant and stored at −70°C. Plasma viral RNA levels were determined by real-time PCR (ABI Prism 7700 sequence detection system; Perkin-Elmer) using reverse-transcribed viral RNA as templates. Viral RNA extraction, reverse transcription, and cDNA amplification was done as previously described (26). Viral p27 antigenemia was measured by using an SIV core antigen assay kit (Coulter) and by following the manufacturer's protocol. The amount of proviral DNA in axillary lymph node cells was determined by PCR as previously described (50).

Lymphocyte immunophenotyping. EDTA-treated blood samples were stained with fluorochrome-conjugated monoclonal antibodies (anti-CD3, anti-CD4, anti-CD8, and anti-CD20) and analyzed by flow cytometry (FACSort; Becton Dickinson) as previously described (26).

Amino acid sequence analyses. Amino acid sequences of the V1/V2 and V3 loops of gp120s from various HIV-1 isolates were compared by the BestFit alignment program from Genetics Computer Group.

RESULTS

Immunization. To accomplish the multiple objectives of this study, animals were divided into the five different vaccine groups indicated in Fig. 1: control (including naïve and mock-immunized), Polyvalent-DH12, Polyvalent, Monovalent-89.6, and Monovalent-DH12. Since all of the macaques ultimately were to be challenged with a SHIV bearing the $HIV\text{-}1_{DH12}$ envelope glycoprotein, animals in the Polyvalent-DH12- and Monovalent-DH12-vaccinated groups modeled potential resistance to a homologous virus challenge, whereas those in the

Virus Strains	DH12	AD8	89.6	BAL	LAI	RF	V1/V2
DH12		72	61.1	61.1	62.3	55.7	125-196
AD8	80.0		76	72.1	75.8	67.7	125-192
89.6	65.7	77.1		70	73.5	71.4	125-198
BAL	80.0	94.3	74.3		72	65.1	107-182
LAI	70.6	82.4	67.6	79.4		67	126-194
RF	82.9	80.0	68.6	80.0	67.6		135-219
V3	296-330	292-326	298-332	282-316	294-329	319-353	Amino Acid

FIG. 2. Comparative amino acid sequence analyses of the gp120 variable loops V1/V2 and V3 of the HIV-1 isolates used for the immunization. The amino acid residue numbers in the V1/V2 and V3 loops included in the analyses are indicated on the right side and the bottom of the figure, respectively. The percent amino acid sequence identity for the V1/V2 and V3 loops are shown on the upper right and lower left side of the diagonal line, respectively.

Polyvalent and Monovalent-89.6 groups measured the response to heterologous virus.

Immunizations with recombinant vaccinia viruses followed by boosts with recombinant proteins have previously been shown to elicit superior immune responses compared to vaccination with either vaccinia virus or subunit proteins alone (12, 13, 21, 24, 25). We have employed such a live-vector prime followed by protein boost vaccination approach in this study. Animals were immunized twice (weeks 0 and 6) with recombinant vaccinia viruses (WR strain) that express full-length HIV-1 gp160(s) (Fig. 1, bottom). This was followed by three immunizations (weeks 15, 24, and 39) with purified recombinant gp120(s), which were tagged with six histidine residues (gp120H) to facilitate their purification. In the Monovalent-89.6 or Monovalent-DH12 groups, macaques were immunized with recombinant vaccinia viruses expressing gp160 from either HIV-$1_{89.6}$ or HIV-1_{DH12}, respectively. In the Polyvalent-DH12 group, animals were vaccinated with a mixture of six different recombinant vaccinia viruses expressing gp160s from the HIV-1 isolates AD8, Bal, LAI, RF, 89.6 and DH12. Macaques in the Polyvalent group were immunized with a mixture of five envelope glycoprotein immunogens that did not include DH12. HIV-1 isolates AD8 and Bal have been classified into R5, LAI and RF have been classified into X4, and 89.6 and DH12 have been classified into X4R5 coreceptor usage groups. This combination of immunogens was chosen to ascertain whether a preferential response might be elicited against envelope glycoproteins with specific coreceptor requirements. For mock immunizations, animals were vaccinated with recombinant vaccinia virus vTF7-3, which expresses T7 RNA polymerase (18).

The amino acid (aa) sequences of the hypervariable V1/V2 and V3 loops of the gp120s from the six HIV-1 isolates used in this study are quite heterogeneous (Fig. 2). In general, the sequence of the V1/V2 loops was more diverse than that of the V3 loops. For example, the V1/V2 loop of the DH12 isolate (aa 125 to 196) showed a range of amino acid identity of 55.7% with RF and 72% with AD8. In contrast, the V3 loop of DH12 (aa 296 to 330) was 65.7 and 82% identical to 89.6 and RF, respectively.

