JOURNAL OF VIROLOGY, Mar. 2005, p. 3358–3369 Vol. 79, No. 6
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.6.3358–3369.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Protection of Rhesus Monkeys against Infection with Minimally Pathogenic Simian-Human Immunodeficiency Virus: Correlations with Neutralizing Antibodies and Cytotoxic T Cells

Gerald V. Quinnan, Jr.,[1]* Xiao-Fang Yu,[2] Mark G. Lewis,[3,4] Peng Fei Zhang,[1] Gerd Sutter,[5] Peter Silvera,[3] Ming Dong,[1] Anil Choudhary,[1] Phuong T. N. Sarkis,[2] Peter Bouma,[1] Zhiqiang Zhang,[1] David C. Montefiori,[6] Thomas C. VanCott,[7]† and Christopher C. Broder[1]

Uniformed Services University of the Health Sciences, Bethesda,[1] Johns Hopkins University School of Public Health, Baltimore,[2] Southern Research Institute, Frederick,[3] BIOQUAL, Inc.,[4] and Military HIV Program, Rockville,[7] Maryland; Division of Virology, Paul-Ehrlich-Institut, Langen, Germany[5]; and Department of Surgery, Laboratory for AIDS Vaccine Research and Development, Duke University Medical Center, Durham, North Carolina[6]

Received 9 August 2004/Accepted 26 October 2004

We studied the capacity of active immunization of rhesus monkeys with HIV-1 envelope protein (Env) to induce primary virus cross-reactive neutralizing antibodies to prevent infection following intravenous challenge with simian-human immunodeficiency virus (SHIV). Monkeys were immunized with the human immunodeficiency type 1 (HIV-1) strain R2 Env. Initially, the Env was expressed in vivo by an alphavirus replicon particle system, and then it was administered as soluble oligomeric gp140. Concurrently, groups of monkeys received expression vectors that encoded either simian immunodeficiency virus (SIV) *gag/pol* genes or no SIV genes in vivo to test the additional protective benefit of concurrent induction of virus-specific cell-mediated immune (CMI) responses. Groups of control monkeys received either the *gag/pol* regimen or sham immunizations. The antibodies induced by the Env immunization regimen neutralized diverse primary HIV-1 strains. Similarly, potent CMI responses were induced by the *gag/pol* regimen, as measured by gamma interferon enzyme-linked immunospot assays. Differences in the responses among groups of monkeys strongly suggested that there was interference between the Env and g*ag/pol* immunization regimens. Complete protection of some of the monkeys against infection after intravenous challenge with the partially pathogenic $SHIV_{DH12R\ (Clone\ 7)}$ was associated independently with both neutralizing antibody and CMI responses. Protection was associated with $SHIV_{DH12\ (Clone\ 7)}$ serum neutralizing antibody titers of ≥1:80 or with cellular immune responses corresponding to >2,000 spot forming cells per 10^6 peripheral blood mononuclear cells. Immunization was also associated with a reduction in the magnitude and duration of virus load. Induction of cross-reactive, primary HIV-1-neutralizing antibodies is feasible and, when potent, may result in complete protection against infection with a heterologous challenge virus strain.

Development of an effective vaccine for prevention of human immunodeficiency virus type 1 (HIV-1) infections has been a daunting challenge. Neutralizing antibodies may be a critical component of immunity required for protection (11, 12). However, induction of potent neutralizing antibody responses by means of candidate vaccines has been difficult (10). Previously, we described the induction of broadly cross-reactive neutralizing responses in small animals (19). It was of interest, therefore, to determine if similar responses could be induced in nonhuman primates and if they could protect against experimental challenge. Here we report results demonstrating the efficacy of such neutralizing antibody responses in the protection of rhesus monkeys from experimental challenge with recombinant simian-human immunodeficiency virus (SHIV).

The HIV-1 envelope glycoprotein (Env) used for immunizations in this study is designated strain R2 (56, 74). The *env* gene encoding this protein was obtained from an unusual HIV-1-infected donor who had neutralizing antibodies that are more highly cross-reactive among primary HIV-1 strains of multiple subtypes than has been observed in sera of other individuals with HIV-1 infection (20, 29, 70). The Env encoded by the R2 gene appears to be in a "triggered" conformation, in that it can mediate infection in the absence of CD4 and displays sensitivity to neutralization by antibodies that usually exhibit little neutralizing activity unless used in the presence of a CD4 binding site ligand (74). Neutralization epitopes that are functional on primary strains of HIV-1 are commonly sensitive to protein conformational changes (48). The approach we used for immunization was intended to present the R2 Env in its native conformation, displaying the epitopes that characterize its triggered state. To this aim, primary immunization was accomplished by using a Venezuelan equine encephalitis virus

* Corresponding author. Mailing address: Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814. Phone: (301) 295-3173. Fax: (301) 295-1933. E-mail: gquinnan@usuhs.mil.

† Present address: Advanced Bioscience Laboratories, Inc., Kensington, MD 20895.

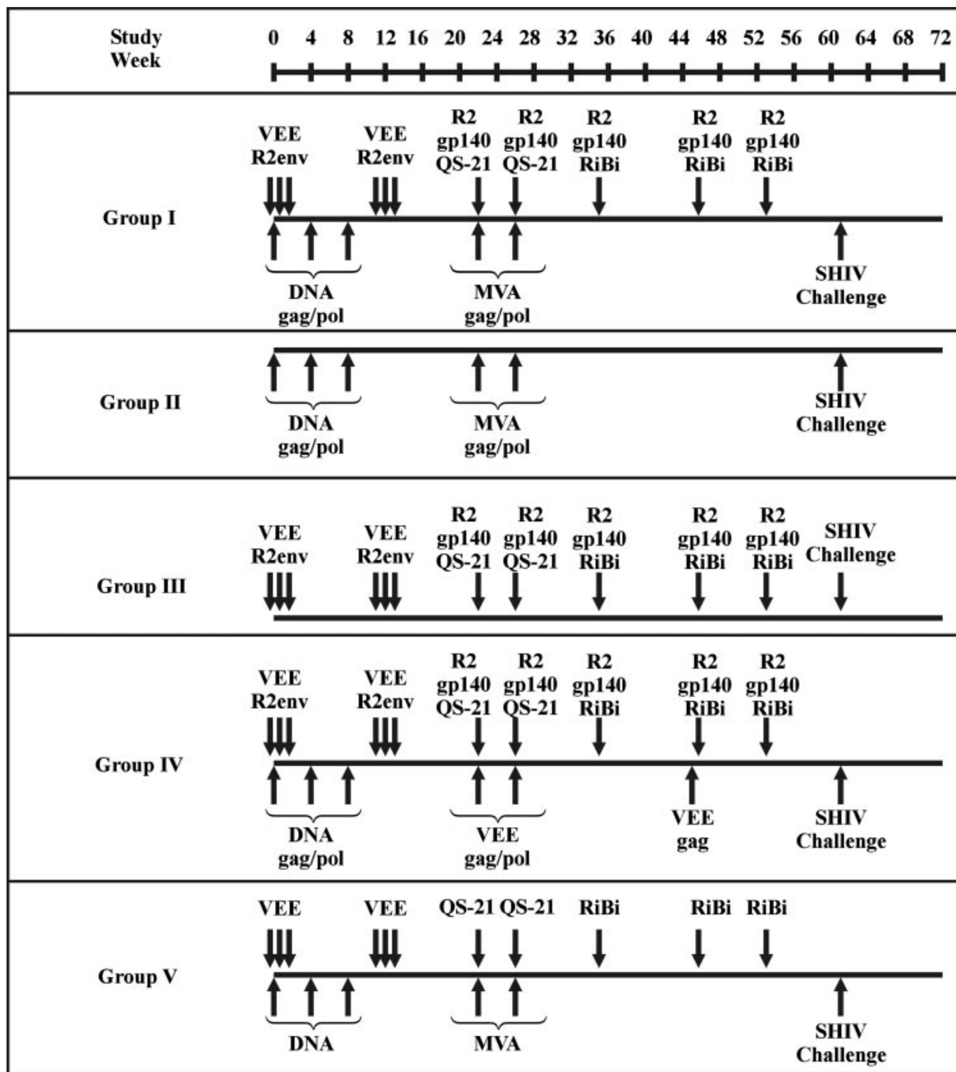

FIG. 1. Schematic diagram of immunization and challenge regimens administered to Chinese-origin *Macaca mulatta*. Six adult rhesus monkeys were included in each group. VEE-R2env indicates VEE-RP expressing HIV-1env$_{R2}$ gene. VEE indicates control replicons not expressing foreign genes. VEE-R2env and VEE replicon particle preparations were administered at doses of $10^{6.5}$ FFU/ml, 0.5 ml subcutaneously in the inguinal region and 0.5 ml intravenously at each time point. Sequential doses were administered on alternate sides. DNA *gag/pol* indicates the codon-optimized SIV$_{mac239}$ *gag/pol* gene in DNA plasmid expression vector. Doses of 5 mg in 1 ml each were given intramuscularly in the leg. R2 gp140 indicates gp140$_{R2}$ purified from supernatants of cell cultures infected with vaccinia virus expressing the glycoprotein. It was administered in 300-μg doses in QS-21 or 400-μg doses in RiBi adjuvant, as indicated, intramuscularly in the leg. MVA *gag/pol* indicates MVA expressing SIV$_{mac251\ (Clone\ J5)}$ *gag/pol*. MVA is the control MVA. Doses of MVA were 5×10^8 PFU given intradermally in the lateral thigh. VEE-*gag/pol* and VEE-*gag* indicate VEE replicons expressing SIV$_{mac251\ (Clone\ J5)}$ *gag/pol* or *gag* gene-coding sequences, respectively. Doses of 10^6 or 10^7 FFU, respectively, were given, half intravenously and half intradermally in the inguinal area.

replicon particle (VEE-RP) system that has been highly effective in induction of neutralizing antibodies against a number of glycoproteins of other viruses (17, 55) VEE-RPs produced by this system are taken up by dendritic cells when they are inoculated into animals and transported to lymphoid tissues, where transgenes are expressed with high efficiency (42). Booster doses of R2 Env were administered as a truncated form, gp140$_{R2}$, in adjuvant. The gp140$_{R2}$ includes the complete gp120 subunit and the ectodomain of gp41. The gp140$_{R2}$ protein was produced in cell culture by using a vaccinia expression system (8, 21, 22) and was purified biochemically to select for the oligomeric Env (23). The combined VEE-RP and soluble oligomer immunization approach offered flexibility for repeated immunization with conformationally intact R2 Env. The regimens were effective for induction of immune responses and prevention of infection after experimental SHIV challenge.

