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Multiclade Human Immunodeficiency Virus Type 1 Envelope Immunogens Elicit Broad Cellular and Humoral Immunity in Rhesus Monkeys

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The development of a human immunodeficiency virus type 1 (HIV-1) vaccine that elicits potent cellular and humoral immune responses recognizing divergent strains of HIV-1 will be critical for combating the global AIDS epidemic. The present studies were initiated to examine the magnitude and breadth of envelope (Env)-specific T-lymphocyte and antibody responses generated by vaccines containing either a single or multiple genetically distant HIV-1 Env immunogens. Rhesus monkeys were immunized with DNA prime–recombinant adenovirus boost vaccines encoding a Gag-Pol-Nef polyprotein in combination with either a single Env or a mixture of clade-A, clade-B, and clade-C Envs. Monkeys receiving the multiclade Env immunization developed robust immune responses to all vaccine antigens and, importantly, a greater breadth of Env recognition than monkeys immunized with vaccines including a single Env immunogen. All groups of vaccinated monkeys demonstrated equivalent immune protection following challenge with the pathogenic simian-human immunodeficiency virus 89.6P. These data suggest that a multicomponent vaccine encoding Env proteins from multiple clades of HIV-1 can generate broad Env-specific T-lymphocyte and antibody responses without antigenic interference. This study demonstrates that it is possible to generate protective immune responses by vaccination with genetically diverse isolates of HIV-1.

The extreme genetic diversity of the human immunodeficiency virus type 1 (HIV-1) envelope (Env) poses a daunting challenge for the creation of an effective AIDS vaccine (16). While Env is the principal target for HIV-1-specific antibody responses, it also serves as a potent T-cell immunogen (15). An ideal HIV-1 vaccine should elicit potent cellular and humoral immunity capable of recognizing a diversity of viral isolates (19, 23). However, the extraordinary genetic variation of HIV-1 Env worldwide may make it impossible to create an effective vaccine using only a single Env gene product.

While many of the promising AIDS vaccine candidates currently under investigation in nonhuman primates and early-phase human clinical trials utilize Env immunogens derived from a single HIV-1 primary isolate (10), this approach has significant limitations. Although these vaccines generate potent cellular and humoral immune responses against HIV-1 Env, it is likely that the breadth of immunity elicited by a single Env immunogen will not effectively confer protection against divergent strains of HIV-1. It is, however, not feasible to undertake the development of multiple country- or clade-specific vaccines. Moreover, such region-specific vaccines would likely not protect against unrelated strains that might be newly introduced into a population.

One strategy for creating a single HIV-1 vaccine for worldwide use is to employ representative immunogens from multiple clades of HIV-1 in a single vaccine formulation (22). Such a multiclade vaccine would contain Env immunogens relevant to the majority of HIV-1 infections worldwide and could be feasibly tested. However, it is not clear whether a multicomponent vaccine encoding antigens from various clades of HIV-1 would elicit antiviral immunity greater than or equal to that of a vaccine employing a single Env immunogen, and whether a complex mixture of immunogens would result in antigenic interference and diminished immune protection (13).

The present studies utilized the simian-human immunodeficiency virus (SHIV)–rhesus monkey model to investigate the breadth and magnitude of immunity elicited by a DNA prime–recombinant adenovirus (rAd) boost vaccine containing Gag-Pol-Nef and either single-clade or multiple-clade Env immunogens. Our findings demonstrate that a multiclade Env vaccine elicits potent cellular and humoral immune responses with greater breadth than can be generated by immunizations performed with a single Env immunogen.

MATERIALS AND METHODS

Immunizations and challenge of rhesus monkeys. Thirty adult Indian-origin rhesus monkeys (*Macaca mulatta*) were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care in accordance with the guidelines of the Institutional Animal Care and Use Committee for Harvard Medical School and the *Guide for the Care and Use of Laboratory Animals*. Monkeys were divided into five groups of six animals each. Each experimental group included two monkeys expressing the major histocompatibility complex class I allele *Mamu-A*01*.

Plasmid DNA and rAd vaccine vectors were constructed as previously de-

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TABLE 1. Experimental groups and plasmid DNA immunization schedule[a]

Group	Amt (mg) of plasmid		
	SIV Gag-Pol-Nef	HIV-1 Env	Sham
1) High clade B Env	4.5	4.5 clade B	
2) Low clade B Env	4.5	1.5 clade B	3.0
3) High clade C Env	4.5	4.5 clade C	
4) Clade A+B+C Env	4.5	1.5 clade A	
		1.5 clade B	
		1.5 clade C	
5) Control			9.0

[a] Animals received three priming immunizations at weeks 0, 4, and 8.

scribed (14, 15) and administered by intramuscular injection using a needle-free Biojector system and a no. 3 syringe (Bioject, Portland, Oreg.) as outlined in Tables 1 and 2. Each plasmid DNA or rAd vaccine vector was split into two aliquots of 0.5 ml each and delivered into each quadriceps muscle. Control monkeys were similarly immunized with sham DNA and sham rAd vectors. At week 42, all monkeys received an intravenous challenge with 50 50% monkey infective doses (MID_{50}) of SHIV-89.6P.