Immune response. Following the first immunizations with vaccinia virus, small lesions of less than 1 cm in diameter, which rapidly healed during the next 2 weeks, were observed. After the second vaccinia virus immunization, extremely small skin lesions appeared in some of the animals. No other side effects were observed in the 18 monkeys vaccinated with the WR strain of vaccinia virus. The humoral immune response elicited by this vaccinia virus prime and protein boost regimen was monitored primarily by measuring the level of antibodies binding to HIV-1_{DH12} gp120 in an ELISA. ELISA antibody responses for individual animals are presented in Fig. 3a. No significant differences were observed between the animals in any particular vaccine group. The levels of anti-gp120 antibodies increased after each immunization and then declined until a subsequent boost was administered. Of note, however, was the very low antibody levels for the Monovalent-89.6 vaccine group on week 18 (3 weeks after the first gp120H boost). This is better seen in Fig. 3b, which shows the average endpoint antibody titers for each group of monkeys. While gp120-specific antibodies did not appear until after the first protein boost for the Monovalent-89.6 group, antibodies were detected immediately after the second vaccinia virus immunization in the other vaccine groups. In these latter animals, antibody levels increased about 1,000-fold after the second vaccinia virus immunization and reached titers over 1:100,000 following the first protein boost. At this time, the titer for the Monovalent-89.6 group was about 10-fold lower. However, after the second protein boost, the titers for all of the vaccine groups were virtually indistinguishable. The difference in antibody titers in the Monovalent-89.6 and the other vaccine groups may be due to two factors. First, analyses of gp160 expression by each of the recombinant vaccinia viruses in cultured HeLa cells indicated that the level of 89.6 gp160 expressed was approximately twofold lower than that of the other envelope proteins (data not shown). Second, when ELISAs were conducted using plates coated with gp120s from different HIV-1 isolates, preferential binding to homologous proteins was observed (i.e., antibodies generated against the DH12 gp120 bound more efficiently to DH12 gp120 than to 89.6 gp120, and vice versa [unpublished observation]).

Protection of animals against SIV or HIV-1 infection correlates with the presence of NAbs, not gp120 binding activity as measured by ELISA, Western immunoblot, or immunoprecipitation assays (48). Since the immunized animals were to be challenged with $SHIV_{DH12}$, assays monitoring NAbs directed against either $SHIV_{DH12}$ or HIV-$1_{AD8\text{-}DH12}$ (AD8-DHenv [10]), a chimeric HIV-1 containing the *env* gene from the DH12 isolate, were carried out. As shown in Table 1, anti-DH12 NAbs initially appeared after the first gp120H protein boost in the Monovalent-DH12-vaccinated monkeys. Thus, the monomeric gp120H used was able to elicit or recall an antibody response capable of neutralizing virus. None of the other groups, including the Polyvalent-DH12 group, produced any neutralizing at this time. Following the second gp120H boost, NAb directed against virus bearing the DH12 gp120 also became detectable in the Polyvalent-DH12 group. Animal 95P001, in particular, generated very high levels of neutralizing activity. On the day of virus challenge, only the animals in the vaccine groups immunized with the DH12 envelope glycoprotein (Polyvalent-DH12 and Monovalent-DH12) were produc-