MATERIALS AND METHODS

Study design. The design of the present study is outlined in Fig. 1. The animals in groups I, III, and IV received an HIV-1 Env immunization regimen. Animals in groups I, II, and IV received simian immunodeficiency virus (SIV) *gag/pol* immunization regimens. The *gag/pol* regimen included primary immunizations with a DNA expression vector that produced the SIV Gag and Pol proteins (27)

and booster immunizations with either modified vaccinia virus Ankara (MVA; groups I and II) or VEE-RP (group IV) that expressed an SIV *gag/pol* gene (67). Similar regimens have been shown by others to induce potent CD8 T-cell responses (30, 46). The booster doses administered to the group IV monkeys involved VEE-RPs that expressed the same SIV *gag/pol* as the MVA vector. Thus, in groups I and IV we tested the combined effects of the Env and *gag/pol* regimens, in group II we tested the effect of the *gag/pol* regimen alone, and in group III we tested the effect of the Env regimen alone. Group V received only sham immunizations. The monkeys were challenged with a SHIV at week 61 of the protocol and observed for 12 additional weeks.

In vivo expression vectors. VEE-RP-HIV-1env_{R2} vectors were prepared as described previously, by using pRepX-R2gp160ΔCT, pCV, and pGPm as templates for in vitro transcription of RNA (19). VEE-RP-HIV-1env_{R2} was administered in doses of $10^{6.5}$ focus forming units (FFU) at weeks 0, 1, 2, 10, 12, and 14 of the study. VEE-RP-SIV*gag/pol* and VEE-RP-SIV*gag* were prepared by cloning of the $SIV_{mac251\ (clone\ J5)}$ *gag/pol* or *gag* gene sequences into pRepX and then processing as for VEE-RP-HIV-1env_{R2}. Doses given were $10^{6.0}$ or $10^{7.0}$ FFU, respectively, with half given intravenously and half given subcutaneously in the inguinal area. MVA was prepared as previously described (30). The dose of 5×10^8 PFU in 0.5 ml was administered intradermally in the lateral thigh. The DNA plasmid vaccine, VR-SIV*gag/pol*, was constructed by inserting a codon-optimized SIV *gag/pol* gene (protein sequence derived from SIV_{mac239} *gag/pol*, GenBank accession no. M33262) into the VR1012 vector (27). The plasmid was amplified in TOP10 cells (Invitrogen Corp., Carlsbad, Calif.) and purified by using an endotoxin-free DNA purification kit (QIAGEN, Valencia, Calif.).

Neutralization assays. Neutralization assays were performed by using envelope-pseudotyped luciferase reporter viruses as previously described (51, 74, 75). The inhibitory effects of sera from immunized monkeys were determined in comparison to the mean inhibitory effects, if any, of sera from monkeys that received sham immunization (group V). Sera that inhibited ≥50% of the control group mean were considered to be neutralizing. Many of the envelope genes and viruses used have been previously described (51–53, 56, 57, 73–75). The *env* gene sequences of the HIV-1 strains 89.6, DH12, and CA1-136 were synthesized by PCR by using plasmid DNA (strain 89.6) (40), plasmid DNA containing a molecular viral clone (strain DH12) (52), or genomic DNA from infected human peripheral blood mononuclear cells (PBMCs; strain CA1-136) as a template (57), and by using proofreading DNA polymerase. The *env* sequences of the SHIV strains 89.6p, SF162p3, and DH12R (clone 7) were synthesized by PCR by using genomic DNA from infected PBMCs as templates. The respective DNA sequences were cloned into an expression vector and screened for function of expressed protein on pseudotyped viruses, as previously described (56, 57).

Neutralization of replication-competent virus was measured as a function of reduction in luciferase reporter gene expression after multiple rounds of virus replication in 5.25.EGFP.Luc.M7 cells. This cell line is a genetically engineered clone of CEMx174 that expresses multiple entry receptors (CD4, CXCR4, and GPR15/Bob) and was transduced to express CCR5 (6). The cells also possess Tat-responsive reporter genes for luciferase (Luc) and green fluorescence protein. Neutralization titers were the dilutions at which relative luminescence units were reduced by 50% compared to virus control wells.

Production of $gp140_{R2}$. The $gp140_{R2}$ coding sequence was prepared by insertion of two translational termination codons following the lysine residue at amino acid position 692, just prior to the predicted gp41 transmembrane region, and of arginine-to-serine substitutions at positions 517 and 520 to disrupt the protease cleavage signal (15, 56). The gene was subcloned into the vaccinia vector pMCO2, linking it to a strong synthetic vaccinia virus early-late promoter (13). A recombinant vaccinia virus encoding $gp140_{R2}$ (vAC4) was generated by using standard methodology (7). Recombinant $gp140_{R2}$ glycoprotein was produced by infecting BS-C-1 cells, and oligomeric $gp140_{R2}$ was purified from culture supernatants by using lentil lectin Sepharose 4B affinity and size exclusion chromatography (21, 23). The oligomeric $gp140_{R2}$ has been extensively analyzed and has been shown by size exclusion chromatography to be approximately 40% trimer and 60% dimer. It has also been shown to bind CD4, undergo CD4-induced conformational change, bind CCR5, possess epitopes reactive with a wide array of anti-Env monoclonal antibodies (MAbs), and display nine of nine CD4i epitopes in the absence of CD4 (C. C. Broder and G. V. Quinnan, unpublished data).

For initial immunizations, $gp140_{R2}$ was prepared in QS-21 adjuvant (Antigenics Inc., Framingham, Mass.). Each animal was given 300 μg of $gp140_{R2}$ and 150 μg of QS-21 in a total volume of 1 ml in two divided doses intramuscularly in the hind legs. For the final three immunizations, 400 μg of oligomeric $gp140_{R2}$ was combined with 1 ml of RiBi adjuvant (Corixa Corp., Seattle, Wash.) and then administered in divided doses intramuscularly in the hind legs. Group V monkeys received identical volumes of adjuvant without $gp140_{R2}$.

ELISPOT assays. Cellular immune responses were monitored by measuring gamma interferon (IFN-γ) secretion of monkey PBMCs stimulated with SIV Gag peptide pools in an enzyme-linked immunospot (ELISPOT) assay (35). The peptide pools were obtained by dividing 125 overlapping 15-mer peptides spanning the SIV 239 *gag* region (catalogue no. 6204; National Institutes of Health [NIH] AIDS Research and Reference Reagent Program) into nine sequential pools of 13 to 14 peptides each. Filter plates (Millipore Corp., Billerica, Mass.) were coated with anti-human/monkey IFN-γ antibody (MAb GZ-4; Mabtech AB, Nacka Strand, Sweden). Freshly isolated PBMCs from each monkey were added to the coated plates at a density of 2×10^5 cells per well. Each PBMC sample was tested with each of the nine SIV Gag peptide pools. The final concentration of each peptide was 2 μg/ml. After overnight incubation, the cells were rinsed from the plates, and biotinylated anti-human/monkey IFN-γ antibody (MAb 7-B6-1; Mabtech) was added to the wells and incubated overnight. The plates were then washed and incubated with complexed avidin-peroxidase (Vectastain ABC kit; Vector Labs, Burlingame, Calif.) and developed with NovaRed substrate (Vector Labs). Spots were counted by using an automated reader (Zellnet Consultants, Fort Lee, N.J.). Numbers of spot-forming cells (SFC)/10^6 cells for each set of wells were averaged. A response was considered positive if the number of SFC/10^6 cells was at least 55 and at least four times the background value.

Animals. The 30 adult Chinese rhesus macaques (*Macaca mulatta*) used in this study were captive bred. Before their inclusion in the study, all animals were screened and confirmed to be free of antibodies to SIV, simian retrovirus, and simian T-cell leukemia virus type 1. The animals were housed at the Southern Research Institute, Frederick, Md., or at BIOQUAL, Inc., Rockville, Md., in accordance with American Association for Accreditation of Laboratory Animal Care standards.

Peripheral blood. PBMCs were isolated from EDTA-treated venous blood by density gradient separation with Ficoll-Hypaque (Ficoll-Paque Plus; Amersham Biosciences, Piscataway, N.J.). Cells were washed twice with Hanks' balanced salt solution without $Ca2^+$ or $Mg2^+$ (GIBCO, Carlsbad, Calif.).

Flow cytometry. Peripheral blood lymphocyte subset analysis was performed on a fluorescence-activated cell sorter-scan flow cytometer (Becton-Dickinson, Mountain View, Calif.) by using a panel of mouse anti-human MAbs known to cross-react with macaque receptors. The lymphocyte subsets were stained with anti-CD-4 (Leu-3a-FITC; Becton-Dickinson) and anti-CD-3/CD-8 (Leu-2a-PerCP; Becton-Dickinson). Staining was performed on whole blood preparations. Analysis was performed on lysed and paraformaldehyde-fixed cells.

SIV viral RNA quantification. SIV viral RNA was quantified by using a procedure described by Suryanarayana et al. (66). Plasma was added in 500-ml aliquots to 1 ml of Dulbecco's phosphate-buffered saline and spun for 1 h at 10,000 rpm. The viral pellet was then lysed by using RNASTAT-60 (Tel-Test B). The samples were then amplified as previously described, with the exception of the primers and probe (66). The primers used were SIV-F (5′-AGTATGGGC AGCAAATGAAT-3′) and SIV-R (5′-TTCTCTTCTGCGTGAATGC-3′), and the probe used was SIV-P (6FAMAGAT-TTGGATTAGCAGAAAGCCTGTT GGA-TAMRA).

Serum enzyme immunoassay. An antigen capture enzyme immunoassay was used to determine serum immunoglobulin (Ig) responses. Immulon II plates (Dynex Technologies, Inc., Chantilly, Va.) were coated with 100 μl of human HIV-1 immune globulin per well at a dilution of 1:1,000 in phosphate-buffered saline containing 1% NP-40 overnight at 4°C. After plates were blocked with nonfat dry milk (BLOTTO) at 37°C for 2 h, 100 μl of HIV-1 IIIB strain gp140, at a concentration of 50 ng/ml in 5% nonfat dry milk with Tween, was added to each well. The gp140 was purified from medium of cell cultures infected with recombinant vaccinia virus as previously described (21). The plates were incubated at 37°C for 1 h. After plates were washed, serially diluted monkey serum was added to the wells, and the plates were incubated at 37°C for 1 h. Reactions were further developed by using biotinylated anti-human IgG. Positive and negative control sera were included in each assay. Sera were assigned titers equal to the highest dilutions that produced reactions twice the level of the negative control serum.