IFN-γ ELISPOT assays. Gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays were performed as previously described (15). Freshly isolated peripheral blood lymphocytes (PBL) were plated in triplicate at 2×10^5/well in a 100-μl final volume with either medium alone or peptide pools. Peptide pools covered the entire SIVmac239 Gag, Nef, and Pol proteins, and the HIV-1 clade-A, clade-B, clade-C, and 89.6P Env proteins. Each pool was composed of 15-amino-acid-peptides overlapping by 11 amino acids, except for the HIV-1 89.6P Env pool, which was composed of 20-amino-acid peptides overlapping by 10 amino acids. Pol and Env peptides were each split into two separate pools such that each pool contained no more than 130 peptides. Each peptide in a pool was present at a concentration of 1 μg/ml. The mean number of spots from triplicate wells was calculated for each animal and adjusted to represent the mean number of spots per 10^6 PBL. Data are presented as the mean number of antigen-specific spots per 10^6 PBL from six monkeys per group.

HIV-1 envelope antibody enzyme-linked immunosorbent assay (ELISA). Vaccine Research Center (VRC) plasmids 5304, 2801, and 5308 (which encode HIV-1 gp145 clade-A, clade-B, and clade-C Env, respectively) were expressed in 293 cells and purified for the major protein product. Immunol-2 HB microtiter plates (Thermo Labsystems, Milford, Mass.) were coated with optimized concentrations of the recombinant antigens (37.5 ng/well) overnight at 4°C. Serial dilutions of monkey plasma were made in duplicate wells and incubated for 2 h at 37°C. Biotin-labeled anti-monkey immunoglobulin G (IgG), IgA, and IgM (Rockland Immunochemicals, Gilbertsville, Pa.) were added for 1 h at 37°C. Streptavidin-conjugated horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to wells for 30 min at room temperature, followed by TMB substrate (Kirkegaard & Perry Laboratories) for 30 min at room temperature. End point titers for each animal were established as the last dilution with a preimmunization corrected optical density of >0.2. Data are presented as the geometric mean titer from six monkeys per group ± standard error of the mean (SEM).

Virus isolates and neutralization assays. A total of 30 HIV-1 isolates were studied: 11 clade-B, 11 clade-C, and 8 clade-A isolates. Viruses were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, except as specifically noted below. All clade-B viruses were primary isolates except the T-cell line-adapted HxB2, which is a molecular clone of HIV-IIIB. BR07 was provided by Dana Gabuzda of the Dana-Farber Cancer Institute. It is a chimeric infectious molecular clone of NL4-3 that contains a nearly full-length Env gene that was cloned directly from brain tissue of an AIDS patient (24). Clade-B primary isolate 6101, previously called P15 (1), and clade-C viruses DU123, DU151, S007, and S080 were provided by David Montefiori (Duke University Medical Center). The clade-C viruses were obtained from HIV-1-infected patients in South Africa (Du prefix) or Malawi (S prefix) and have been described previously (2). TV1 (clade C) was provided by David Montefiori and Estrelita Janse Van Rensburg (University of Stellenbosch, Stellenbosch, South Africa). GS14 is an infectious molecular clone of an Ethiopian clade-C virus that was provided by Francine McCutchan and colleagues from the U.S. Military HIV Research program. Clade-A viruses DJ263 and 44951 were primary virus isolates provided by researchers from the U.S. Military HIV Research program. The UG29 isolate had been previously passaged in H9 cells and would therefore be considered a T-cell line-adapted virus.

Virus neutralization assays were performed by using a single-round-of-infection flow cytometric assay using previously described methods (18). This assay detects HIV-1-infected T cells by intracellular staining for HIV-1 p24 Gag antigen (p24-Ag). A protease inhibitor is used to prevent secondary rounds of virus replication. The percent virus neutralization mediated by each immune plasma sample was derived by calculating the reduction in the number of p24-Ag-positive cells in the test wells with immune sera compared to the number of p24-Ag-positive cells in wells containing preimmune plasma from the corresponding animal. Plasma from the six sham-immunized monkeys was included for analysis, and these data are shown in Results. All plasma samples were also tested against an amphotropic murine leukemia virus (MuLV) to test for non-HIV-1-specific plasma effects. The MuLV reporter viruses encoded green fluorescent protein (GFP), and infected T cells were detected by expression of GFP rather than by expression of p24-Ag (18).

Quantitation of plasma viral RNA levels and $CD4^+$ T-lymphocyte counts. Plasma viral RNA levels were measured by an ultrasensitive branched DNA amplification assay with a lower detection limit of 125 copies per ml (Bayer Diagnostics, Berkeley, Calif.). Peak plasma viral load was measured on day 16 post-SHIV-89.6P challenge in all vaccinated and control monkeys. Set point plasma viral RNA levels were calculated as the median of values measured at six time points between days 85 and 169 postchallenge. The percentage of $CD4^+$ T lymphocytes in the peripheral blood of infected monkeys was determined by monoclonal antibody staining and flow cytometric analysis. Briefly, freshly isolated PBL were stained with allophycocyanin-conjugated anti-CD3 (FN18), phycoerythrin-conjugated anti-CD4 (19Thy5D7), and fluorescein isothiocyanate-conjugated anti-CD8 (SK1; BD Biosciences, Mountain View, Calif.). Samples were acquired by using a FACSCalibur flow cytometer, and data were analyzed by using CellQuest software (BD Biosciences).

Statistical analysis. The nonparametric Wilcoxon rank sum test was used to compare $CD4^+$ T lymphocytes, peak viral RNA, and set point viral RNA between monkeys in the nonvaccinated and vaccinated groups. All tests were two-sided.