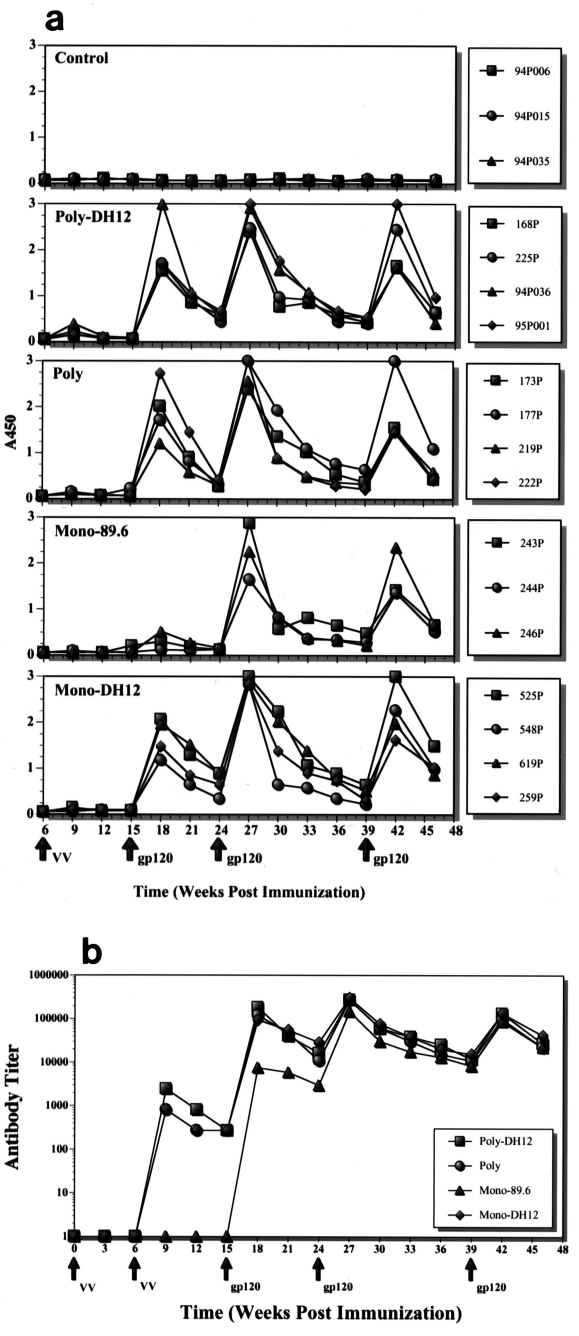

FIG. 3. Antibody response during the course of immunization. (a) The antibody levels in the plasma samples of individual animals were analyzed by ELISA. Twenty nanograms of HIV-1_{DH12} gp120 was coated in each well and results from one plasma sample dilution (1:2,430) are shown. (b) Average endpoint antibody titers for the vaccine groups immunized with envelope glycoproteins. The times at which the animals were immunized with either recombinant vaccinia virus (VV) or gp/120 are indicated by the arrows.

ing antibodies that neutralized $SHIV_{DH12}$. Two macaques, one in the Polyvalent-DH12 group (95P001) and the other in the Monovalent-DH12 group (259P), had extremely high titers of neutralizing activity (1:81). Two other animals in the Monovalent-DH12 group (548P and 619P) had intermediate levels of neutralizing activity (1:9), while the other four monkeys had relatively low titers of NAbs [$<$(1:9) or $\leq$(1:3)]. It would appear that the production of NAbs may very well be antigen dose-dependent, judging by the delayed appearance and generally lower titers elicited by polyvalent immunization (8.3×10^6 PFU of the recombinant vaccinia virus expressing DH12 gp160 and 16.7 μg of DH12 gp120) than by vaccination with monovalent DH12 Env (5×10^7 PFU of the recombinant vaccinia virus and 100 μg of gp120).

Virus challenge. Seven weeks after the final gp120H boost, all 18 immunized animals plus 2 naïve macaques were challenged intravenously with 100 $TCID_{50}$ of $SHIV_{DH12}$, produced in macaque PBMC. In general, viral RNA in the plasma became detectable on week 1, peaked on week 2, and then declined (Fig. 4a). The majority of the viral burden was observed during the first 4 weeks after challenge. After week 12 (the monitoring continued up to 32 weeks postinfection), all of the animals had plasma virus loads below the level of detection. $CD4^+$ T-lymphocyte numbers in the blood did not change significantly (data not shown), as the challenge virus used is not pathogenic.

The total plasma virus load measured during the first 12 weeks of infection is compiled in Fig. 4b. In the control group, four out of five animals (94P015, 94P035, 068P, and 93P049) produced large amounts of viral RNA. As shown in Fig. 4a, these same animals had peak virus loads of 2×10^6 to 9×10^6 RNA copies per ml of plasma on week 2. Unexpectedly, one of the control monkeys (94P006) had a lower total virus load even though it had viral RNA levels similar to those of the other control macaques on week 1 (Fig. 4a). In contrast to the control group, plasma viremia was undetectable in two of the four animals in the Monovalent-DH12 group throughout the 32-week observation period (macaques 548P and 259P). One additional monkey in this group (619P) exhibited a plasma viremia barely detectable over background on week 2 (Fig. 4a). The fourth animal in the Monovalent-DH12 group (525P) produced relatively small amounts of viral RNA during the first 12 weeks of infection (approximately 280-fold less than that measured in control monkeys [$\sim 4.8 \times 10^6$ copies/ml]). The difference in plasma viremia between the control and Monovalent-DH12 groups was statistically significant ($P = 0.016$ by the Wilcoxon rank sum test). In the Polyvalent-DH12 group, one macaque (95P001) had no detectable viremia, two animals (168P and 94P036) produced small amounts of virus, and a single monkey (225P) experienced a viremia indistinguishable from the control group. In the Polyvalent group, three animals produced relatively small quantities of virus, and only one animal (219P) had a viremia similar to that measured in the control group. Although the macaques in the Polyvalent group generated no detectable NAb against $SHIV_{DH12}$, significant protection against the virus challenge occurred, suggesting that a nonhumoral immune response induced by the vaccine (e.g., envelope-specific cytotoxic T-lymphocyte [CTL] activity) might be responsible for the low plasma virus loads observed. This response is to be contrasted with the animals in the Monovalent-89.6 group, two of which developed high virus loads and one which had a relatively low viremia.