Cells and culture conditions. Human HeLa (CCL-2), HeLaS3 (CCL-2.2), $HuTk^-$ 143B (CRL 8303), and HEK293T (CRL11554) cells and simian BSC-1 (CCL-26) and CV-1 (CCL-70) cells were obtained from the American Type Culture Collection, Manassas, Va. HuTk-, BSC-1, and CV-1 cells were maintained in Eagle's minimum essential medium supplemented with 10% bovine calf serum (BCS) and 2 mM L-glutamine and antibiotics. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% BCS and 2 mM L-glutamine and antibiotics. HeLaS3 cells were maintained in vented spinner bottles at 37°C in Eagle's minimum essential spinner medium, 5% horse serum, and 2 mM glutamine. HeLa cell monolayers were maintained in Dulbecco's

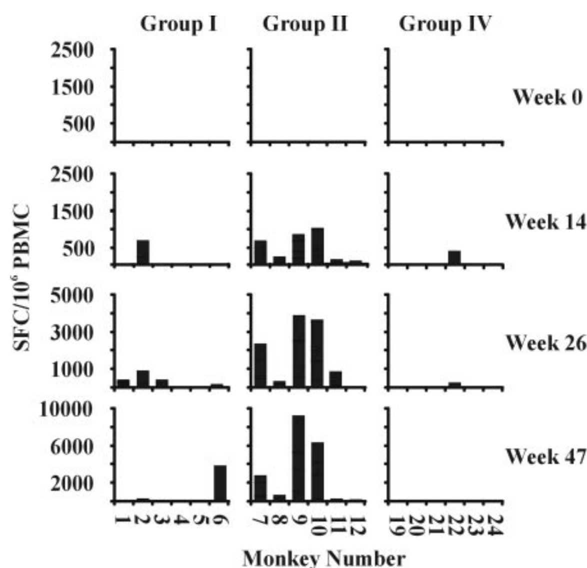

FIG. 2. SIV Gag peptide-specific cellular immune responses of monkeys to immunization with DNA and MVA vectors expressing the SIV-*gag*/*pol* gene. Responses were measured in IFN-γ ELISPOT assays, and results are shown as numbers of SFC per 10^6 PBMC. Responses were considered positive if there were greater than 55 SFC/10^6 PBMC and results were at least four times the background level. Only those responses that were rated as positive are included in the graphs. PBMC were stimulated in aliquots with nine different pools, each consisting of a mixture of 15-mer peptides. Collectively, the pools included sequences homologous to the full Gag amino acid sequence. Heights of bars indicate cumulative number of SFC for all positive responses, with background subtracted, at the time points indicated.

modified Eagle's medium (Quality Biologicals, Gaithersburg, Md.) supplemented with 10% BCS and 2 mM L-glutamine. Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Roller bottle cultures of BSC-1 cells were maintained in Eagle's minimum essential medium supplemented with 10% BCS at 37° at 0.5 revolutions/min.

RESULTS

Virus-specific cell-mediated immune responses. The virus-specific cell-mediated immune responses of the monkeys were monitored by using an IFN-γ ELISPOT assay that tested responses to nine different pools of overlapping peptides representative of the entire Gag sequence (Fig. 2). Animals in group II (*gag*/*pol* only group) developed T-cell responses against multiple Gag peptide pools that were apparent following the DNA vector priming doses, between weeks 0 and 14 (note that the responses shown in Fig. 2 are the sums of the responses observed against individual peptide pools). There was further enhancement of specific T-cell responses by the booster doses of MVA (note that the scale of the abscissa in Fig. 2 varies from date to date). The frequency of responses observed at week 14 in groups I and IV, which received both Env and *gag*/*pol* regimens, was significantly less than the frequency in group II (chi-square test, $P = 0.036$), and the magnitude of the responses was greater in group II. These results suggest that concurrent administration of the VEE-RP-HIV-1*env* to the animals receiving the DNA-*gag*/*pol* vector inhibited induction of the primary immune responses against the Gag T-cell epitopes expressed by the DNA vector. Four of the monkeys in group I had anti-Gag T-cell responses following the MVA-SIV*gag*/*pol* booster (compare week 26 to earlier weeks), while none of the monkeys in group IV developed anti-Gag T-cell responses following the VEE-RP-SIV*gag*/*pol* booster. Possible reasons for the nonresponse in group IV included the possibility that anti-VEE immunity may have been induced by previous VEE-RP administration and that the length of the *gag*/*pol* coding sequence may have exceeded the gene size that could be expressed efficiently by the VEE-RP. Indeed, the yield of VEE-RP-SIV*gag* in FFU was about 10-fold higher than the yield of VEE-RP-SIV*gag*/*pol* (data not shown), consistent with the possibility that the large size of the *gag*/*pol* insert might limit replicon function. To further evaluate these possibilities, monkeys in group IV were given VEE-RP-SIV*gag* on week 45. Again, no anti-Gag responses were detected, consistent with the possibility that anti-VEE immunity may have inhibited infections with the VEE-RP. By week 47 of the study, all animals in group II had detectable anti-Gag T-cell responses, while only two animals in group I and no animal in group IV had detectable responses.

Antibody responses. All monkeys in groups I, III, and IV developed antibody responses to immunization. Binding antibody responses, as measured by enzyme-linked immunosorbent assay (ELISA), are shown in Fig. 3. Antibody responses were monitored by ELISA over the first 28 weeks of the immunization regimen. The geometric mean titers obtained by the monkeys in groups I, III, and IV were 1:28,500, 1:185,800, and 1:49,000, respectively. In each group, the geometric mean titer remained essentially constant after week 16 or 24. In groups I and IV there was no increase compared to week 16 titers in geometric mean titers following the administration of gp140 in QS21 adjuvant. In group III there was some change in the mean titer observed, which corresponded to a fourfold change in one monkey and a twofold change in another monkey, while the four remaining monkeys in the group experienced no change. Statistical analyses were performed by using the log-transformed titers for each monkey, as shown in Fig. 3. The geometric mean titer obtained for group III monkeys at week 28 was significantly higher, by a two-tailed Student's *t* test (done in Excel), than the geometric mean titer obtained for groups I ($P = 0.0035$) or IV ($P = 0.044$).

Neutralizing antibody responses are shown in Fig. 4. Evolution of neutralizing antibodies against homologous HIV-1_{R2}, HIV-1_{SF162}, and $SHIV_{DH12R\ (Clone\ 7)}$ are shown in Fig. 4A. Responses were observed in all three groups immunized with Env immunogens following the series of VEE-RP-HIV-1*env* immunizations. Few monkeys had increases in antibody titers following doses of $gp140_{R2}$ in QS-21 adjuvant at weeks 22 and 26 of the study. These neutralizing antibody results through week 28 were consistent with the results obtained by ELISAs, indicating that no booster effect was apparent following administration of soluble gp140 in QS21 adjuvant. Therefore, we elected to try the administration of additional doses of $gp140_{R2}$ in RiBi adjuvant at weeks 33, 48, and 59 of the study. Since augmentation of the amount of protein to be administered was also feasible, the dose of gp140 administered was increased from 300 to 400 μg/dose. Significant responses were then observed after the week 33 doses. We have no explanation for the differences in responses seen when the $gp140_{R2}$ was given in QS-21 or RiBi adjuvant, although it is possible that the higher

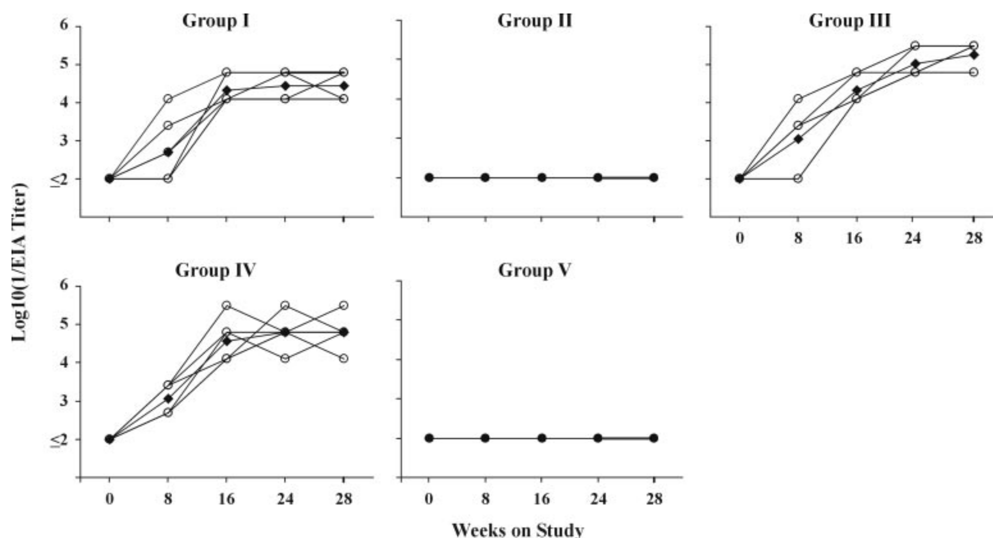

FIG. 3. Env_{R2} gp140 binding antibody responses of rhesus macaques immunized with VEE-RP expressing gp160Δ CT_{R2} and then boosted with soluble $gp140_{R2}$ in QS21 adjuvant. Oligomeric $gp140_{R2}$ was used as solid phase reagent in an ELISA. Open symbols indicate responses of individual monkeys, and closed symbols indicate geometric mean titers for each group.

dose of Env used in conjunction with RiBi may be important. Before the week 48 doses, titers had declined to levels similar to week 16 levels. Elevated responses were again observed following the week 48 doses, with titers reaching levels similar to the week 36 titers.

Significant differences in the neutralizing antibody titers obtained were observed at week 51 among the three Env-immunized groups, as determined by analysis of variance and Student *t* tests, with group III having higher geometric mean responses against each of the three viruses than groups I and IV. The differences were statistically significant at a *P* value of <0.05 in comparison to groups I and IV against HIV-1_{SF162} and $SHIV_{DH12R\ (Clone\ 7)}$ and in comparison to group I against HIV-1_{R2}. These differences between groups could indicate an interfering effect of the *gag/pol* immunization regimen on the capacity of the monkeys to develop neutralizing antibody responses to the Env regimen. Since additional doses of $gp140_{R2}$ in RiBi adjuvant did not appear to boost the titers of neutralizing antibodies above the level obtained at week 36 of the study, a final boost was given on week 59 in anticipation of SHIV challenge on week 61.

The cross-reactivity of the neutralizing antibody responses of the monkeys against other strains of HIV-1 and SHIV was also examined (Fig. 4B and C). Neutralizing activity was observed against two additional subtype B strains and against individual subtypes C, A/G, and F strains (Fig. 3B), but not against subtype E or D strain. In replicating virus neutralization assays, one or more of the selected sera tested neutralized each of the three subtype B strains (Fig. 4*C*). Sera from weeks 36 and 51 were tested for neutralization of $SHIV_{SF162p3}$ and $SHIV_{89.6p}$ (results not shown). A few of the sera inhibited infection of $SHIV_{SF162p3}$ weakly, but none of the sera neutralized $SHIV_{89.6p}$. However, sera from all of the Env-immunized monkeys neutralized HIV-1_{SF162} (Fig. 4A), and sera pooled from group III monkeys neutralized HIV-$1_{89.6}$ (results not shown). Thus, the neutralizing sensitivity or specificity of $SHIV_{SF162p3}$ and $SHIV_{89.6p}$ differed significantly from the HIV-1 strains from which they were derived. Overall, these results demonstrate that the neutralizing antibody responses that were induced were cross-reactive among multiple subtypes of HIV-1.