TABLE 2. Experimental groups and immunization schedule for rAd (wk 26)

Group	No. of particles		
	SIV Gag-Pol rAd	HIV-1 Env rAd	Sham rAd
1) High clade B Env	1.0×10^{12}	1.0×10^{12} clade B	
2) Low clade B Env	1.0×10^{12}	3.3×10^{11} clade B	6.6×10^{11}
3) High clade C Env	1.0×10^{12}	1.0×10^{12} clade C	
4) Clade A+B+C Env	1.0×10^{12}	3.3×10^{11} clade A	
		3.3×10^{11} clade B	
		3.3×10^{11} clade C	
5) Control			2.0×10^{12}

RESULTS

Study design. Thirty adult rhesus monkeys were divided into five experimental groups of six animals (Tables 1 and 2). Groups 1 to 4 received three priming immunizations at weeks 0, 4, and 8 with 4.5 mg of plasmid DNA vectors expressing an SIVmac239 Gag-Pol-Nef fusion protein and plasmid DNA vectors expressing various HIV-1 Env proteins. Groups 1 to 3 were immunized with single HIV-1 Env immunogens as follows: group 1 received 4.5 mg of clade-B Env (high clade B), group 2 received 1.5 mg of clade-B Env (low clade B), and group 3 received 4.5 mg of clade-C Env (high clade C). Group 4 monkeys were immunized with a combination of HIV-1 Env immunogens: 1.5 mg each of clade-A Env, clade-B Env, and clade-C Env (clade A+B+C). At week 26, monkeys received a single rAd boost immunization (2.0×10^{12} total particles) with vectors expressing SIVmac239 Gag-Pol and various HIV-1 Env genes consistent with those delivered during DNA priming (Table 2). Group 1 received 1.0×10^{12} particles of clade-B Env

(high clade B), group 2 received 3.3×10^{11} particles of clade-B Env (low clade B), and group 3 received 1.0×10^{12} particles of clade-C Env (high clade C). Group 4 received 3.3×10^{11} particles each of clade-A, clade-B, and clade-C Env (clade A+B+C). Group 5 monkeys were immunized with sham DNA and sham rAd vectors. DNA prime and rAd boost immunizations were delivered by intramuscular injection. All plasmid DNA and rAd vectors expressed codon-modified SIVmac239 and HIV-1 genes for enhanced expression in mammalian cells. All *env* genes used in these vectors were ΔCFI constructs, containing mutations in the cleavage, fusion, and interhelical domains that have previously been shown to enhance expression and immunogenicity (5). The percentage of amino acid identity among the HIV-1 Env immunogens ranged from 71 to 76%, with the clade-B and clade-C Envs demonstrating the greatest divergence.

Cellular immune responses elicited by immunization. The cellular immune responses to SIV Gag and Pol and HIV-1 Envs in immunized monkeys were assessed by pooled peptide IFN-γ ELISPOT assays using freshly isolated PBL. Moreover, the extent of cross-clade reactivity of vaccine-elicited Env-specific cellular immune responses was determined by measuring PBL IFN-γ ELISPOT responses to clade-A, clade-B, and clade-C Env peptide pools. Because these monkeys were to be challenged with SHIV-89.6P, we also evaluated T-cell recognition of a peptide pool representing the clade-B 89.6P Env. Monkeys receiving the high- and low-dose clade-B Env plasmid DNA immunogen generated cellular immune responses to all Env peptide pools tested (Fig. 1, top panel). The responses to both the clade-B and 89.6P (heterologous clade B) Env peptide pools were of a higher frequency than those observed against the clade-A or clade-C Env pools. Monkeys receiving the high-dose clade-C Env immunogen also developed cellular immune responses to all Env peptide pools tested, but with clade-C Env responses higher than those to clade-A, clade-B, or 89.6P Envs. Importantly, comparable cellular immune responses to clade-A, clade-B, clade-C, and 89.6P HIV-1 Env peptide pools were observed in PBL of monkeys receiving the multiclade plasmid DNA immunogens.

The DNA-primed cellular immune responses of all vaccinated monkeys were dramatically augmented following the boost immunization with the rAd vaccines (Fig. 1, middle panel). While responses to all Env peptide pools were observed in monkeys receiving the high-dose clade-B Env rAd boost immunization, spot-forming cell (SFC) responses clade-B and 89.6P peptides were higher than those to clade-A or -C peptides ($P = 0.06$ and 0.04, respectively, by the Wilcoxon rank sum test). The Env-specific cellular immune responses of the low-dose clade-B Env-immunized monkeys were comparable to those of monkeys receiving the high-dose clade-B Env immunogens. Thus, lowering the dose of the Env plasmid and rAd vaccines by two-thirds did not result in major reductions in immunogenicity. The animals boosted with the high-dose clade-C Env rAd construct also showed increases in T-cell reactivity to all Env peptide series, but responses to clade-C peptides were significantly higher than those to clade-A or -B peptides ($P = 0.04$ for both). In contrast, multiclade Env-immunized monkeys exhibited no bias in Env-specific cellular immune responses. Following the boost immunization with the clade-A, clade-B, and clade-C Env rAd constructs, the monkeys developed responses to the clade-A, clade-B, clade-C, and 89.6P Env peptide pools that were of comparable magnitude (Fig. 1, middle panel). Furthermore, the magnitude of each individual clade-specific ELISPOT response in these monkeys was comparable to the optimal clade-specific response elicited in monkeys receiving a single-clade Env immunogen. Finally, the vaccine-elicited Env-specific T-cell responses in all groups of monkeys were durable, persisting at a high frequency up to the time of viral challenge (Fig. 1, bottom panel).