The protection against the $SHIV_{DH12}$ challenge observed in the eight animals immunized with HIV-1_{DH12} envelope glycoprotein (Polyvalent-DH12 and Monovalent-DH12 groups) was

TABLE 1. Neutralization antibody titers against DH12

Time of neutralization assay	Vaccine group and antibody titer for each animal[a]				
	Control	Polyvalent-DH12	Polyvalent	Monovalent-89.6	Monovalent-DH12
3 wk after first gp120H boost	None	None	None	None	525P (1:3) 548P (1:9) 619P (1:27) 259P (1:3)
3 wk after second gp120H boost	None	168P (none[b]) 225P (1:10) 94P036 (1:10) 95P001 (1:50)	None	None	525P (none[b]) 548P (1:10) 619P (1:50) 259P (1:50)
Day of challenge	None	168P [<(1:3[c])] 225P (1:3) 94P036 [<(1:3[c])] 95P001 (1:81)	None	None	525P [<(1:9[c])] 548P (1:9) 619P (1:9) 259P (1:81)

[a] The antibody titer (in parentheses) is the dilution of serum that results in complete neutralization of 100 $TCID_{50}$ in MT-4 cells.
[b] The lowest dilution tested was 1:10.
[c] Only two of the quadruplicate samples showed complete neutralization.

significantly better than in animals that did not (Polyvalent and Monovalent-89.6; $P = 0.039$). It should be noted that the virus loads in the individual Polyvalent-DH12-, Polyvalent-, and Monovalent-89.6-vaccinated groups were not statistically different from that of the control group ($P > 0.15$ after Bonferroni correction for multiple comparisons) despite the marked reduction of virus loads in several of the animals in the Polyvalent-DH12 and Polyvalent groups. This undoubtedly reflects the small number of animals in each group, since the viral load in the three groups as a whole (11 animals) was significantly lower than that of the control group ($P = 0.027$). Viral p27 antigenemia was detected only on week 2 in all of the animals with high ($>10^5$ RNA copies/ml) plasma viral RNA loads (data not shown).

To determine, in fact, whether or not a virus infection had occurred in animals with undetectable or barely detectable plasma viremia (monkeys 95P001, 548P, 619P, and 259P), lymph node biopsies were collected at week 2 postinfection and analyzed for proviral DNA by PCR. Viral DNA was readily amplified from samples of 100,000 or 4,000 lymphocytes, prepared from lymph node specimens collected from two control group animals (94P015 and 94P035) (data not shown). In contrast, no proviral DNA was detected in specimens from macaques 95P001, 548P, and 259P. Proviral DNA could be amplified from the 100,000 cells but not from the 4,000 cells from the lymph node of animal 619P, suggesting that a low level of virus replication had occurred. Despite limited viral replication in lymph node cells, this animal was able to control its plasma virus load to barely detectable levels.

The strength of the postvirus challenge anamnestic antibody responses against DH12 gp120, measured by gp120 ELISA, did not necessarily correlate with the virus load measurements (Fig. 5a). Among four vaccinated animals with high plasma viremia (225P, 219P, 244P, and 246P), only one exhibited a significant anamnestic response (225P). Strong anamnestic responses were observed in only two other monkeys (94P036 and 525P), both of which had low plasma virus loads. All three animals with strong anamnestic responses were members of either the Polyvalent-DH12 or Monovalent-DH12 group. This suggests that the anamnestic responses were primarily directed against the epitopes specific to DH12 envelope glycoprotein (i.e., the variable regions of DH12 gp120). In contrast, negligible or no anamnestic responses occurred in the three macaques (95P001, 548P, and 259P) in which no plasma viral RNA or lymph node proviral DNA was detected. Relatively weak anamnestic responses were observed in animals 168P and 619P. For animal 619P, this weak response suggested that virus did replicate to some degree and was consistent with the low but detectable levels of proviral DNA and plasma viremia described earlier. The anamnestic responses in all of the Polyvalent vaccine group animals were also quite low and only one of three animals in the Monovalent-89.6 group (246P) exhibited a significant anamnestic response, although all three macaques in this vaccine group had a substantial plasma viremia. Anti-gp120 antibodies were first detected 5 weeks postinfection in control group animals, but only at much lower plasma dilutions (1:90) (data not shown).