SHIV challenge virus in vivo titration. In order to test our hypothesis regarding the efficacy of neutralizing antibodies, it was necessary to identify a SHIV that was neutralized by the antibodies induced by our Env immunization regimen. Among the three SHIV strains tested, only $SHIV_{DH12R\ (Clone\ 7)}$ was neutralized consistently. Virus derived from clone 7 of pathogenic $SHIV_{DH12R}$ was provided by M. Martin (NIH, Bethesda, Md.) (32, 33). The virus was propagated in rhesus monkey PBMCs, and its infectivity was determined in MT4 cells. An in vivo titration of the virus pool was conducted in adult rhesus monkeys of Chinese origin. Two animals each received 1,000, 100, 10, 1, 0.1, or 0.01 times the 50%-tissue culture infectious dose ($TCID_{50}$) by the intravenous route. Assays were performed on plasma for viral RNA and on peripheral blood lymphocytes for ratios of $CD4^+/CD8^+$ cells (Fig. 5). By 1 or 2 weeks after inoculation, all monkeys that received $\geq$10 $TCID_{50}$ became positive for viral RNA in plasma, while none of the monkeys that received lower doses became positive (Fig. 5A). Peak viral loads occurred at 3 weeks after inoculation and ranged between $10^{4.8}$ and $10^{5.9}$ copies/ml. Five of the monkeys converted to viral RNA negative by 8 weeks, while one monkey remained viral RNA positive until euthanized 3 months after inoculation. The monkey with persistent viremia had a persistent alteration in the CD4/CD8 ratio (Fig. 5B), which was accompanied by a decline to near zero levels of $CD4^+$ cells (data not shown). Aliquots of the virus pool that were used in the in vivo titration study were administered in doses of 1,000 $TCID_{50}$ to two Indian-origin rhesus monkeys to determine whether the degree of pathogenicity of the virus pool derived from $SHIV_{DH12R\ (Clone\ 7)}$ was related to the origin of monkeys being tested. These two monkeys of Indian origin developed transient viremia similar to the majority of the monkeys of Chinese origin used in the titration study. These results indicated that the challenge pool of $SHIV_{DH12R\ (Clone\ 7)}$ was con-

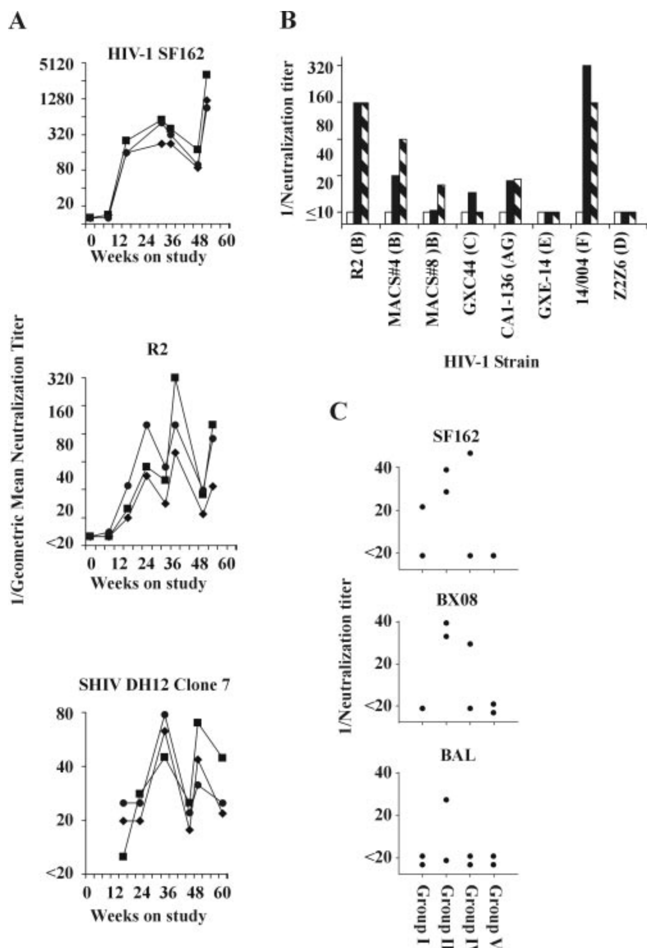

FIG. 4. Neutralizing antibody responses induced by immunization of rhesus monkeys with R2 envelope immunogens. (A and B) Assays were performed by using pseudotyped luciferase reporter viruses. In panel A, sera from sequential time points during the study period were assayed for neutralization of HIV-1_{SF162}, HIV-1_{R2}, and $SHIV_{DH12R\ (Clone\ 7)}$. Results shown are geometric means for sera from animals in group I (♦), group III (■), and group IV (▲). In panel B, pools of sera from five or six monkeys in each of groups II (open bars), group III (filled bars), and group IV (striped bars) were tested in pseudotyped virus neutralization assays against the HIV-1 strains shown. Neutralization titers are the endpoint dilutions that resulted in >50% inhibition of virus infection. (C) HIV-1 neutralization measured by using replication-competent viruses and selected sera from monkeys in groups I, III, and IV. Neutralization titers are the endpoint dilutions of sera that mediated >80% inhibition of virus infection. Sera used for panels B and C were from week 51 of the study.

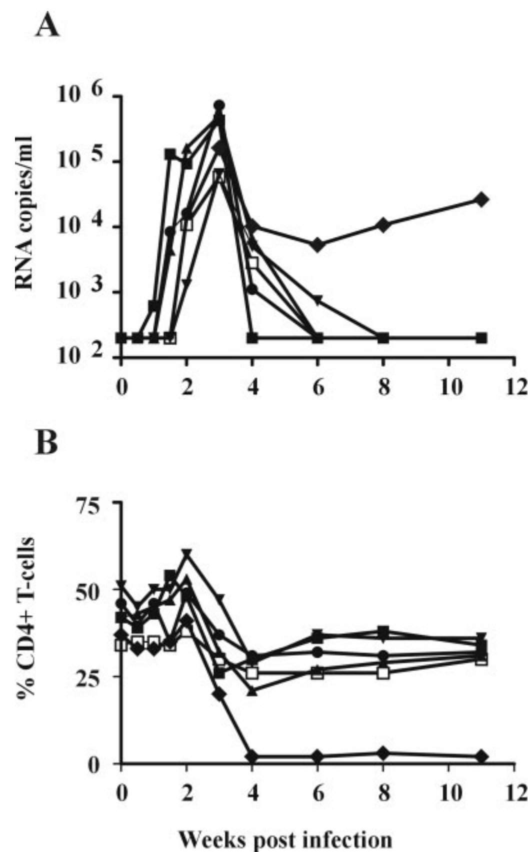

FIG. 5. Titration of $SHIV_{DH12R\ (Clone\ 7)}$ pool in Chinese-origin rhesus monkeys. Individual monkeys were inoculated with 1,000 (■, ▲), 100 (▼, ♦), or 10 (●, □) $TCID_{50}$ of $SHIV_{DH12R\ (Clone\ 7)}$. Additional monkeys received inocula in each of the following amounts (two monkeys for each dose): 1, 0.1, and 0.01 $TCID_{50}$; results for these monkeys are not shown, since none developed infection detected by our testing. (A) Viral RNA copies per milliliter of plasma at times after inoculation, as determined by reverse transcription-PCR. (B) CD4/CD8 ratios at indicated times after infection.

sistently infectious and moderately pathogenic for rhesus monkeys of Chinese origin and produced similar consequences in rhesus monkeys of Indian origin.

Monkey challenge study. Each monkey in the challenge study was inoculated intravenously with 100 $TCID_{50}$ of $SHIV_{DH12R\ (Clone\ 7)}$. Viremia was observed in each of the control animals in group V within 1 or 2 weeks, with peak levels occurring at 2 or 3 weeks after inoculation (Fig. 6, left side panels). Peak levels varied between 10^3 to $10^{6.7}$ copies/ml. Viremia was no longer detected in any group V monkey by 5 weeks after inoculation. Viremia was detected in four of five monkeys in group I, two of six in group II, four of six in group III, and five of six in group IV (Fig. 6, left column). For each group of immunized monkeys, the average area under the curve, representing virus RNA copies per milliliter of plasma over time postchallenge, was significantly reduced compared to group V ($P < 0.01$ by a two-tailed Student's t test) (Fig. 7A), and the mean duration of viremia was significantly reduced compared to duration in group V ($P < 0.05$ by a two-tailed Student's t test) (Fig. 7B). Significant declines in numbers of $CD4^+$ cells per milliliter of blood occurred transiently in only one animal in group V (Fig. 6, middle column). All of the animals with detected viremia developed $SHIV_{DH12R\ (Clone\ 7)}$-neutralizing antibody responses to infection (Fig. 6, right column). In addition, one of the animals in group II and the animal in group IV without detected viremia also had neutralizing antibody responses. Monkey sera were also tested for occurrence of anti-p27 antibody responses by ELISAs (results not shown). All of the monkeys with postchallenge neutralizing responses also developed anti-p27 responses. Overall, the results indicated that all monkeys became infected with $SHIV_{DH12R\ (Clone\ 7)}$ except for one monkey in group I, three monkeys in group II, and two monkeys in group III. In addi-

