

Vaccination of Rhesus Macaques with Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin Env V3 Elicits Neutralizing Antibody-Mediated Protection against Simian-Human Immunodeficiency Virus with a Homologous but Not a Heterologous V3 Motif

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Although the correlates of vaccine-induced protection against human immunodeficiency virus type 1 (HIV-1) are not fully known, it is presumed that neutralizing antibodies (NAb) play a role in controlling virus infection. In this study, we examined immune responses elicited in rhesus macaques following vaccination with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin expressing an HIV-1 Env V3 antigen (rBCG Env V3). We also determined the effect of vaccination on protection against challenge with either a simian-human immunodeficiency virus (SHIV-MN) or a highly pathogenic SHIV strain (SHIV-89.6PD). Immunization with rBCG Env V3 elicited significant levels of NAb for the 24 weeks tested that were predominantly HIV-1 type specific. Sera from the immunized macaques neutralized primary HIV-1 isolates in vitro, including HIV-1_{BZ167/X4}, HIV-1_{SF2/X4}, HIV-1_{CI2/X4}, and, to a lesser extent, HIV-1_{MNP/X4}, all of which contain a V3 sequence homologous to that of rBCG Env V3. In contrast, neutralization was not observed against HIV-1_{SF33/X4}, which has a heterologous V3 sequence, nor was it found against primary HIV-1 R5 isolates from either clade A or B. Furthermore, the viral load in the vaccinated macaques was significantly reduced following low-dose challenge with SHIV-MN, and early plasma viremia was markedly decreased after high-dose SHIV-MN challenge. In contrast, replication of pathogenic SHIV-89.6PD was not affected by vaccination in any of the macaques. Thus, we have shown that immunization with an rBCG Env V3 vaccine elicits a strong, type-specific V3 NAb response in rhesus macaques. While this response was not sufficient to provide protection against a pathogenic SHIV challenge, it was able to significantly reduce the viral load in macaques following challenge with a nonpathogenic SHIV. These observations suggest that rBCG vectors have the potential to deliver an appropriate virus immunogen for desirable immune elicitation.

Development of a preventive vaccine against human immunodeficiency virus type 1 (HIV-1) is urgently needed to control the spread of the virus worldwide. Although the immunological parameters that correlate with protective immunity against natural infection with HIV-1 are not fully known, it is assumed that a preventive vaccine must elicit potent, broadly reactive immunity against divergent strains of HIV-1 (25, 36, 42). Several recent studies have demonstrated that induction of virus-specific T-cell responses can confer protective immunity in nonhuman primate models, and these responses may also play a role in controlling HIV-1 replication in humans (6, 18, 19, 31, 33, 34, 38, 45, 48). Vaccine constructs containing viral *env* genes, in addition to *gag* and *pol*, have been shown to effec-

tively control replication of challenge viruses (2, 5, 10), suggesting that neutralizing antibody (NAb) responses might also contribute to protection against pathogenic infection or disease progression. Passive transfer of serum immunoglobulin from chimpanzees experimentally infected with several different HIV-1 isolates has been shown to block the establishment of a simian immunodeficiency virus (SIV)-HIV chimeric simian-human immunodeficiency virus (SHIV) infection in pig-tailed macaques (37, 46). It is not known, however, whether vaccines that actively elicit a potent NAb response can provide protection in nonhuman primates challenged with SHIV.

Previously, we demonstrated that recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG), which secretes a chimeric protein consisting of the V3-neutralizing epitope of HIV-1 and α -antigen (rBCG Env V3), can induce HIV-1-specific NAb in a small-animal model (9, 15, 16). BCG was selected as a vaccine vehicle because it has several characteristics that are considered efficacious for developing a candidate

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HIV-1 vaccine (1, 49), including the ability to induce long-lasting immune responses (7). It is generally accepted that a candidate vaccine against HIV-1 must also be easily administered and affordable in developing countries, and it must be compatible with other commonly administered vaccines (35). If effective, a BCG-based recombinant HIV-1 (rBCG-HIV-1) vaccine would fulfill many of these critical requirements.