Postinfection humoral immune responses were also examined by Western blot analysis (Fig. 5b). All animals in the control group, including the macaque with low virus loads (94P006), developed antibodies against Gag proteins by 8 weeks postinfection. Antibodies against both p27 and p17 were detected in these animals. By 24 weeks postinfection, antibodies against gp120 were also detected, although the reactivity was considerably weaker than the reactivity measured in vaccinated monkeys. In contrast to the control animals, only one of four macaques in the Monovalent-DH12 group (525P) developed antibodies against p17. This was the only animal with clearly demonstrable plasma viremia. Although monkey 619P had proviral DNA in the lymph node, no antibodies against Gag proteins were generated. In the Polyvalent-DH12 group, the three animals with plasma viremia all developed antibodies to p17, although the response was barely demonstrable in animal 168P. Interestingly, no antibodies were detected against p27. As expected, monkey 95P001, which experienced no plasma viremia, failed to mount an anti-Gag antibody response. In the Polyvalent vaccine group, three of the four animals generated antibodies against both p17 and p27. The

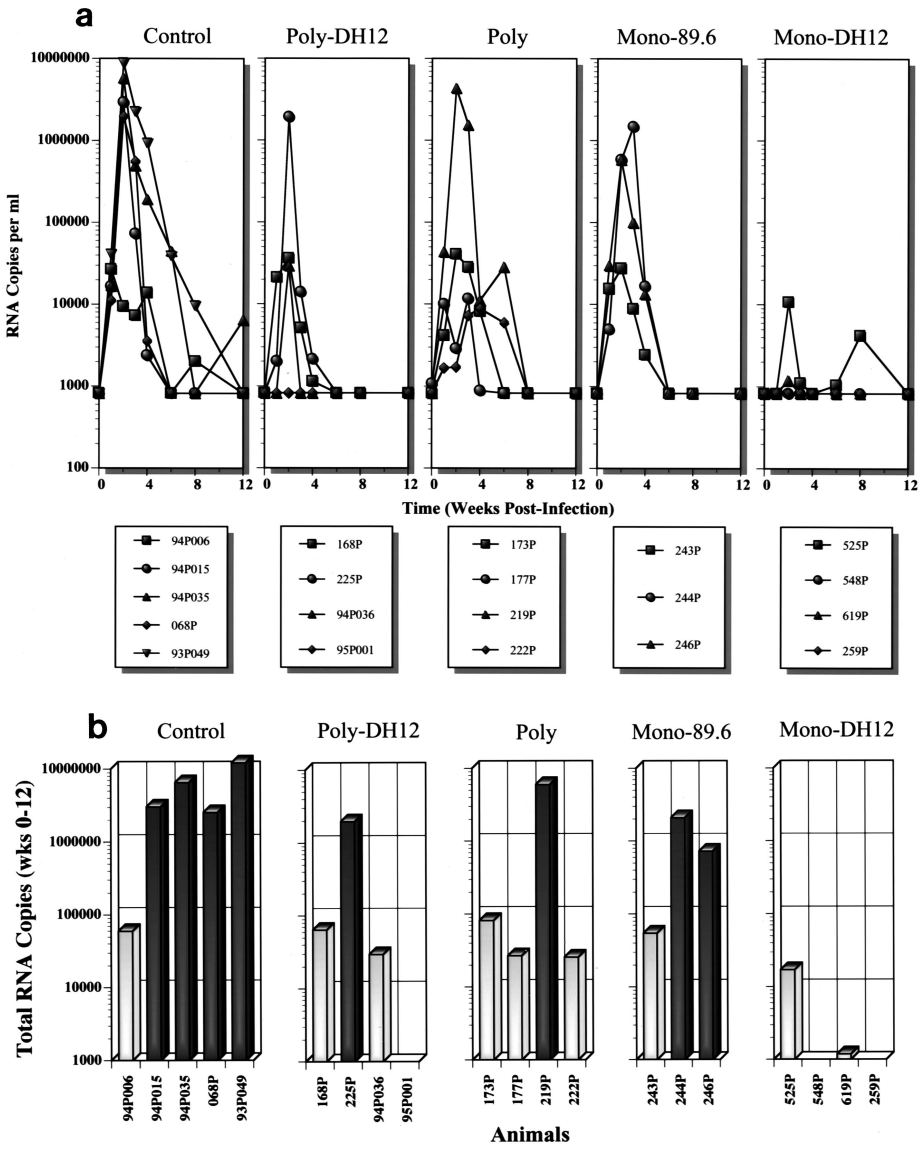

FIG. 4. Plasma viral RNA loads in animals subsequent to $SHIV_{DH12}$ challenge. (a) Viral RNA copies in the plasma of infected animals, determined by quantitative real time reverse transcription-PCR, during the first 12 weeks after the virus infection. (b) Total plasma viral load (arithmetic sum) during the first 12 weeks of virus infection.

fourth macaque, 173P, made no antibody to either p27 or p17, although it sustained a robust plasma viremia. In the Monovalent-89.6 group, weak antibody responses against p17 were detected in animals 243P and 246P, whereas monkey 244P developed no anti-Gag antibodies despite having relatively high virus loads. At present, the factors determining whether or not animals immunized with HIV-1 envelope glycoproteins generate antibodies against Gag proteins during a subsequent virus infection are not known. Although a general correlation between plasma viremia and an antibody response to Gag proteins existed, several exceptions (e.g., 244P and 173P) were observed.