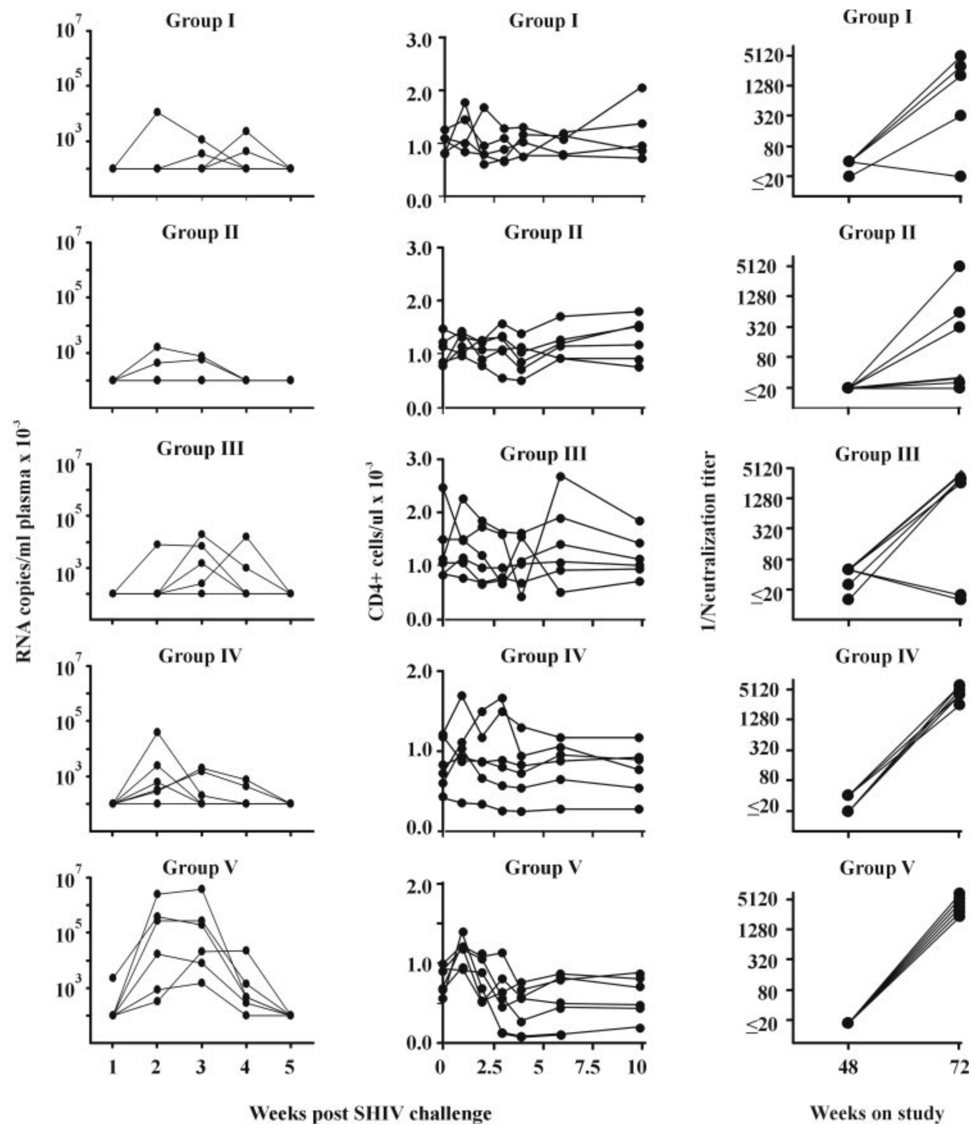

FIG. 6. Responses of immunized and control monkeys to intravenous challenge with $SHIV_{DH12R\ (Clone\ 7)}$. (Left column graphs) Viral RNA copies per milliliter of plasma, determined as described in the legend to Fig. 4. (Middle column) Numbers of $CD4^+$ cells per milliliter of peripheral blood. (Right column) Changes in $SHIV_{DH12R\ (Clone\ 7)}$ neutralization titers from before challenge to 12 weeks after SHIV challenge.

tion, immunized monkeys that became infected had attenuated levels of viremia, compared to levels in group V monkeys.

Correlations between prechallenge immune responses and protection from SHIV infection. Relationships were examined between prechallenge immune responses and occurrence of infection. The four monkeys that received the *gag/pol* immunization regimen, and in which we found no evidence of infection postchallenge, were monkeys 6, 7, 9, and 10. These four monkeys had cell-mediated immune (CMI) responses that were 4.3- to 14.3-fold higher in magnitude than any of the monkeys in groups I, II, and IV in which we detected infection (Fig. 2). The association of high-potency CMI responses with an absence of detectable infection postchallenge is consistent with the notion that cytotoxic T lymphocytes (CTL) are a protective mechanism in these monkeys.

There were three monkeys that received Env immunization in which we did not detect infection postchallenge. The animal in group I that remained uninfected was number 6. This animal also had a significant T-cell response, as just discussed. However, neither of the animals in group III that remained uninfected had received *gag/pol* immunization. We could not assay for Env-specific T-cell responses in our animals. Most CTL responses in HIV infections are directed against nonenvelope proteins, but escape mutation at an Env-specific CTL epitope early in infection has been documented (5, 63). Nevertheless, we cannot exclude the possibility that Env-specific CTL may have contributed to the control of infection in these animals (5, 47). The two animals in group III that received the Env immunization regimen in which we did not detect evidence of SHIV infection were numbers 16 and 18. The prechallenge anti-$SHIV_{DH12R\ (Clone\ 7)}$-neutralizing antibody titers in mon-

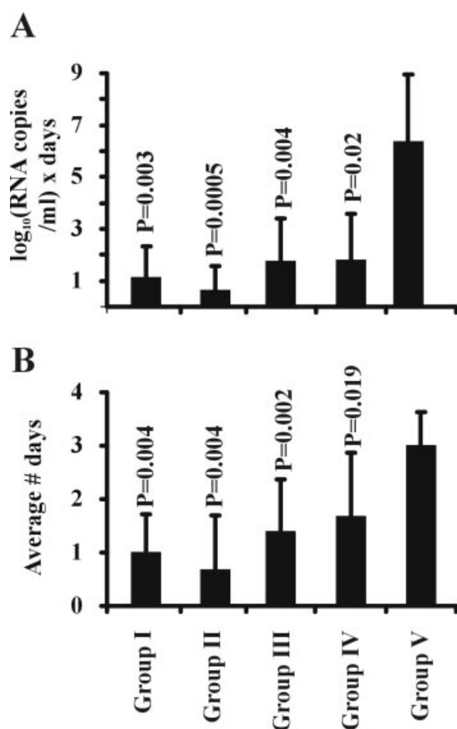

FIG. 7. Analyses of differences between virus loads of immunized and control monkeys. (A) Areas under curves for each monkey were calculated, and mean areas were determined. (B) Mean number of days of positivity in assay for plasma viral RNA per group is shown. Results in each panel were compared by a Student's *t* test with Excel.

keys 6, 16, and 18 were 1:40, 1:80, and 1:80, respectively, as shown in Fig. 8. No other monkey had a prechallenge $SHIV_{DH12\ (Clone\ 7)}$ neutralization titer of ≥1:80. In addition, as discussed above, the highest binding and neutralizing antibody responses were observed in group III, which also had the highest level of apparent protection of the Env-immunized groups. In all the studies we report here, a total of 14 nonimmune rhesus monkeys have been inoculated with aliquots containing ≥10 $TCID_{50}$ of the pool of $SHIV_{DH12R\ (Clone\ 7)}$ used in this study, and all have become infected, with virus loads exceeding those observed in our immunized animals. The differences in rates of protection in our Env-immunized animals with neutralization titers of ≥1:40 (3 of 12 animals) compared to the total control experience (0 of 14) is significant by chi-square analysis (P = 0.047). Considering the evidence that concurrent administration of the *gag*/*pol* immunization regimen may have interfered with Env immunization, the best estimate of the protection rate afforded by levels of neutralizing antibodies in the range of 1:40 to 1:80 may be reflected by the group III experience, with 33% apparent protection. Our interpretation is consistent with the published findings of the protection of rhesus monkeys from $SHIV_{DH12R}$ infection by passive immunization with Ig, and protection against $SHIV_{89.6p}$ challenge with MAbs. The experience of Shibata et al. in passive immunization with HIV-1-immune IgG demonstrated protection at serum neutralization titers about twofold higher than we achieved in monkeys that appeared to be protected (60). In other passive immunization studies of MAbs with or without human IgG, Mascola et al. and Shibata et al. demonstrated 80% protection against pathogenic SHIV at serum titers of 1:579 to 1:2,800 and 50% protection at serum titers of 1:69 to 1:153 (43–45, 60).

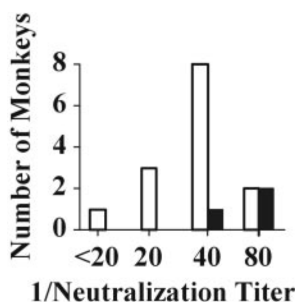

FIG. 8. Correlation between $SHIV_{DH12R(Clone\ 7)}$-neutralizing antibody titer and protection from infection after intravenous challenge with the same SHIV. Titers were determined on testing of sera taken at week 51 of the study.

DISCUSSION

Prevention of infection in experimental HIV-1 animal models is the elusive goal of preclinical efficacy studies of experimental HIV-1 vaccines. The strongest protection that has been observed to date in vaccine studies in animal models has involved either passive immunization with IgG and/or MAbs that neutralize HIV-1 or active immunization to induce neutralizing antibodies (28, 44, 45, 60). The previous study that demonstrated the efficacy of neutralizing antibodies induced by active immunization involved an immunogen that is expected to induce a narrowly specific immune response. That study demonstrated cross-neutralization only against a strain of HIV-1 derived from the same donor as the vaccine and protection against a strain of SHIV constructed from the same late isolate from that donor (28). We were able to induce neutralizing antibodies in rhesus monkeys with broad cross-reactivity against heterologous strains of HIV-1 and with neutralizing activity against a heterologous strain of SHIV. The anti-$SHIV_{DH12R\ (Clone\ 7)}$ neutralization titers approached the levels that were associated with protection in the earlier studies of passive immunization. We found that animals with higher levels of serum neutralizing activity, near the range previously associated with protection, were protected from infection in our study. Moreover, the challenge virus used in our study was not closely related to the strain used for immunization. Our study demonstrates the feasibility of induction of broadly cross-reactive neutralizing antibodies by vaccination for protection against HIV-1 infection.

The HIV-1 Env, designated strain R2, that was used for the induction of neutralizing antibodies in monkeys in the present study is unusual. It is derived from a donor with broadly cross-reactive neutralizing antibodies and has properties that suggest that it exhibits a conformation that HIV-1 Env proteins usually exhibit only after binding of the primary receptor for HIV-1, CD4 (19, 56, 70, 74). Immunogens very similar to those used here, and based on the same Env, induce neutralizing antibodies in small animals with cross-reactivity profiles very similar to those induced in monkeys in the present study (19). The epitopes that are recognized by the neutralizing antibodies

induced by this Env are unknown. However, multiple, conserved neutralization epitopes are induced by CD4 binding, and it is possible that antibodies with multiple neutralization specificities are induced by the R2 Env. While the cross-reactivity of the neutralizing response induced by R2 is not universal, it is substantial. In contrast to the previous report of Heeney et al., in which variable loop peptides were used to induce antibodies that neutralized and protected against a homologous SHIV (28), the approach of the present study has the potential to result in protection against diverse strains of HIV-1. Such protection will be required of an HIV-1 vaccine for use in humans.

The principal hypothesis addressed by this study was that neutralizing antibodies, in the presence or absence of potent cellular immunity, would mediate protection against intravenous challenge infection with SHIV. Several factors limited the extent to which we could test this hypothesis. Specifically, there was immunological interference between the regimens used to induce neutralizing antibodies and CMI, it appeared that the monkeys did not respond as well to Env administered in QS21 compared to RiBi adjuvant, and the attempt to boost CMI responses by administration of VEE-RPs expressing SIV*gag/pol* did not induce the expected responses. Our data strongly indicate that coadministration of VEE replicons expressing HIV-1 Env and plasmid DNA expressing SIV*gag/pol* resulted in nearly complete inhibition of the CMI responses induced by the administration of DNA expressing *gag/pol* alone and was consistent with some level of interference by the plasmid DNA expressing Gag/Pol with the neutralizing antibody response to the HIV-1 Env. The result was that we could only test the protective effects of the CMI and neutralizing antibody responses independently. Our data do not permit us to determine whether the interference was vector or HIV-1 antigen dependent. Interference with CMI responses attributed to coimmunization with multiple HIV antigens has been reported (39, 54). Monkeys given Env in the form of soluble gp140 in QS21 adjuvant following primary immunization with VEE replicons expressing Env from the same donor appeared to experience minimal, if any, booster response, while responses to gp140 in RiBi adjuvant appeared somewhat better. This finding resulted in the extension of the duration and added complexity of the immunization regimen used. The reason for the apparent difference between the two adjuvants is unclear, although similar results have been obtained by others (M. Lewis, personal communication). The failure of monkeys to develop an enhanced CMI response to SIV Gag/Pol antigens after boosting with VEE replicons expressing the SIV*gag/pol* gene is enigmatic. Good CMI responses to SIV Gag/Pol antigens after immunization with similar VEE-RPs have been observed (18). In our assessment, the failure of the same monkeys to respond subsequently to the administration of VEE-RPs expressing the SIV*gag* gene favors the possibility that an immune response to VEE may have interfered with the response. However, we were not able to test for direct evidence of interfering VEE-specific immune responses in this study. Development of a better understanding of our observation may facilitate subsequent efforts to use alphavirus replicons as vaccine delivery systems. The various difficulties we encountered exemplify problems that may be encountered when applying multiple or sequential immunization modalities or when attempting to induce diverse effector mechanisms against a single agent. Despite these difficulties, we were able to test the efficacy of neutralizing antibody and CMI responses, independently, against SHIV challenge but as separate rather than combined effector mechanisms.