Results using other vaccine modalities, in particular, live attenuated SIV vaccines, have raised concerns about the potential for reversion to pathogenicity (3, 4), suggesting that many SIV strains may be potentially virulent. In this study, we used two distinct strains of challenge virus: SHIV-MN (29), which contains V3 sequences homologous to rBCG Env V3, and SHIV-89.6PD (12, 20, 28, 41), which is heterologous in the V3 region and highly pathogenic. We examined whether vaccination with rBCG Env V3 could effectively elicit NAb responses in rhesus macaques and whether it might effectively induce protective immunity against challenge with either SHIV-MN or SHIV-89.6PD.

MATERIALS AND METHODS

Animals. The macaques (*Macaca mulatta*) used in this study originated from China and were purchased through Japan SLC Ltd., Shizuoka, Japan. The animals were maintained according to standard operating procedures established for the evaluation of human vaccines at the Tsukuba Primate Center, National Institute of Infectious Diseases, Tsukuba, Ibaragi, Japan. The study was conducted in the P3 facility for monkeys in the Murayama Branch, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan, and in accordance with requirements specified in the laboratory biosafety manual of the World Health Organization.

Construction of the rBCG Env V3 immunogen. rBCG substrain Tokyo was produced by transfection of BCG-Tokyo 172 cells with plasmid pSO246 as described previously (21, 22, 30). The XhoI site of this plasmid was used to insert a mycobacterial codon-optimized DNA fragment encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence (NTRKSIHIGPGRAFYTGS), which has a neutralization sequence identical to that of HIV-1_{MN} (16, 23, 39, 52). The resulting rBCG vector was designated rBCG Env V3. By semiquantitation of a chimeric protein consisting of the V3 peptides and α -K protein (9), the concentration of the secreted protein was estimated to range from 1 to 3 μ g/ml in the culture filtrate of rBCG Env V3 (data not shown).

Viruses. Viruses used in challenge experiments were kindly provided by Y. Lu, Harvard AIDS Institute, Cambridge, Mass. The SHIV-MN virus stock was prepared in concanavalin A-activated macaque peripheral blood mononuclear cells (PBMC) from normal animals, and the amount of virus was quantified by SIV p27 antigen enzyme-linked immunosorbent assay (ELISA) (Coulter Co., Hialeah, Fla.). The tissue culture infective dose (TCID₅₀) of the stock was measured on CEMx174 cells (AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, Md.). Stocks of HIV-1_{MN} and HIV-1_{IIB} (AIDS Research and Reference Reagent Program) were prepared by propagating 100 50% TCID₅₀ of each virus in phytohemagglutinin-activated normal human PBMC, as described previously (17). The primary isolate, HIV-1_{MNp}, was kindly provided by J. Sullivan, University of Massachusetts Medical School, Worcester, Mass. All other viruses were obtained from the AIDS Research and Reference Reagent Program. Cell-free virus stocks were stored at -130°C until they were used.

V3-specific ELISA. HIV-1 V3 peptide-based ELISAs were used for titration and quantification of serum antibodies for detection as described previously (14). In brief, 96-well ELISA plates (MaxiSorp; Nunc A/S, Roskilde, Denmark) were coated with 100 μ l of peptide MN (DKRIHIGPGRAFYT) /well in 50 mM carbonate buffer (pH 9.3) at 5 μ g/ml overnight at 4°C . The wells were washed and treated with 5% nonfat milk in phosphate-buffered saline for 1 h at 37°C . Duplicate samples containing either control or test macaque serum at appropriate dilutions were then added at 100 μ l/well, and the plates were incubated for 1 h at 37°C . The wells were washed and incubated with a detection antibody solution consisting of peroxidase-conjugated goat anti-monkey immunoglobulin G (IgG) antibody (EY laboratories Inc., San Mateo, Calif.) at 100 μ l/well for 1 h at 37°C . After final washes with 0.05% Tween-20-phosphate-buffered saline

(PBST), peroxidase substrate was added, and the reaction was stopped by the addition of 0.5 M H_2SO_4 .