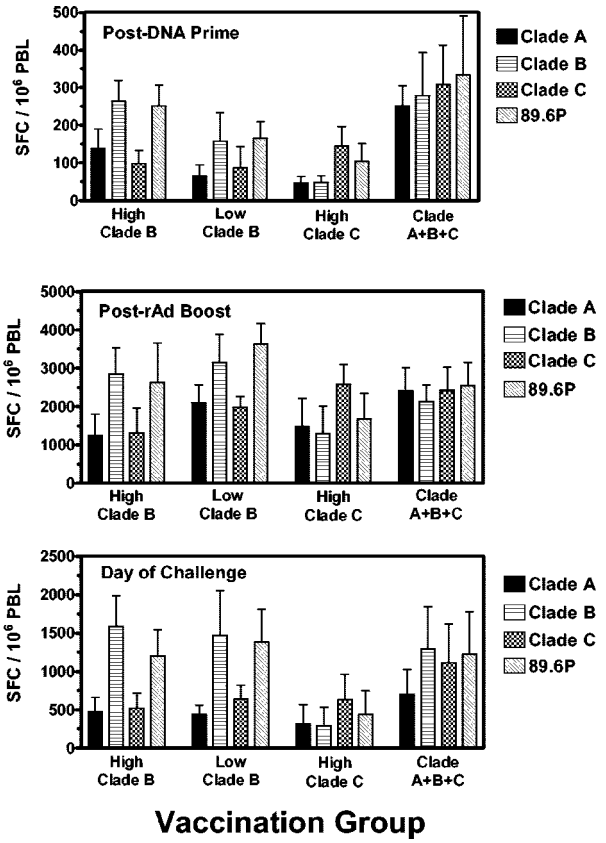

FIG. 1. Vaccine-elicited cellular immune responses to HIV-1 clade-A, clade-B, clade-C, and 89.6P Env antigens by PBL of rhesus monkeys following DNA prime and rAd boost immunizations. PBL were freshly isolated at weeks 12 (post-DNA prime), 27 (post-rAd boost), and 42 (day of challenge) postimmunization and were assessed for IFN-γ ELISPOT responses following stimulation with peptide pools spanning the indicated HIV-1 Env proteins. Data are presented as the mean number of antigen-specific SFC per 10^6 PBL ± SEM from six monkeys per group.

Cellular immune responses to SIV Gag and Pol were observed in all vaccinated monkeys following the DNA priming immunizations (data not shown) as well as following the rAd boost immunizations (Fig. 2). Importantly, PBL of monkeys receiving the multiclade Env immunizations developed ELISPOT responses to these SIV proteins that were comparable in magnitude to those observed in PBL from monkeys receiving single-clade Env immunogens. Thus, immunizing monkeys with the complex pool of SIV Gag-Pol and multiclade HIV-1 Env immunogens elicited cellular immune responses to all the vaccine components without evidence of antigenic interference. No significant differences in the magnitude or breadth of the peak vaccine-elicited cellular immune responses were ob-

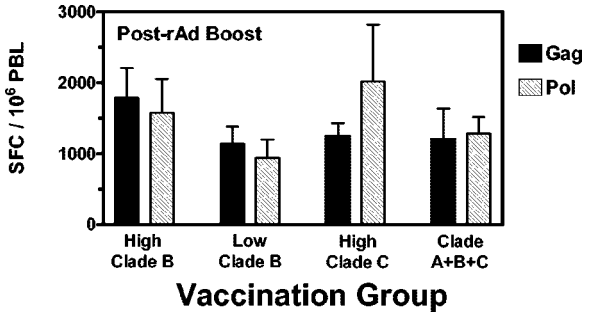

FIG. 2. Vaccine-elicited cellular immune responses to SIV Gag and Pol by PBL of rhesus monkeys following DNA prime–rAd boost immunizations. PBL were freshly isolated at week 27 postimmunization (1 week following rAd boost) and assessed for IFN-γ ELISPOT responses following stimulation with peptide pools spanning the SIV Gag and Pol proteins. Data are presented as the mean number of antigen-specific SFC per 10^6 PBL ± SEM from six monkeys per group.

served between the *Mamu-A*01*$^+$ and *Mamu-A*01*$^-$ monkeys in each immunization group.

Antibody responses elicited by immunization. The magnitude and breadth of humoral immune responses elicited by single-clade and multiclade Env immunizations in these monkeys were investigated following rAd administration. Plasma samples were tested for antibody binding activity to the clade-A, clade-B, and clade-C gp145 Env proteins by ELISA. Monkeys receiving the high-dose clade-B Env immunogens generated antibody responses that bound all three Env proteins (Fig. 3); however, the highest antibody titers were those against the clade-B Env protein ($P = 0.002$ and 0.13 versus clade-A and -C Env proteins, respectively, by the Wilcoxon rank sum test). A similar pattern of antibody reactivity was observed in monkeys receiving the low-dose clade-B Env immunizations, and lowering the dose of Env immunogen by two-thirds did not result in a substantial reduction in immunogenicity. Monkeys receiving the high-dose clade-C Env immunogens similarly developed antibody responses that recognized all three Env antigens, but titers against the clade-C Env protein were significantly higher than those against clade-A or -B Env proteins ($P = 0.004$ and 0.002, respectively). In contrast, monkeys immunized with the mixture of clade-A, clade-B, and clade-C Env immunogens demonstrated comparable antibody responses to all three Env proteins.