Breadth of NAb response. Since one of the major aims of this study was to determine whether broader NAb response might be elicited by immunizing animals with a mixture of HIV-1 envelope glycoproteins, the neutralization sensitivity of viruses other than $HIV\text{-}1_{DH12}$ was evaluated (Fig. 6). Assays were performed with plasma samples collected 3 weeks after the final gp120H boost, using either human PBMC or the MT-2 continuous T-cell line. No neutralizing activity was detected in the plasma collected from control group macaques. Animals in the Monovalent-DH12 group developed NAbs that were highly specific for $HIV\text{-}1_{DH12}$; their antisera failed to neutralize any of the other test viruses, including relatively neutralization-sensitive $HIV\text{-}1_{MN}$. Antisera from the macaques immunized with the 89.6 envelope glycoprotein exhibited a slightly broader NAb response than those vaccinated with DH12, being able to neutralize virus strains bearing both the homologous 89.6 and heterologous MN gp120s.

In general, animals in the Polyvalent-DH12 and Polyvalent

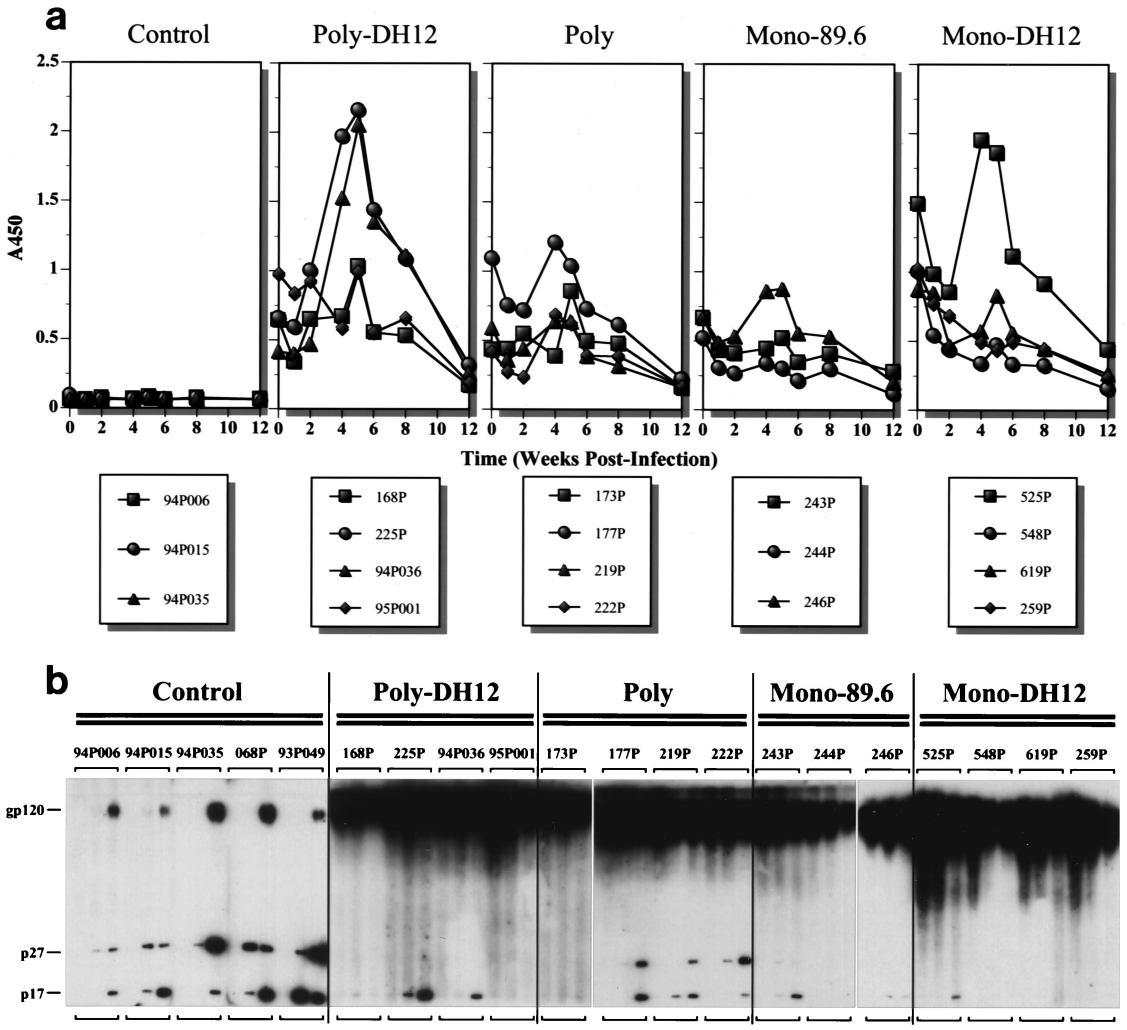

FIG. 5. Antibody responses after $SHIV_{DH12}$ challenge. (a) The antibody levels in the plasma samples of individual animals were analyzed by ELISA as described for Fig. 3. A450, absorbance at 450 nm. (b) Western immunoblot detection of antibodies against HIV-1 gp120 and SIV Gag proteins. The antiserum collected at times 0, 8, and 24 weeks postinfection from each animal were analyzed. Bands corresponding to HIV-1 gp120 and SIV p27 and p17 are indicated on the left.