IFN- γ ELISPOT assay. Enzyme-linked immunospot (ELISPOT) assays were performed using the method developed by Mothe and Watkins of the Wisconsin University Primate Center and described elsewhere (18, 33). In brief, 96-well flat-bottom plates (U-CyTech-BV, Utrecht, The Netherlands) were coated with anti-gamma interferon (IFN- γ) monoclonal antibody before being washed with PBST and blocked with bovine serum albumin. Freshly isolated PBMC were mixed with either concanavalin A or 2 μ M V3 peptide and were then incubated for 16 h at 37°C in 5% CO_2 in anti-IFN- γ -coated plates. Once the plates had been washed, rabbit anti-IFN- γ polyclonal biotinylated detector antibodies were added, and the plates were incubated. Gold-labeled anti-biotin IgG solution (U-CyTech-BV) was added to the plates after they were washed with PBST. The plates were then incubated for 1 h at 37°C . Developed wells were imaged, and spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Carl Zeiss, Oberkochen, Germany). An SFC was defined as a large black spot with a fuzzy border (33).

In vitro virus neutralization assays. GHOST cell neutralization assays were performed as previously described (8). Briefly, GHOST cells expressing either CXCR4 or CCR5 were used as targets for HIV-1 infection (50, 54). The cells were analyzed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, Calif.), and 15,000 events were scored. The mean number of fluorescent GHOST cells determined from negative controls plus 2 standard deviations was considered the cutoff for a positive sample. Purified human immunoglobulin (Nihon Pharmaceutical Co., Tokyo, Japan) and saline were included as additional controls.

M8166 cell-based virus neutralization assays were also performed as described previously (16, 47). In brief, the in vitro neutralization activity of purified macaque IgG was determined using 100 TCID₅₀ of either HIV-1_{MN} or SHIV-MN in cultures of M8166 cells. The results were compared with parallel cultures to which preimmune serum IgG was added. Neutralization was expressed as percent inhibition of HIV-1 p24 or SIV p27 antigen production in the culture supernatants. Purified normal macaque IgG was used as a control.

Quantification of cell-associated viral load. Levels of cell-associated virus were quantified by limiting dilution of PBMC (from 10^6 to 1 cells), and the virus was cocultured with M8166 cells as described previously (17). Virus released into the culture supernatant was measured by SIV p27 antigen ELISA (Coulter). The smallest number of PBMC required to produce a positive culture was considered the end point, and the titer of infectious virus was expressed as TCID₅₀ per 10^6 PBMC.

PCR detection of proviral HIV-1 infection of rhesus macaques. PBMC with SHIV were detected by DNA PCR using a primer pair that spans the C2-V3 sequence of HIV-1_{IIB}, followed by Southern blotting with an SE1 probe, 5'-G CAGAAGAAGAGGTAGTAATTAGAT-3' (nucleotides 7019 to 7043) (47). The positions of the oligonucleotides are numbered relative to the HIV-1_{HXB2} isolate in the ENTREZ database (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.). Viral DNA was quantified by comparison with standards derived from 8E5/LAV cells, which contain one copy of HIV-1 proviral DNA per cell (AIDS Research and Reference Reagent Program).

Competitive PCR quantitation of SHIV RNA in plasma. Quantitative, competitive reverse transcription-PCR was performed using a competitor RNA and a DNA template as previously described (18, 32, 44). The detection limit of this assay was 500 RNA copies/ml in monkey plasma (18, 32).

Sequencing of HIV-1 Env C2-V3 sequence. To determine the sequence of the HIV-1 Env C2-V3 region, mRNA was extracted from stock virus and cDNA was synthesized using a Micro-FastTrack version 2.0 kit (Invitrogen, Carlsbad, Calif.) and a cDNA cycle kit (Invitrogen) according to the manufacturer's instructions. The PCR products were cloned into a pCR II vector with a dual promoter using a TA cloning kit (Invitrogen) (47). Sequence analysis was performed using a Big Dye terminator cycle-sequencing FS kit (Perkin-Elmer, Foster City, Calif.) and automated ABI 310 sequencer (Perkin-Elmer) with Sp6 and T7 sequence primers (Invitrogen). Sequence data were compared with published HIV-1 sequences in GenBank (National Center for Biotechnology Information, National Institutes of Health).