The neutralizing antibody responses we obtained were more potent and broadly cross-reactive against primary strains of HIV-1 than has been reported elsewhere. The proportion of primary HIV-1 strains neutralized in our study was 11 of 13. These results included mostly strains that are not known to be particularly neutralization sensitive. Neutralization titers ranged from 1:20 to 1:320. All tests included comparisons to sham immunized control sera, and neutralizing activity at the higher titers observed were many times greater in potency than any nonspecific activity present in control sera. The concurrent development of neutralizing activity against multiple strains at the same time that the neutralizing activity against the R2 and SF162 strains was increasing progressively to high titers provided a clear demonstration of the cross-reactive nature of the responses we observed. Our results are distinct from those reported by others in studies of macaque immunization. Recent efforts to use synthetic peptides or recombinant proteins in creative ways that may induce effective neutralizing antibody responses have not substantially improved on the disappointing results obtained with similar approaches used in early HIV vaccine studies (9, 23, 25, 28, 38, 41, 68, 69). Neither has the use of a virus-like particle vaccine or in vivo expression systems achieved potent primary virus-neutralizing antibody responses (2, 4, 16, 24, 37, 49, 50, 58, 59, 72). The outcome obtained in such studies always included one of the following: no neutralizing antibodies were detected, neutralizing antibodies were obtained that were specific for the homologous immunizing strain or cross-reacted with only T-cell line-adapted strains, or neutralizing antibodies were not observed until after the monkeys were challenged with SHIV. Fouts et al. reported induction of cross-reactive primary virus-neutralizing antibodies after the immunization of monkeys with Env cross-linked with soluble CD4 (26). However, there is no consensus as to whether the inhibitory effect they reported is mediated by antibodies against HIV. The most promising results reported elsewhere to date involve the use of SF162 strain Env with variable region 2 (V2) partially deleted (3, 14, 15, 64, 65). The V2 deletion enhances the capacity of the Env to induce cross-reactive neutralizing antibodies in rabbits, and neutralizing responses induced in two monkeys, ranging from 56 to 95% neutralization of primary isolates at 1:10 serum dilutions, were reported (64). Monkeys immunized with SF162 strain Env with the V2 deletion were partially protected against challenge with a nonpathogenic SHIV expressing Env homologous to the immunogen (14, 15). Cross-reactivity against a partially pathogenic, heterologous SHIV challenge virus is another notable difference between the data obtained with the SF162Δ V2 immunogen and our results. The immunization regimen we used induced responses that were substantially greater than any of these reported previously.

The regimen we used involved multiple immunizations with VEE-RP preparations. We have not yet determined whether the regimen induced antibodies that neutralize the VEE replicons. The failure of VEE replicons that expressed *gag/pol* or *gag* to boost anti-*gag/pol* CMI responses is consistent with the

possibility that earlier immunizations with VEE-RP Env may have induced VEE-neutralizing antibodies. This question is also relevant to the possibility of using VEE-RP to further boost neutralizing antibody responses and will be addressed in future studies.

There is presently no ideal challenge model for the evaluation of the efficacy of candidate HIV vaccines. The model most commonly employed uses pathogenic SHIV in rhesus monkeys (36, 71). However, recombinant SHIVs are not naturally pathogenic for rhesus monkeys but have been adapted in some cases by serial passage in vivo in order to enable manifest greater virulence in monkeys. Use of such pathogenic SHIVs has been favored in studies attempting to assess the efficacy of immunization regimens that are designed to induce potent CTL responses (71). Pathogenic SHIVs are used because of concern that the effects of CTL responses on infections by nonpathogenic SHIVs may not be relevant to the protection of humans from HIV infection. This concern is based in part on the observation that CTL responses can attenuate early SIV replication but not prevent infection with highly pathogenic SIV in rhesus monkeys and that such attenuated infections progress to immunodeficiency (71). The SHIV titration study we report here indicates that the clonally derived $SHIV_{DH12R\ (Clone\ 7)}$ was probably less pathogenic than the pathogenic $SHIV_{DH12R}$ pool from which it was derived (33, 34). Nevertheless, the objective of using immunization to induce neutralizing antibodies is to prevent the establishment of infection, not to attenuate initial or later stages of infection. Thus, it may not be pertinent to use highly pathogenic challenge viruses if protection from infection can be discerned equally well with less pathogenic SHIVs. Therefore, we used this $SHIV_{DH12R\ (Clone\ 7)}$ preparation, since it permitted us to test the hypothesis that neutralizing antibodies can indeed protect against SHIV challenge infection.

Our studies demonstrate protective effects of both Gag-specific CMI and neutralizing antibodies against the SHIV challenge virus used. The protective effect of *gag/pol* immunization overall was reflected in the infection rate of 4 of 17 monkeys. However, since the Env immunization regimen clearly interfered with induction of CTL, consideration of the infection rate in group II probably provides a more meaningful estimate of the protection rate. Three of six monkeys in group II were protected. The protective effect of CTL is based on the capacity of memory T cells to respond to antigen stimulation associated with virus replication in vivo. Suppression of the manifestations of infection was associated with the most potent CMI responses and probably reflected the effects of such responses in the prevention of manifestations of infection with a challenge virus of such a low to moderate pathogenicity. The level of protection we observed is greater than that which has been reported by others using similar immunization regimens (1, 2). Whether such responses might contribute to complete suppression of clinically evident HIV-1 infections in humans has yet to be determined.

Evidence that neutralizing antibodies protected against SHIV infection was also notable. Protection against infection was observed in 3 of 17 monkeys that received the Env immunization regimen and 2 of 6 monkeys that received the Env regimen alone. We were unable to test the possibility that Env-specific CMI contributed to protection against SHIV challenge because of the absence of available pools of peptides corresponding to sequences of the immunizing or challenge virus strains used in the study. Nevertheless, the finding that protection was observed only in monkeys mounting the highest levels of neutralization of the challenge virus is suggestive that neutralization contributed to protection. Indeed, the breadth of cross-reactivity and the magnitude of the neutralizing antibody responses we induced were notable. Our study protocol induced neutralizing responses that were active against 11 of 13 primary HIV strains tested, only two of which, SF162 and BXO8, are considered to be somewhat neutralization-sensitive strains. The demonstration that such responses can be induced in monkeys fosters hope that more potent and more cross-reactive responses may be induced by modified or enhanced immunization regimens. The neutralizing antibodies induced did not cross-react with $SHIV_{89.6p}$ or $SHIV_{SF162p3}$, although they did neutralize $HIV\text{-}1_{89.6}$ and $HIV\text{-}1_{SF162}$. The alteration of the neutralization properties of SHIV consequent to adaptation to monkeys is likely to be relevant to the use of these viruses as models for human infections (31, 61, 62). In this study, we demonstrate for the first time that active immunization to induce neutralizing antibodies protected rhesus monkeys from experimental challenge from moderately pathogenic, heterologous SHIV against which the neutralizing response was cross-reactive. It is likely that subsequent studies involving regimens that induce more potent and broadly cross-reactive responses will result in the levels and breadth of protection sufficient to justify studies in human populations.

ACKNOWLEDGMENTS

We express our appreciation to Felicia Funderburk for administrative assistance in the conduct of this project. We are grateful to Malcolm Martin, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md., for providing $SHIV_{DH12R\ (Clone\ 7)}$ and for advice regarding work with the virus. We are also grateful to Charlotte Kensil, Antigenics (Framingham, Mass.), for providing QS-21 adjuvant. We appreciated advice from Jeffrey Ahlers, Josephine Cox, Robert Doms, Nancy Haigwood, Vanessa Hirsch, James Hoxie, R. Paul Johnson, John Mascola, and Nancy Miller.

Funding for this project was provided by NIH grants 1 PO1 AI48280 and 1 RO1 AI37438 and by European Commission grant QLK2-CT-2002-01867.

REFERENCES

1. **Amara, R. R., J. M. Smith, S. I. Staprans, D. C. Montefiori, F. Villinger, J. D. Altman, S. P. O'Neil, N. L. Kozyr, Y. Xu, L. S. Wyatt, P. L. Earl, J. G. Herndon, J. M. McNicholl, H. M. McClure, B. Moss, and H. L. Robinson.** 2002. Critical role for Env as well as Gag-Pol in control of a simian-human immunodeficiency virus 89.6P challenge by a DNA prime/recombinant modified vaccinia virus Ankara vaccine. J. Virol. **76:**6138–6146.
2. **Amara, R. R., F. Villinger, S. I. Staprans, J. D. Altman, D. C. Montefiori, N. L. Kozyr, Y. Xu, L. S. Wyatt, P. L. Earl, J. G. Herndon, H. M. McClure, B. Moss, and H. L. Robinson.** 2002. Different patterns of immune responses but similar control of a simian-human immunodeficiency virus 89.6P mucosal challenge by modified vaccinia virus Ankara (MVA) and DNA/MVA vaccines. J. Virol. **76:**7625–7631.
3. **Barnett, S. W., S. Lu, I. Srivastava, S. Cherpelis, A. Gettie, J. Blanchard, S. Wang, I. Mboudjeka, L. Leung, Y. Lian, A. Fong, C. Buckner, A. Ly, S. Hilt, J. Ulmer, C. T. Wild, J. R. Mascola, and L. Stamatatos.** 2001. The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. J. Virol. **75:**5526–5540.
4. **Barouch, D. H., S. Santra, M. J. Kuroda, J. E. Schmitz, R. Plishka, A. Buckler-White, A. E. Gaitan, R. Zin, J. H. Nam, L. S. Wyatt, M. A. Lifton, C. E. Nickerson, B. Moss, D. C. Montefiori, V. M. Hirsch, and N. L. Letvin.** 2001. Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. J. Virol. **75:**5151–5158.

5. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. **3:**205–211.
6. **Brandt, S. M., R. Mariani, A. U. Holland, T. J. Hope, and N. R. Landau.** 2002. Association of chemokine-mediated block to HIV entry with coreceptor internalization. J. Biol. Chem. **277:**17291–17299.
7. **Broder, C. C., and P. L. Earl.** 1999. Recombinant vaccinia viruses: design, generation, and isolation. Mol. Biotechnol. **13:**223–245.
8. **Broder, C. C., P. L. Earl, D. Long, S. T. Abedon, B. Moss, and R. W. Doms.** 1994. Antigenic implications of human immunodeficiency virus type 1 envelope quaternary structure: oligomer-specific and -sensitive monoclonal antibodies. Proc. Natl. Acad. Sci. USA **91:**11699–11703.
9. **Buckner, C., L. G. Gines, C. J. Saunders, L. Vojtech, I. Srivastava, A. Gettie, R. Bohm, J. Blanchard, S. W. Barnett, J. T. Safrit, and L. Stamatatos.** 2004. Priming B cell-mediated anti-HIV envelope responses by vaccination allows for the long-term control of infection in macaques exposed to a R5-tropic SHIV. Virology **320:**167–180.
10. **Burton, D. R.** 2002. Antibodies, viruses and vaccines. Nat. Rev. Immunol. **2:**706–713.
11. **Burton, D. R., R. C. Desrosiers, R. W. Doms, M. B. Feinberg, R. C. Gallo, B. Hahn, J. A. Hoxie, E. Hunter, B. Korber, A. Landay, M. M. Lederman, J. Lieberman, J. M. McCune, J. P. Moore, N. Nathanson, L. Picker, D. Richman, C. Rinaldo, M. Stevenson, D. I. Watkins, S. M. Wolinksky, and J. A. Zack.** 2004. Public health. A sound rationale needed for phase III HIV-1 vaccine trials. Science **303:**316.
12. **Burton, D. R., R. C. Desrosiers, R. W. Doms, W. C. Koff, P. D. Kwong, J. P. Moore, G. J. Nabel, J. Sodroski, I. A. Wilson, and R. T. Wyatt.** 2004. HIV vaccine design and the neutralizing antibody problem. Nat. Immunol. **5:**233–236.
13. **Carroll, M. W., and B. Moss.** 1995. *E. coli* beta-glucuronidase (GUS) as a marker for recombinant vaccinia viruses. BioTechniques **19:**352–354, 356.
14. **Cherpelis, S., X. Jin, A. Gettie, D. D. Ho, S. W. Barnett, I. Shrivastava, and L. Stamatatos.** 2001. DNA-immunization with a V2 deleted HIV-1 envelope elicits protective antibodies in macaques. Immunol. Lett. **79:**47–55.
15. **Cherpelis, S., I. Shrivastava, A. Gettie, X. Jin, D. D. Ho, S. W. Barnett, and L. Stamatatos.** 2001. DNA vaccination with the human immunodeficiency virus type 1 SF162ΔV2 envelope elicits immune responses that offer partial protection from simian/human immunodeficiency virus infection to $CD8^+$ T-cell-depleted rhesus macaques. J. Virol. **75:**1547–1550.
16. **Cho, M. W., M. K. Lee, C. H. Chen, T. Matthews, and M. A. Martin.** 2000. Identification of gp120 regions targeted by a highly potent neutralizing antiserum elicited in a chimpanzee inoculated with a primary human immunodeficiency virus type 1 isolate. J. Virol. **74:**9749–9754.
17. **Davis, N. L., K. W. Brown, and R. E. Johnston.** 1996. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. J. Virol. **70:**3781–3787.
18. **Davis, N. L., I. J. Caley, K. W. Brown, M. R. Betts, D. M. Irlbeck, K. M. McGrath, M. J. Connell, D. C. Montefiori, J. A. Frelinger, R. Swanstrom, P. R. Johnson, and R. E. Johnston.** 2000. Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. J. Virol. **74:**371–378.
19. **Dong, M., P. F. Zhang, F. Grieder, J. Lee, G. Krishnamurthy, T. VanCott, C. Broder, V. R. Polonis, X. F. Yu, Y. Shao, D. Faix, P. Valente, and G. V. Quinnan, Jr.** 2003. Induction of primary virus-cross-reactive human immunodeficiency virus type 1-neutralizing antibodies in small animals by using an alphavirus-derived in vivo expression system. J. Virol. **77:**3119–3130.
20. **D'Souza, M. P., G. Milman, J. A. Bradac, D. McPhee, C. V. Hanson, and R. M. Hendry.** 1995. Neutralization of primary HIV-1 isolates by anti-envelope monoclonal antibodies. AIDS **9:**867–874.
21. **Earl, P. L., C. C. Broder, D. Long, S. A. Lee, J. Peterson, S. Chakrabarti, R. W. Doms, and B. Moss.** 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. J. Virol. **68:**3015–3026.
22. **Earl, P. L., R. W. Doms, and B. Moss.** 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc. Natl. Acad. Sci. USA **87:**648–652.
23. **Earl, P. L., W. Sugiura, D. C. Montefiori, C. C. Broder, S. A. Lee, C. Wild, J. Lifson, and B. Moss.** 2001. Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. J. Virol. **75:**645–653.
24. **Earl, P. L., L. S. Wyatt, D. C. Montefiori, M. Bilska, R. Woodward, P. D. Markham, J. D. Malley, T. U. Vogel, T. M. Allen, D. I. Watkins, N. Miller, and B. Moss.** 2002. Comparison of vaccine strategies using recombinant *env-gag-pol* MVA with or without an oligomeric Env protein boost in the SHIV rhesus macaque model. Virology **294:**270–281.
25. **Eller, N., H. Golding, S. Inoue, P. Beining, J. Inman, N. Matthews, D. E. Scott, and B. Golding.** 2004. Systemic and mucosal immunity in rhesus macaques immunized with HIV-1 peptide and gp120 conjugated to Brucella abortus. J. Med. Primatol. **33:**167–174.
26. **Fouts, T., K. Godfrey, K. Bobb, D. Montefiori, C. V. Hanson, V. S. Kalyanaraman, A. DeVico, and R. Pal.** 2002. Crosslinked HIV-1 envelope-CD4 receptor complexes elicit broadly cross-reactive neutralizing antibodies in rhesus macaques. Proc. Natl. Acad. Sci. USA **99:**11842–11847.
27. **Hartikka, J., M. Sawdey, F. Cornefert-Jensen, M. Margalith, K. Barnhart, M. Nolasco, H. L. Vahlsing, J. Meek, M. Marquet, P. Hobart, J. Norman, and M. Manthorpe.** 1996. An improved plasmid DNA expression vector for direct injection into skeletal muscle. Hum. Gene Ther. **7:**1205–1217.
28. **Heeney, J. L., V. J. Teeuwsen, M. van Gils, W. M. Bogers, C. De Giuli Morghen, A. Radaelli, S. Barnett, B. Morein, L. Akerblom, Y. Wang, T. Lehner, and D. Davis.** 1998. β-Chemokines and neutralizing antibody titers correlate with sterilizing immunity generated in HIV-1 vaccinated macaques. Proc. Natl. Acad. Sci. USA **95:**10803–10808.
29. **Hioe, C. E., S. Xu, P. Chigurupati, S. Burda, C. Williams, M. K. Gorny, and S. Zolla-Pazner.** 1997. Neutralization of HIV-1 primary isolates by polyclonal and monoclonal human antibodies. Int. Immunol. **9:**1281–1290.
30. **Horton, H., T. U. Vogel, D. K. Carter, K. Vielhuber, D. H. Fuller, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, D. C. Montefiori, V. Erfle, R. C. Desrosiers, N. Wilson, L. J. Picker, S. M. Wolinsky, C. Wang, D. B. Allison, and D. I. Watkins.** 2002. Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. J. Virol. **76:**7187–7202.
31. **Hsu, M., C. Buckner, J. Harouse, A. Gettie, J. Blanchard, J. E. Robinson, and C. Cheng-Mayer.** 2003. Antigenic variations in the CD4 induced sites of the CCR5-tropic, pathogenic SHIVsf162p3 gp120 variants. J. Med. Primatol. **32:**211–217.
32. **Igarashi, T., C. Brown, A. Azadegan, N. Haigwood, D. Dimitrov, M. A. Martin, and R. Shibata.** 1999. Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. Nat Med. **5:**211–216.
33. **Igarashi, T., Y. Endo, G. Englund, R. Sadjadpour, T. Matano, C. Buckler, A. Buckler-White, R. Plishka, T. Theodore, R. Shibata, and M. Martin.** 1999. Emergence of a highly pathogenic simian/human immunodeficiency virus in a rhesus macaque treated with anti-CD8 mAb during a primary infection with a nonpathogenic virus. Proc. Natl. Acad. Sci. USA **96:**14049–14054.
34. **Igarashi, T., H. Imamichi, C. R. Brown, V. M. Hirsch, and M. A. Martin.** 2003. The emergence and characterization of macrophage-tropic SIV/HIV chimeric viruses (SHIVs) present in CD4+ T cell-depleted rhesus monkeys. J. Leukoc. Biol. **74:**772–780.
35. **Kumar, A., W. Weiss, J. A. Tine, S. L. Hoffman, and W. O. Rogers.** 2001. ELISPOT assay for detection of peptide specific interferon-gamma secreting cells in rhesus macaques. J. Immunol. Methods **247:**49–60.
36. **Letvin, N. L.** 1998. Progress in the development of an HIV-1 vaccine. Science **280:**1875–1880.
37. **Letvin, N. L., Y. Huang, B. K. Chakrabarti, L. Xu, M. S. Seaman, K. Beaudry, B. Korioth-Schmitz, F. Yu, D. Rohne, K. L. Martin, A. Miura, W. P. Kong, Z. Y. Yang, R. S. Gelman, O. G. Golubeva, D. C. Montefiori, J. R. Mascola, and G. J. Nabel.** 2004. Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. J. Virol. **78:**7490–7497.
38. **Letvin, N. L., S. Robinson, D. Rohne, M. K. Axthelm, J. W. Fanton, M. Bilska, T. J. Palker, H. X. Liao, B. F. Haynes, and D. C. Montefiori.** 2001. Vaccine-elicited V3 loop-specific antibodies in rhesus monkeys and control of a simian-human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate envelope. J. Virol. **75:**4165–4175.
39. **Leung, L., I. K. Srivastava, E. Kans., H. Legg, Y. Sun, C. Greer, D. C. Montefiori, J. zur Megede, and S. W. Barnett.** 2004. Immunogenicity of HIV-1 Env and Gag in baboons using a DNA prime/protein boost regimen. AIDS **18:**991–1001.
40. **Li, J., C. I. Lord, W. Haseltine, N. L. Letvin, and J. Sodroski.** 1992. Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. J. Acquir. Immune Defic. Syndr. **5:**639–646.
41. **Liao, H. X., B. Etemad-Moghadam, D. C. Montefiori, Y. Sun, J. Sodroski, R. M. Scearce, R. W. Doms, J. R. Thomasch, S. Robinson, N. L. Letvin, and B. F. Haynes.** 2000. Induction of antibodies in guinea pigs and rhesus monkeys against the human immunodeficiency virus type 1 envelope: neutralization of nonpathogenic and pathogenic primary isolate simian/human immunodeficiency virus strains. J. Virol. **74:**254–263.
42. **MacDonald, G. H., and R. E. Johnston.** 2000. Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. J. Virol. **74:**914–922.
43. **Mascola, J. R., M. G. Lewis, G. Stiegler, D. Harris, T. C. VanCott, D. Hayes, M. K. Louder, C. R. Brown, C. V. Sapan, S. S. Frankel, Y. Lu, M. L. Robb, H. Katinger, and D. L. Birx.** 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J. Virol. **73:**4009–4018.
44. **Mascola, J. R., M. G. Lewis, T. C. VanCott, G. Stiegler, H. Katinger, M. Seaman, K. Beaudry, D. H. Barouch, B. Korioth-Schmitz, G. Krivulka, A. Sambor, B. Welcher, D. C. Douek, D. C. Montefiori, J. W. Shiver, P. Poignard, D. R. Burton, and N. L. Letvin.** 2003. Cellular immunity elicited by

human immunodeficiency virus type 1/ simian immunodeficiency virus DNA vaccination does not augment the sterile protection afforded by passive infusion of neutralizing antibodies. J. Virol. **77:**10348–10356.