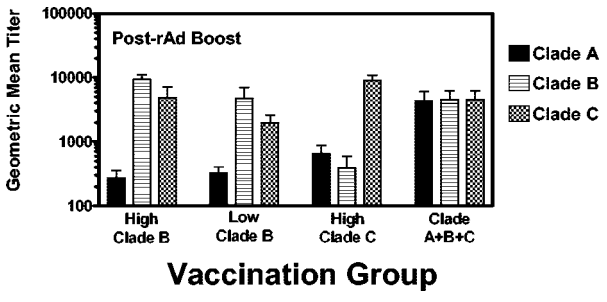

FIG. 3. Titers of antibodies to HIV-1 clade-A, clade-B, or clade-C Env proteins in plasma from rhesus monkeys following DNA prime–rAd boost immunizations. Plasma samples were obtained at week 28 postimmunization (2 weeks following rAd boost), and titers of anti-gp145 antibody to the indicated HIV-1 Env proteins were determined by ELISA. Data are presented as the mean geometric titer from six monkeys per group.

Plasma samples obtained following the rAd boost immunizations were also tested for neutralizing activity against panels of 30 clade-A, clade-B, and clade-C HIV-1 isolates (Fig. 4). While plasma from all groups of vaccinated monkeys demonstrated modest levels of neutralization against some HIV-1 isolates, the antibodies of monkeys immunized with a single-clade Env immunogen exhibited the highest neutralizing activity against viruses of the same clade. Thus, plasma from the clade-B-immunized animals displayed little activity against clade-A or -C viruses, and plasma from clade-C-immunized animals did not neutralize clade-B viruses. However, there was some cross-neutralization of clade-A viruses by the clade-C vaccine plasma. Importantly, monkeys immunized with multiclade Env developed antibodies with neutralizing activity against some HIV-1 strains from all three clades, and there was no decrement in the potency of neutralization compared to single-Env immunization. Neutralizing activity against viruses expressing the clade-A, clade-B, or clade-C Envs utilized in the plasmid DNA and rAd vaccines was not assessed in these experiments. Two controls were performed to demonstrate that the modest levels of virus neutralization observed were due to HIV-1-specific antibodies. The mean neutralization activity of plasma obtained from sham-vaccinated monkeys was consistently less than 20% (Fig. 4). In addition, the mean activity of plasma from each of the vaccine groups against a MuLV Env pseudovirus was less than 20% (Fig. 4, top panel). These data indicate that the multiclade Env immunization regimen elicited humoral immune responses of increased breadth compared to responses elicited by immunization with a single Env, and with no evidence of antigenic interference.

Protection against SHIV-89.6P challenge. All monkeys received an intravenous challenge with 50 MID_{50} of SHIV-89.6P on week 42, 16 weeks following the rAd boost immunization. At 2 weeks after viral challenge, robust cellular immune responses to HIV-1 Env and SIV Gag and Pol were detected in all groups of experimentally vaccinated monkeys but not in control monkeys (Fig. 5). Peak plasma viral RNA levels were observed in all monkeys on day 16 following challenge; median levels detected were 7.54 (control), 5.66 (high clade B Env), 6.83 (low clade B Env), 6.20 (high clade C Env), and 6.46 (clade A+B+C Env) $\log_{10}$ copies of viral RNA (Fig. 6, upper panel). Thus, a significant reduction in the peak viral load following SHIV-89.6P challenge was observed in all groups of experimentally vaccinated monkeys compared with nonvaccinated control monkeys (*P* values ranged from 0.002 to 0.004 by the Wilcoxon rank sum test). However, no significant differences in peak viral RNA levels were observed when monkeys receiving the single-clade Env or multiclade Env immunizations were compared.

All groups of experimentally vaccinated monkeys also demonstrated lower set point plasma viral RNA levels than control monkeys, with median levels of 4.77 (control), 2.30 (high-dose clade-B Env), 2.63 (low-dose clade-B Env), 2.28 (high-dose clade-C Env), and 2.69 (clade A+B+C Env) $\log_{10}$ viral RNA copies per ml measured between days 85 and 169 postchallenge (Fig. 6, lower panel). The set point viral RNA levels were significantly lower than those of the control animals for the high-dose clade-B Env group and the high-dose clade-C group