groups exhibited significantly broader NAb responses than those in the two monovalent vaccine groups ($P = 0.0054$ by the Wilcoxon rank sum test). There was, however, significant animal-to-animal variation as well as differences in the ability to elicit NAbs with different envelope glycoproteins. While macaques 95P001 and 219P generated NAbs that could neutralize up to five and four different isolates, respectively, some of the other animals were able to neutralize only two isolates, which invariably included the very sensitive $HIV\text{-}1_{MN}$ (monkeys 225P, 94P036, and 222P). None of the vaccinated macaques could neutralize $HIV\text{-}1_{AD8}$, possibly because it may be an intrinsically neutralization-resistant primary isolate. Alternatively, the $HIV\text{-}1_{AD8}$ envelope glycoprotein may be relatively nonimmunogenic and may be unable to elicit detectable levels of NAb. Another possibility is that antigenic competition from other envelope glycoproteins included in the mixture may have muted an immune response. We are not presently able to distinguish between these alternative explanations. In this regard, only one macaque (219P) of the eight animals in the Polyvalent-DH12 and Polyvalent vaccine groups was able to neutralize $SHIV_{89.6}$. This result is in contrast to that obtained with animals in the Monovalent-89.6 group, where plasma from all three macaques neutralized $SHIV_{89.6}$. This would indicate that the 89.6 envelope glycoprotein is immunogenic, $SHIV_{89.6}$ is neutralizable, and antigenic competition may have muted the immune response against the 89.6 envelope glycoprotein. Alternatively, the lower amounts of 89.6 antigen administered to the monkeys in the Polyvalent-DH12 and Polyvalent vaccine groups (i.e., either 1/6 or 1/5 of the dose given to the Monovalent-89.6 group, respectively) may have been insufficient to elicit detectable anti-$SHIV_{89.6}$ NAbs.

Because antisera from monkeys 95P001 and 219P exhibited the broadest cross-reactive neutralizing activity, their ability to neutralize primary HIV-1 isolates not included in the vaccine mixture was examined. Six isolates from clade B (P15, P27, 1168, 1196, Pvo, and Tro) (5) and two isolates from clade C (DU151 and DU123) were selected for analysis. All were R5 isolates obtained during early seroconversion, and the neutral-

		1/dilution (50% CPE)				% p24/p27 Reduction		
Groups	Animal	SHIV HXB2	SHIV 89.6	HIV-1 RF	HIV-1 MN	HIV-1 Bal	HIV-1 AD8	SHIV DH12
Control	94P006							
	94P015							
	94P035							
Poly-DH12	168P			49	123	94		
	225P				201			86
	94P036				101			82
	95P001	71		33	554	99		98
Poly	173P			43	153	89		
	177P			107	454	96		
	219P		87	60	267	99		
	222P			30	289			
Mono-89.6	243P		30		526			
	244P		22		40			
	246P		220		20			
Mono-DH12	525P							78
	548P							97
	619P							90
	259P							95

FIG. 6. Neutralization activity against various HIV-1 and SHIV strains. The antisera collected 3 weeks after the final gp120 boost (4 weeks prior to the challenge) were analyzed for neutralizing activity. NAb against SHIV-HXB2, SHIV-89.6, HIV-1_{RF}, and HIV-1_{MN} were tested using cell-killing reduction assays in MT-2 cells while HIV-1_{Bal}, HIV-1_{AD8}, and $SHIV_{DH12}$ were tested using p24 reduction assays with human PBMC as target cells. In the cell killing assays, the number indicates 1 divided by the dilution of the serum samples that yield 50% reduction in cytopathic effects. In the p24 reduction assays, the number indicates the percentage of reduction in Gag protein production at the dilution of serum of 1:4.

ization assay was performed in human PBMC with a 1:4 dilution of the antisera. No significant neutralizing activity was detected with either antiserum (data not shown).