45. **Mascola, J. R., G. Stiegler, T. C. VanCott, H. Katinger, C. B. Carpenter, C. E. Hanson, H. Beary, D. Hayes, S. S. Frankel, D. L. Birx, and M. G. Lewis.** 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat. Med. **6:**207–210.
46. **McConkey, S. J., W. H. Reece, V. S. Moorthy, D. Webster, S. Dunachie, G. Butcher, J. M. Vuola, T. J. Blanchard, P. Gothard, K. Watkins, C. M. Hannan, S. Everaere, K. Brown, K. E. Kester, J. Cummings, J. Williams, D. G. Heppner, A. Pathan, K. Flanagan, N. Arulanantham, M. T. Roberts, M. Roy, G. L. Smith, J. Schneider, T. Peto, R. E. Sinden, S. C. Gilbert, and A. V. Hill.** 2003. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. Nat. Med. **9:**729–735.
47. **McMichael, A. J., and R. E. Phillips.** 1997. Escape of human immunodeficiency virus from immune control. Annu. Rev. Immunol. **15:**271–296.
48. **Moore, J. P., Y. Cao, L. Qing, Q. J. Sattentau, J. Pyati, R. Koduri, J. Robinson, C. F. Barbas III, D. R. Burton, and D. D. Ho.** 1995. Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. J. Virol. **69:**101–109.
49. **Notka, F., C. Stahl-Hennig, U. Dittmer, H. Wolf, and R. Wagner.** 1999. Construction and characterization of recombinant VLPs and Semliki-Forest virus live vectors for comparative evaluation in the SHIV monkey model. Biol. Chem. **380:**341–352.
50. **Ourmanov, I., M. Bilska, V. M. Hirsch, and D. C. Montefiori.** 2000. Recombinant modified vaccinia virus ankara expressing the surface gp120 of simian immunodeficiency virus (SIV) primes for a rapid neutralizing antibody response to SIV infection in macaques. J. Virol. **74:**2960–2965.
51. **Park, E. J., and G. V. Quinnan, Jr.** 1999. Both neutralization resistance and high infectivity phenotypes are caused by mutations of interacting residues in the human immunodeficiency virus type 1 gp41 leucine zipper and the gp120 receptor- and coreceptor-binding domains. J. Virol. **73:**5707–5713.
52. **Park, E. J., L. K. Vujcic, R. Anand, T. S. Theodore, and G. V. Quinnan, Jr.** 1998. Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. J. Virol. **72:**7099–7107.
53. **Park, I. W., E. Kondo, L. Bergeron, J. Park, and J. Sodroski.** 1996. Effects of human immunodeficiency virus type 1 infection on programmed cell death in the presence or absence of Bcl-2. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **12:**321–328.
54. **Patterson, L. J., N. Malkevitch, J. Zhao, B. Peng, and M. Robert-Guroff.** 2002. Potent, persistent cellular immune responses elicited by sequential immunization of rhesus macaques with Ad5 host range mutant recombinants encoding SIV Rev and SIV Nef. DNA Cell Biol. **21:**627–635.
55. **Pushko, P., M. Parker, G. V. Ludwig, N. L. Davis, R. E. Johnston, and J. F. Smith.** 1997. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. Virology **239:**389–401.
56. **Quinnan, G. V., Jr., P. F. Zhang, D. W. Fu, M. Dong, and H. J. Alter.** 1999. Expression and characterization of HIV type 1 envelope protein associated with a broadly reactive neutralizing antibody response. AIDS Res. Hum. Retrovir. **15:**561–570.
57. **Quinnan, G. V., Jr., P. F. Zhang, D. W. Fu, M. Dong, and J. B. Margolick.** 1998. Evolution of neutralizing antibody response against HIV type 1 virions and pseudovirions in multicenter AIDS cohort study participants. AIDS Res. Hum. Retrovir. **14:**939–949.
58. **Ramsburg, E., N. F. Rose, P. A. Marx, M. Mefford, D. F. Nixon, W. J. Moretto, D. Montefiori, P. Earl, B. Moss, and J. K. Rose.** 2004. Highly effective control of an AIDS virus challenge in macaques by using vesicular stomatitis virus and modified vaccinia virus Ankara vaccine vectors in a single-boost protocol. J. Virol. **78:**3930–3940.
59. **Rasmussen, R. A., R. Hofmann-Lehman, D. C. Montefiori, P. L. Li, V. Liska, J. Vlasak, T. W. Baba, J. E. Schmitz, M. J. Kuroda, H. L. Robinson, H. M. McClure, S. Lu, S. L. Hu, T. A. Rizvi, and R. M. Ruprecht.** 2002. DNA prime/protein boost vaccine strategy in neonatal macaques against simian human immunodeficiency virus. J. Med. Primatol. **31:**40–60.
60. **Shibata, R., T. Igarashi, N. Haigwood, A. Buckler-White, R. Ogert, W. Ross, R. Willey, M. W. Cho, and M. A. Martin.** 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat. Med. **5:**204–210.
61. **Si, Z., M. Cayabyab, and J. Sodroski.** 2001. Envelope glycoprotein determinants of neutralization resistance in a simian-human immunodeficiency virus (SHIV-HXBc2P 3.2) derived by passage in monkeys. J. Virol. **75:**4208–4218.
62. **Si, Z., P. Gorry, G. Babcock, C. M. Owens, M. Cayabyab, N. Phan, and J. Sodroski.** 2004. Envelope glycoprotein determinants of increased entry in a pathogenic simian-human immunodeficiency virus (SHIV-HXBc2P 3.2) passaged in monkeys. AIDS Res. Hum. Retrovir. **20:**163–173.
63. **Soudeyns, H., S. Paolucci, C. Chappey, M. B. Daucher, C. Graziosi, M. Vaccarezza, O. J. Cohen, A. S. Fauci, and G. Pantaleo.** 1999. Selective pressure exerted by immunodominant HIV-1-specific cytotoxic T lymphocyte responses during primary infection drives genetic variation restricted to the cognate epitope. Eur. J. Immunol. **29:**3629–3635.
64. **Srivastava, I. K., L. Stamatatos, E. Kan, M. Vajdy, Y. Lian, S. Hilt, L. Martin, C. Vita, P. Zhu, K. H. Roux, L. Vojtech, C. M. David, J. Donnelly, J. B. Ulmer, and S. W. Barnett.** 2003. Purification, characterization, and immunogenicity of a soluble trimeric envelope protein containing a partial deletion of the V2 loop derived from SF162, an R5-tropic human immunodeficiency virus type 1 isolate. J. Virol. **77:**11244–11259.
65. **Srivastava, I. K., K. VanDorsten, L. Vojtech, S. W. Barnett, and L. Stamatatos.** 2003. Changes in the immunogenic properties of soluble gp140 human immunodeficiency virus envelope constructs upon partial deletion of the second hypervariable region. J. Virol. **77:**2310–2320.
66. **Suryanarayana, K., T. A. Wiltrout, G. M. Vasquez, V. M. Hirsch, and J. D. Lifson.** 1998. Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. AIDS Res. Hum. Retrovir. **14:**183–189.
67. **Sutter, G., and B. Moss.** 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc. Natl. Acad. Sci. USA **89:**10847–10851.
68. **VanCott, T. C., J. R. Mascola, L. D. Loomis-Price, F. Sinangil, N. Zitomersky, J. McNeil, M. L. Robb, D. L. Birx, and S. Barnett.** 1999. Cross-subtype neutralizing antibodies induced in baboons by a subtype E gp120 immunogen based on an R5 primary human immunodeficiency virus type 1 envelope. J. Virol. **73:**4640–4650.
69. **Voss, G., K. Manson, D. Montefiori, D. I. Watkins, J. Heeney, M. Wyand, J. Cohen, and C. Bruck.** 2003. Prevention of disease induced by a partially heterologous AIDS virus in rhesus monkeys by using an adjuvanted multicomponent protein vaccine. J. Virol. **77:**1049–1058.
70. **Vujcic, L. K., and G. V. Quinnan, Jr.** 1995. Preparation and characterization of human HIV type 1 neutralizing reference sera. AIDS Res. Hum. Retrovir. **11:**783–787.
71. **Warren, J.** 2002. Preclinical AIDS vaccine research: survey of SIV, SHIV, and HIV challenge studies in vaccinated nonhuman primates. J. Med. Primatol. **31:**237–256.
72. **Willey, R. L., R. Byrum, M. Piatak, Y. B. Kim, M. W. Cho, J. L. Rossio Jr., Jr., J. Bess Jr., Jr., T. Igarashi, Y. Endo, L. O. Arthur, J. D. Lifson, and M. A. Martin.** 2003. Control of viremia and prevention of simian-human immunodeficiency virus-induced disease in rhesus macaques immunized with recombinant vaccinia viruses plus inactivated simian immunodeficiency virus and human immunodeficiency virus type 1 particles. J. Virol. **77:**1163–1174.
73. **Zhang, M. Y., Y. Shu, S. Phogat, X. Xiao, F. Cham, P. Bouma, A. Choudhary, Y. R. Feng, I. Sanz, S. Rybak, C. C. Broder, G. V. Quinnan, T. Evans, and D. S. Dimitrov.** 2003. Broadly cross-reactive HIV neutralizing human monoclonal antibody Fab selected by sequential antigen panning of a phage display library. J. Immunol. Methods **283:**17–25.
74. **Zhang, P. F., P. Bouma, E. J. Park, J. B. Margolick, J. E. Robinson, S. Zolla-Pazner, M. N. Flora, and G. V. Quinnan, Jr.** 2002. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. J. Virol. **76:**644–655.
75. **Zhang, P. F., X. Chen, D. W. Fu, J. B. Margolick, and G. V. Quinnan, Jr.** 1999. Primary virus envelope cross-reactivity of the broadening neutralizing antibody response during early chronic human immunodeficiency virus type 1 infection. J. Virol. **73:**5225–5230.