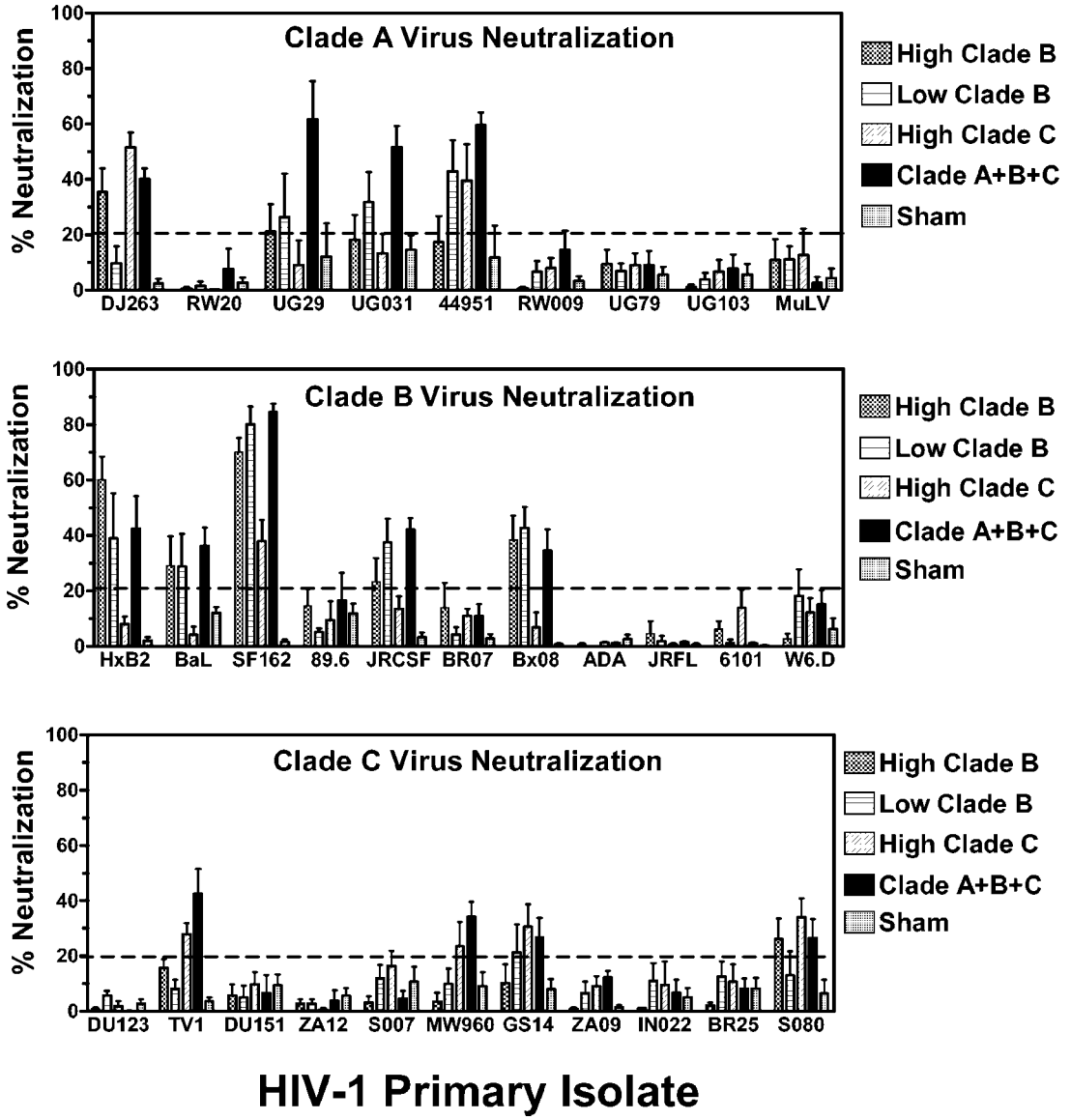

FIG. 4. Antibody neutralizing activity in plasma of rhesus monkeys following DNA prime–rAd boost immunizations. Plasma samples were obtained from vaccinated and control monkeys at week 28 postimmunization (2 weeks following rAd boost) and were tested at a 1:5 dilution for neutralizing activity against panels of clade-A, clade-B, and clade-C HIV-1 isolates. The dashed line represents a reference point of 20% neutralization, as noted in Results. Data are presented as the mean percent neutralizing activity ± SEM from six monkeys per group. Note that the top panel of clade-A viruses also includes a control MuLV Env pseudovirus.

($P < 0.05$ by the Wilcoxon rank sum test). A trend toward significance was observed by comparing the control animals to the low-dose clade-B Env group ($P = 0.06$) and the clade A+B+C Env group ($P = 0.09$). No significant differences in set point viral loads were observed among the groups of monkeys vaccinated with the single-Env or multiclade Env immunogens.

Peripheral blood $CD4^+$ T-lymphocyte counts were also measured for the infected monkeys as a means of evaluating vaccine-mediated protection against SHIV-89.6P-induced disease. Sham-vaccinated control monkeys developed a rapid and persistent decline in $CD4^+$ T-lymphocyte numbers within the first 21 days following challenge (Fig. 7). All groups of experimentally vaccinated monkeys exhibited significant blunting of $CD4^+$ T-lymphocyte loss between days 85 and 169 postchallenge compared with control monkeys (P values ranging from 0.015 to 0.026). While there were no significant differences in $CD4^+$ T-lymphocyte numbers between the groups of vaccinated monkeys during the acute and chronic phases of infection, monkeys in the high-dose clade-B Env and multiclade Env vaccine groups demonstrated the best preservation of this lymphocyte population.

DISCUSSION

A global HIV-1 vaccine must elicit effective immune responses to diverse viral isolates. In fact, broadly cross-reactive HIV-1-specific T-cell immune responses have been described. HIV-1-infected individuals develop T-lymphocyte responses

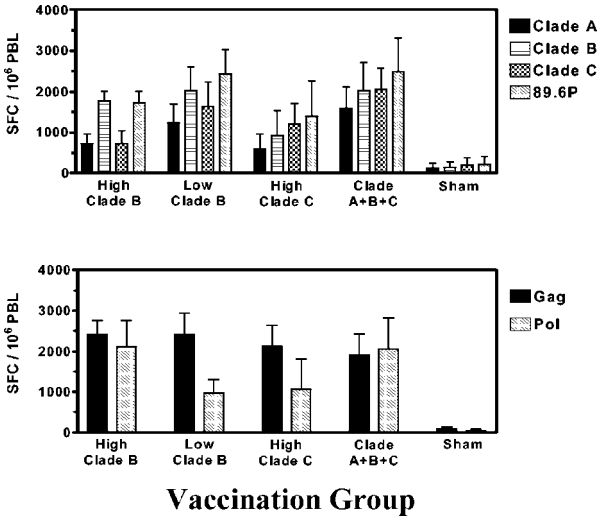

FIG. 5. Cellular immune responses to HIV-1 Env and SIV Gag and Pol by PBL of vaccinated and control rhesus monkeys following SHIV-89.6P challenge. PBL were freshly isolated 2 weeks following challenge and assessed for IFN-γ ELISPOT responses following stimulation with peptide pools spanning the indicated HIV-1 Env proteins or the SIV Gag and Pol proteins. Data are presented as the mean number of antigen-specific SFC per 10^6 PBL ± SEM from six monkeys per group.