Statistical analysis. Calculations of the geometric mean \pm standard deviation (SD) were carried out with a microcomputer. Significance was defined as a *P* value of <0.05 .

RESULTS

Vaccination protocol. Twenty-four male rhesus macaques (R-01 through R-24) were enrolled in the study. Of these, 15 were subcutaneously immunized for 24 weeks with 10 mg of

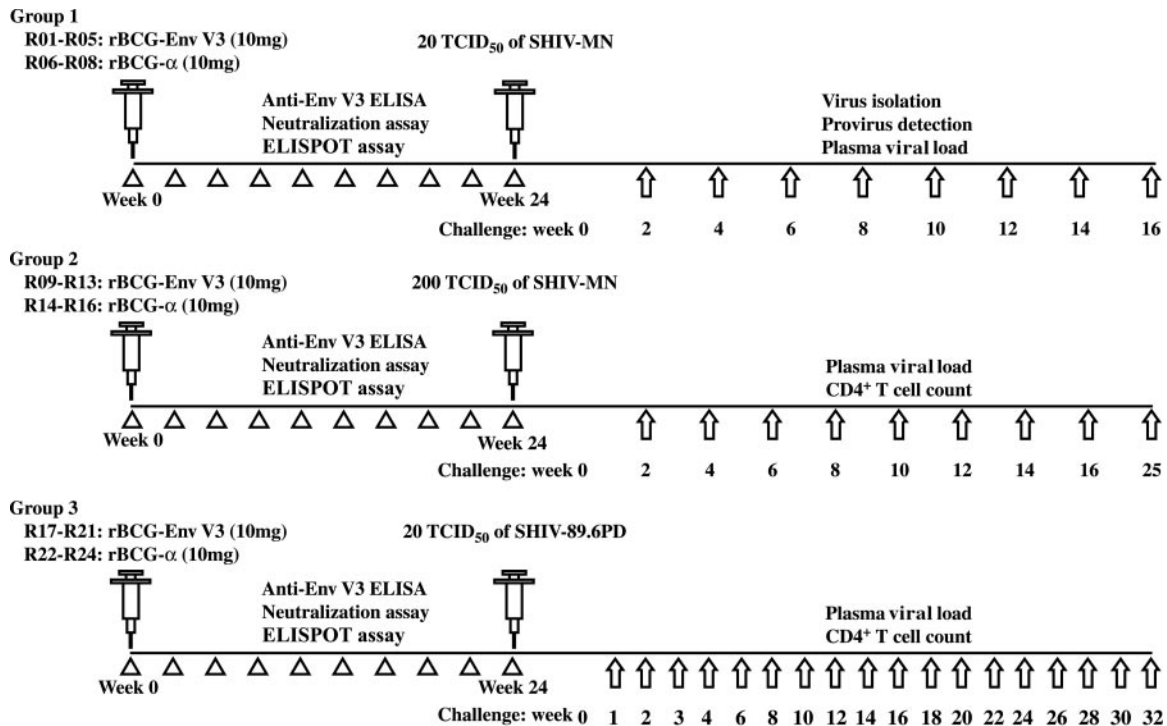


FIG. 1. Schematic representation of the experimental protocol for immunization of rhesus macaques with rBCG Env V3 and challenge with either SHIV-MN or SHIV-89.6PD. A total of 24 macaques were assigned to either the rBCG Env V3 vaccine or rBCG vector control group. The animals each received a single subcutaneous injection and were then split into three groups prior to challenge with either low-dose SHIV-MN, high-dose SHIV-MN, or SHIV-89.6PD.

rBCG Env V3 (16), which expresses and secretes a chimeric protein consisting of α -antigen and the Env V3 region of HIV-1_{MN}. The remaining nine macaques were immunized by the same route and with the same dose of rBCG α -antigen and served as vector controls. All macaques inoculated with rBCG Env V3 remained in good health following vaccination. Three of the 15 immunized macaques experienced transient redness with slight erosion localized at the injection site; however, the reaction spontaneously resolved within 3 months. Following immunization, the 24 macaques were divided into three groups, each group consisting of five immunized animals and three vector controls. The macaques within each group received an intravenous challenge with either SHIV-MN (20 or 200 TCID₅₀) or SHIV-89.6PD (20 TCID₅₀) (Fig. 1).