DISCUSSION

In this study, the recombinant vaccinia virus prime and subunit protein boost approach was used to demonstrate that mixtures of HIV-1 envelope glycoproteins could elicit broader immune responses than vaccinations with individual Env immunogens. Animals in the two monovalent vaccine groups developed NAbs against only one or two HIV-1 isolates, whereas five of eight animals in the polyvalent vaccine groups made NAbs against three or more viral strains ($P = 0.0054$). Unfortunately, however, this increased breadth of neutralization was limited almost entirely to the virus strains used for vaccination. This was best illustrated with the antisera from two macaques (95P001 and 219P) which possessed neutralizing activity against five and four different viruses, respectively, but were unable to neutralize any of eight heterologous primary HIV-1 isolates tested. As far as protective immunity was concerned, resistance to $SHIV_{DH12}$ strongly correlated with the levels of NAbs specifically directed against $SHIV_{DH12}$ at the time of challenge. Disappointingly, the most potent protective humoral responses against the $SHIV_{DH12}$ challenge were elicited only in monkeys vaccinated with preparations containing a DH12 Env component. Nonetheless, it might still be possible to elicit more broadly protective virus neutralizing responses by immunization with a pool of HIV-1 glycoproteins representing a larger cross section of the neutralization subtypes in circulation.

Because polyvalent vaccination regimens have not been extensively used to elicit protective immune responses against primate lentiviruses, some of the results obtained were unexpected. For example, the immunogenicity of the envelope glycoprotein mixture was quite variable in stimulating NAbs in the pigtailed macaques under study. In the case of DH12, four of four recipients of monovalent immunogen and three of four vaccinees given the mixture of Env proteins (including DH12) developed NAbs against $SHIV_{DH12}$, as measured in p27 reduction assays (Fig. 6). This is in contrast to the 89.6 Env protein which elicited NAbs against $SHIV_{89.6}$ in all three recipients of monovalent 89.6 Env but in only one of eight animals when 89.6 Env was administered in a mixture of other envelope glycoproteins. This also appeared to be the case for the AD8 Env, which failed to elicit NAbs in any of eight polyvalent vaccinees, although a comparable monovalent AD8 control group was not included in this study. At present, it is not clear whether these variable responses reflect the innate poor immunogenicity of HIV-1 envelope proteins, possible antigenic competition among the various components of the polyvalent vaccine, or simply the effect of antigen dilution resulting from the administration of the same amount of total Env immunogen as a polyvalent or monovalent vaccine.

As noted earlier, a strong inverse correlation was observed between the levels of vaccine-induced NAbs on the day of virus challenge and the subsequent plasma viremia ($P = 0.0008$ by the Jonckheere-Terpstra test for trend). Specifically, the four animals with either no demonstrable (548P, 259P, and 95P001) or barely detectable (619P) levels of plasma viremia all had NAb titers of 1:9 or greater (Table 1). This result is consistent with a previously published passive immunization study, which reported that NAb titers of approximately 1:8 (based on 100% neutralization assay in MT-4 cells), but not 1:4, conferred complete protection against a 100-$TCID_{50}$ challenge with $SHIV_{DH12}$ (48). Conversely, there was no statistical correlation of the postchallenge viral RNA levels in animals with anti-virus neutralization titers of less than 1:9 and with no detectable NAbs (viz. the monkeys in the Polyvalent and Monovalent-89.6 vaccine groups; $P = 0.53$ by the Wilcoxon rank sum test). These and previously published results strongly suggest the existence of a NAb threshold for complete or near-complete neutralization of the virus inoculum (and/or progeny virions produced during the first replication cycle). If the NAb titer is below the critical threshold, some fraction of the input virus will escape and will be able to establish a productive infection that may be refractory to subsequent cell-mediated immune responses.

Immunization with live virus vectors (e.g., vaccinia virus) or DNA vaccines, which allow de novo synthesis of antigens, are known to elicit CTL responses (7, 52). Although none of the macaques in either the Monovalent-89.6 or Polyvalent (lacking DH12) vaccine groups produced NAbs against $SHIV_{DH12}$, only the virus loads in the Polyvalent group seemed to be markedly lower following the $SHIV_{DH12}$ challenge (Fig. 4). This result suggests the possibility that the monkeys immunized with a mixture of HIV-1 envelope glycoproteins mounted a more effective nonhumoral immune response (possibly of CTL

origin) than the Monovalent-89.6 group. We were unable to monitor possible CTL responses, because priming animals with recombinant vaccinia viruses precluded these vectors from being used to express viral proteins in autologous target cells for subsequent CTL assays. The tetramer binding assay (1, 40) could not be used because the macaques under study had not been previously classified for their major histocompatibility complex class I genotype. If CTLs were indeed responsible for the relatively low plasma viremia in the Polyvalent vaccine group animals, the protective effect observed might reflect the immunologic response of multiple cross-reactive CTL epitopes to the mixture of gp120 variable-loop peptides. Alternatively, such a hypothetical CTL response could be directed against the more highly conserved Env domains within the gp160 cocktail administered to animals in the Polyvalent group. CTL responses to epitopes mapping to lentiviral Gag proteins, but not to envelope glycoproteins, have received primary attention in the context of controlling the primary infection or developing a protective HIV-1 vaccine. It would therefore seem prudent to include mixtures of Gag and possibly other more conserved viral proteins in polyvalent vaccine formulations to elicit more effective immune responses against heterologous virus isolates.

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