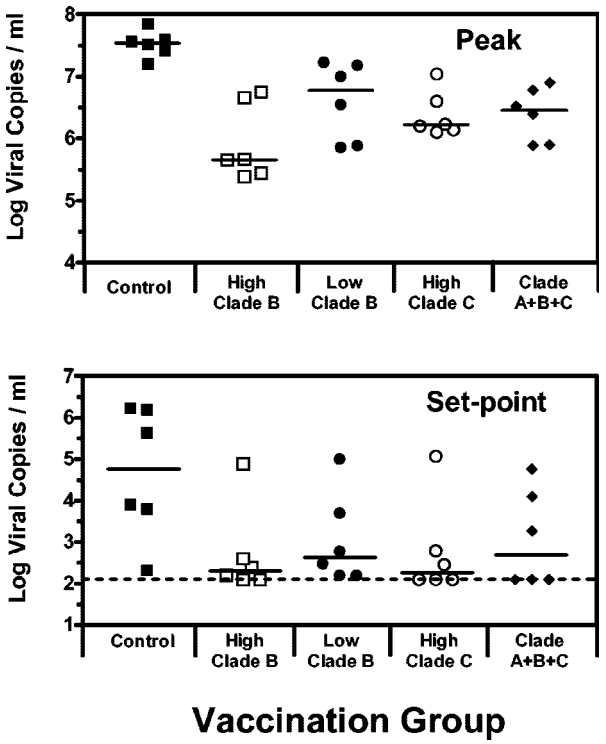

FIG. 6. Plasma viral RNA levels following SHIV-89.6P challenge. The peak plasma viral RNA level for each monkey was measured on day 16 postchallenge. The set point plasma viral RNA level for each monkey was calculated as the median of values detected between days 85 and 169 postchallenge. Log viral copies per milliliter from individual monkeys are shown, with horizontal bars indicating the median value of the six monkeys per experimental group. The detection limit of the assay, 125 copies/ml, is shown by a dashed line.

that recognize viral sequences from a diversity of HIV-1 clades (3). Cross-clade-reactive cytotoxic T lymphocytes (CTL) have also been detected in uninfected volunteers who have been vaccinated with recombinant canarypox constructs (8). However, because these studies employed CTL clones or in vitro-cultured PBL to assess cross-clade T-cell reactivity, the true breadth of these HIV-1-specific immune responses is unknown. In the present study we demonstrate that immunization of rhesus monkeys with a DNA prime–rAd boost vaccine that includes multiple Env immunogens elicits cellular and humoral immune responses that exhibit a greater breadth of Env-specific recognition than that observed in monkeys immunized with single Env immunogens.

PBL from monkeys immunized with single HIV-1 Env immunogens demonstrated a high frequency of cellular immune responses to peptide pools matching the vaccine-encoded Env immunogen, with a lower frequency of responses to peptides of Env proteins not included in the vaccine. These cross-reactive responses may reflect T-lymphocyte recognition of conserved viral epitopes, as well as cross-reactive recognition of variant epitopes that may differ by limited numbers of amino acids (6, 12). The highest degree of heterologous Env recognition in this study was the reactivity of PBL of monkeys immunized with the clade-B HXBc2/BaL Env immunogen against peptide pools representing 89.6P Env, a heterologous clade-B Env (Fig. 1). HXBc2/BaL Env shares 81% amino acid identity with 89.6P Env and only 75 and 72% identity, respectively, with the clade-A and -C Env sequences used in these immunizations. These data suggest that immunizing with single-Env immunogens may elicit the highest frequency of cross-reactive T-cell responses against Envs of viruses of the same clade.

A concern with a vaccine that includes viral proteins from multiple clades of HIV-1 is that interference between these diverse antigens may diminish immune responses. In fact, such antigenic interference has been observed in vaccines that include proteins of multiple pathogens (7, 11). Moreover, studies have shown that complex mixtures of plasmid DNA vaccines can lead to decreased protein expression and immunogenicity in vivo (13, 25). The findings in the present study demonstrate that the inclusion of Env immunogens from several clades of HIV-1 in a single vaccine can increase the breadth of vaccine-elicited Env-specific T-cell and antibody responses. Thus, monkeys immunized with the multiclade Env vaccine developed high frequencies of cellular immune responses and antibody

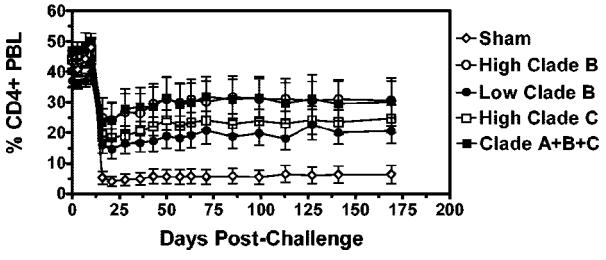

FIG. 7. Peripheral blood $CD4^+$ T lymphocytes post-SHIV-89.6P challenge. The percentage of $CD3^+$ $CD4^+$ T lymphocytes in the peripheral blood of the rhesus monkeys was assessed by flow cytometry through day 169 following SHIV-89.6P infection. Data are presented as the mean percentage of peripheral blood $CD4^+$ T lymphocytes from six monkeys per group ± SEM.