Vaccine-induced HIV-specific immune responses following rBCG Env V3 immunization. (i) **Neutralizing antibodies.** As described above, 15 rhesus macaques were vaccinated with a single subcutaneous inoculation of 10 mg of rBCG Env V3. Induction of HIV-1-specific immunity was measured 24 weeks later in blood samples obtained pre- and postvaccination. All 15 immunized macaques exhibited HIV-1 Env V3 peptide-binding antibody activity by ELISA at serum dilutions ranging from 1:640 to 1:10,240 (Fig. 2). Antibody responses were monophasic, peaking at 4 to 6 weeks and then gradually declining. Serum samples obtained from naïve macaques were consistently negative by ELISA, while postvaccination sera did not react with a control fusion peptide of HIV gp41 (data not shown).

Antibodies were purified from the macaque sera to remove factors that might interfere with certain bioassays (51). The purified antibodies were then tested *in vitro* for the ability to neutralize SHIV-MN infection in M1866 cells (Fig. 3). Antibodies induced in macaques vaccinated with rBCG Env V3 strongly neutralized both the challenge SHIV-MN (grown in rhesus PBMC) and a T-cell line-adapted (TCLA) laboratory strain, HIV-1_{MN}. A mean 50% inhibitory concentration (IC₅₀) of 0.05 to 0.5 μ g of IgG/ml was measured against SHIV-MN, and a mean IC₉₀ of \sim 3.0 μ g of IgG/ml was observed against HIV-1_{MN}. Neutralizing activity was detected in serum samples obtained 4 to 6 weeks after vaccination and was maintained for at least 24 weeks. Preimmune serum IgG from nine macaques immunized with vector alone, and IgG from three additional naïve macaques (data not shown), did not neutralize either virus.

(ii) **Neutralization responses against primary HIV-1 isolates.** To further assess the specificity of antibodies in immune sera, neutralizing activity was evaluated against a panel of seven primary HIV-1 isolates using GHOST cells expressing either CCR5 or CXCR4 (Table 1). Purified IgG from macaques in each of the three immunization groups was able to effectively neutralize HIV-1_{BZ167/X4}, HIV-1_{SF2/X4}, and HIV-1_{CI2/X4} (Table 1 and Fig. 4), with mean IC₅₀ values of 5 to 7, 4 to 7, and 5 to 15 μ g/ml, respectively. By comparison, neutralization of HIV-1_{MNp/X4} required \sim 10-fold more serum IgG, with a mean IC₅₀ of 50 μ g/ml. Three additional isolates, HIV-1_{SF33/X4}, HIV-1_{SF33/R5}, and the clade A isolate HIV-1_{VI131/R5},