responses to all vaccine-encoded Env antigens. The magnitudes of T-lymphocyte responses to the clade-B and clade-C Env peptide pools following the DNA prime and rAd boost with the multiclade Env immunogens were similar to those observed in monkeys receiving the high-dose single clade-B or -C Env vaccine. Furthermore, no deleterious effects on the magnitudes of Gag- or Pol-specific cellular immune responses were detected in the multiclade Env-immunized monkeys. These results support the findings of previous studies in mice demonstrating that multiclade HIV-1 vaccines can elicit robust cellular and humoral immune responses to all vaccine-encoded antigens, with no evidence of antigenic interference (4, 14).

While this immunization strategy can elicit high-titer HIV-1-specific antibody responses, both the potency and the breadth of the neutralizing antibodies induced by these Env immunogens were limited. We employed appropriate controls to confirm the presence of antibody-mediated neutralization by the immune plasma. These controls included the use of preimmune plasma from each animal and an irrelevant MuLV control virus. Although only low levels of neutralization were detected, the specificity of the demonstrated neutralization also lends credibility to the results. For example, the clade-A viruses were more strongly neutralized by plasma from animals that received the clade-A, -B, and -C multiclade Env immunogens than by plasma from animals that received only the clade-B immunogen. Several of the isolates in the panel of clade-B viruses that was used to evaluate the breadth of neutralization are known to be particularly neutralization sensitive (e.g., HxB2, SF162, Bx08). The clade-A virus isolates evaluated in this study also appear to be more sensitive to neutralization than the clade-C isolates (Fig. 4). The panel of 8 clade-A and 11 clade-C viruses used in this study was composed of viruses readily available to us. No a priori criteria were applied in choosing these specific viruses. In fact, we have little prior experience in evaluating vaccine-elicited antibody neutralization of non-clade-B viruses, and it is possible that the clade-A viruses we used in this panel were inherently more sensitive to neutralization than the clade-C viruses.

We have previously demonstrated that DNA prime–rAd boost immunization of rhesus monkeys with vaccine antigens that include 89.6P Env or a clade-B Env fails to elicit neutralizing antibodies against the SHIV-89.6P challenge virus (15, 20). Several other vaccine studies have also demonstrated SHIV-89.6P to be a neutralization-resistant isolate (17, 21). Thus, while we did not evaluate plasma-mediated neutralization of SHIV-89.6P in this study, we are confident that the immunogens that were employed would not elicit neutralizing antibodies to the challenge virus. Neutralizing antibodies to SHIV-89.6P do develop after SHIV challenge, but our prior study of a homologous 89.6P Env immunogen was unable to demonstrate that Env immunization accelerated the development of these antibodies (15, 20). We therefore believe it is highly unlikely that an anamnestic antibody response played a significant role in the clinical protection observed in this study.

It is encouraging that the combination of the clade-A, -B, and -C Env immunogens elicited neutralizing antibodies to some viral isolates not included in the vaccine. Clearly, this neutralization was low in potency, and further efforts will be required to design more-potent Env immunogens. We have yet to examine in depth whether this multiclade Env immunization elicits T-cell cross-reactivity to diverse clade-A, -B, or -C Envs that are not included in the vaccine or whether it simply elicits immune responses to the vaccine antigens that are additive; however, given our observation that single-clade Env immunization elicits immune responses that are highly cross-reactive with Env sequences from the same clade, it may be expected that T lymphocytes of multiclade Env-immunized monkeys will exhibit a high frequency of cross-reactive cellular immune responses to heterologous clade-A, -B, and -C Envs.

The present data show that multiclade Env immunization does not diminish vaccine-elicited immune protection against SHIV-89.6P infection. Monkeys receiving DNA prime–rAd boost vaccines encoding either a single Env or multiple-clade Env immunogens demonstrated equivalent viral containment during acute and chronic infection, and comparable preservation of $CD4^+$ T lymphocytes. We have previously demonstrated that DNA prime–rAd boost vaccine-elicited protection against SHIV-89.6P infection was associated with an anamnestic antigen-specific cellular rather than a neutralizing-antibody response (15). It is therefore not surprising that no significant differences in clinical protection were evident between the various groups of vaccinated monkeys; they all demonstrated robust prechallenge cellular immune responses to SIV Gag and Pol, as well as some degree of cellular immune cross-reactivity to 89.6P Env. In fact, the ELISPOT responses to 89.6P Env increased rapidly in the PBL of all groups of Env-vaccinated monkeys following challenge, suggesting that vaccine-elicited T lymphocytes capable of recognizing 89.6P Env epitopes expanded in response to the replicating virus (Fig. 5).

The present study demonstrates that the inclusion of viral proteins from multiple clades of HIV-1 is a viable approach for a global HIV-1 vaccine. Whether this strategy proves superior to the use of immunogens created from ancestral or consensus HIV-1 viral sequences for increasing the breadth of immune recognition of HIV-1 isolates remains to be determined (9). Nevertheless, this study illustrates the feasibility of constructing vaccine immunogens that address the problem of the extreme variability of HIV-1 isolates throughout the world.

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