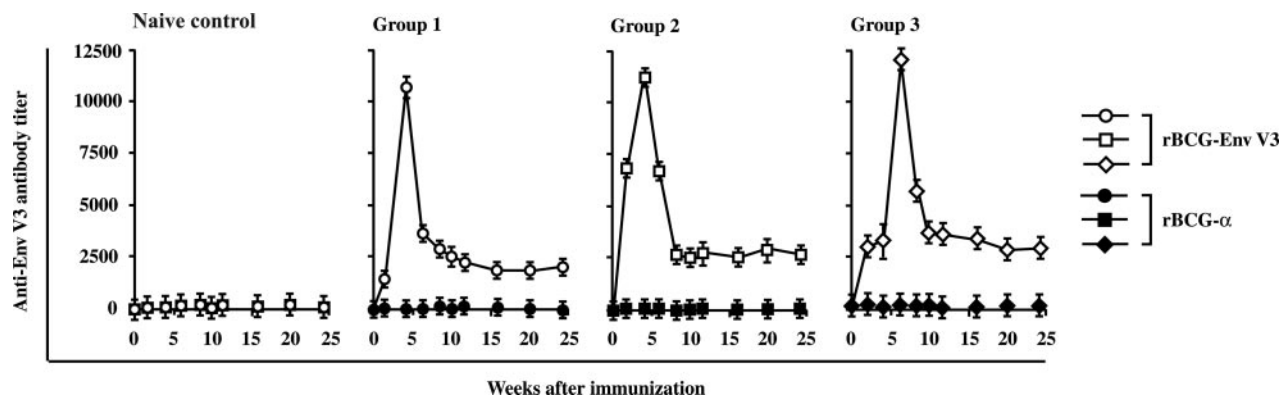


FIG. 2. Serum anti-V3 antibody titers determined by peptide-based ELISA. Preimmune and immune sera from macaques inoculated with rBCG Env V3 were collected and stored at -80°C until they were used. Sera from naïve macaques were used as controls. Data using preimmune sera were within the control levels (data not shown). The results are expressed as the means \pm SD of four independent assays.

were not neutralized with serum IgG concentrations up to 50 $\mu\text{g/ml}$ (Table 1). Preimmune sera had no neutralizing activity against any of the isolates. Thus, antibodies present in sera from the immunized macaques were able to neutralize primary HIV-1 isolates, including HIV-1_{BZ167}, HIV-1_{SF2}, and HIV-1_{CI2}, in assays using GHOST cells that express CXCR4 with 10- to 50-fold-higher sensitivity than that of the dual-tropic (X4-R5) TCLA strain HIV-1_{MNP}. Among the neutralization-sensitive viruses, the V3 sequence motifs of HIV-1_{BZ167} and

HIV-1_{SF2} shown in Fig. 5 did not correlate with the observed neutralization profiles of HIV-1 Env V3.

(iii) **V3 peptide-specific T-cell responses.** Table 2 offers a comparison of the virus-specific T-cell response levels determined by IFN- γ ELISPOT analysis in immunized animals with the neutralization data provided in Fig. 2. Of the 15 animals immunized with rBCG Env V3 (180 and 160 SFC/ 10^6 PBMC at 6 weeks postimmunization [p.i.], respectively), only R-09 and R-10 showed very low levels of SFC activities at the time of

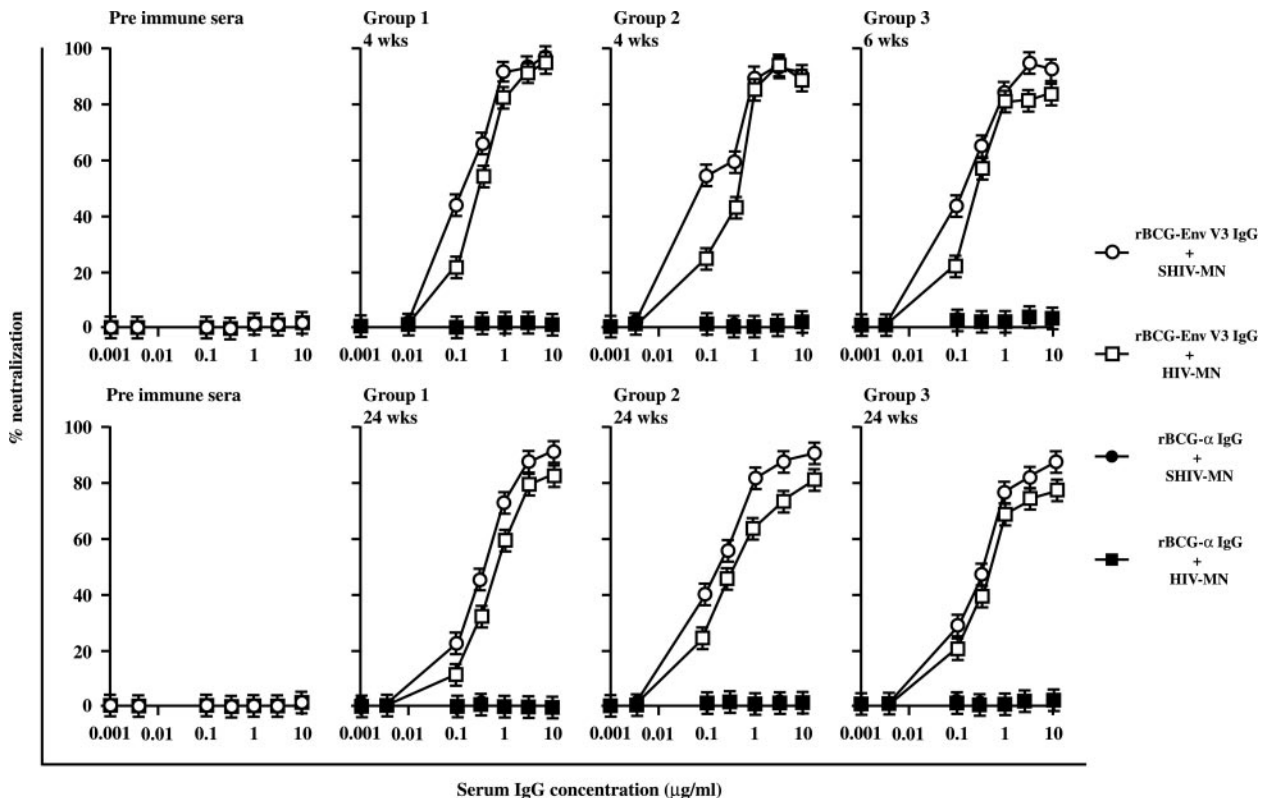


FIG. 3. HIV-1-specific neutralization antibody responses in macaques vaccinated with rBCG Env V3. Analysis of in vitro neutralization of SHIV-MN by anti-rBCG-HIV-1 antibodies using M8166 cell-based virus neutralization assays. Serum IgG was purified from preimmune or immune sera of macaques inoculated with rBCG Env V3 at the indicated times. The results are expressed as the means \pm SD of four independent assays.

TABLE 1. 50% neutralization calculated on the basis of neutralization curves^a

Serum sample	Neutralizing activity (μg)						
	BZ167/X4	MNp/X4	SF2/X4	SF33/X4	SF33/R5	VI1313/R5	C12/X4
Group 1	6.5	50	7	>50	>50	>50	10
Group 2	5	50	4	>50	>50	>50	5
Group 3	7	50	6.5	>50	>50	>50	15
Pre immunization sera of groups 1, 2, and 3	>50	>50	>50	>50	>50	>50	>50

^a The neutralization assays with the various viruses were carried out in GHOST cells expressing either CXCR4 (X4) or CCR5 (R5) as indicated in Fig. 4. BZ167, MNp, SF2, SF33, and CI-2 are HIV-1 clade B viruses. VI1313 is an HIV-1 clade A virus.

SHIV challenge (120 and 110 SFC/10⁶ PBMC at 24 weeks p.i., respectively) (Table 2). In contrast, <100 SFC/10⁶ PBMC were observed in other immunized animals, and <20 SFC/10⁶ PBMC were observed in controls. Thus, the V3 region antigen in the rBCG Env V3 proved unable to induce significant levels of virus-specific T-cell responses in immunized animals.

Challenge with low-dose SHIV-MN. The first group of eight macaques (R-01 through R-08), consisting of five animals that received rBCG Env V3 and three that received control rBCG α-antigen, were intravenously challenged with low-dose SHIV-MN (20 TCID₅₀) at 24 weeks p.i. The cell-associated virus load was measured in PBMC cocultures, and proviral copy numbers were estimated by DNA PCR using primary PBMC genomic DNA. The level of plasma viremia in each macaque was quantified by competitive reverse transcription-

PCR to assess infection and virus replication for 16 weeks after virus challenge (Table 3).

Control macaques vaccinated with the vector alone (R-06 through R-08) were positive in all three viral-load assays 2 weeks after SHIV-MN challenge and remained positive for a follow-up period of 10 weeks. Because only low levels of viral RNA (<10⁴ RNA copies/ml) were transiently detected 2 weeks postchallenge, all three assays (virus isolation, plasma RNA, and proviral DNA) were used for virus detection. Using these criteria, we observed that all three parameters remained negative after low-dose SHIV-MN challenge in three of five macaques vaccinated with rBCG Env V3 (R-02, R-04, and R-05). However, macaque R-01 was transiently positive in all three assays for virus infection at 4 weeks. Another macaque immunized with rBCG Env V3 (R-03) exhibited a sharp in-

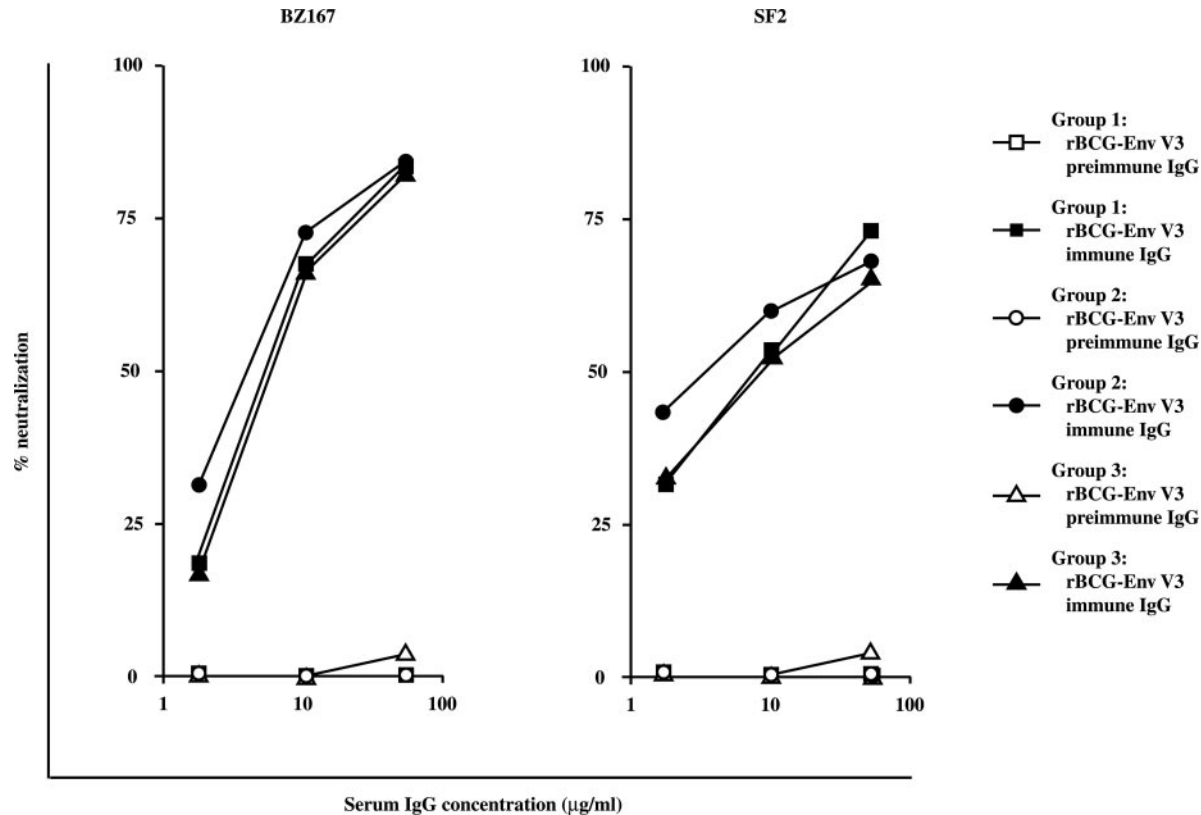


FIG. 4. Neutralization of HIV-1_{BZ167} and HIV-1_{SF2} in GHOST-X4 cells by immune sera from macaques vaccinated with rBCG Env V3. Dilutions of immune sera (closed symbols) and preimmune sera (open symbols) were tested in duplicate, and the percent neutralization was calculated using the mean value. The dose-response curves represent the means of three independent assays.

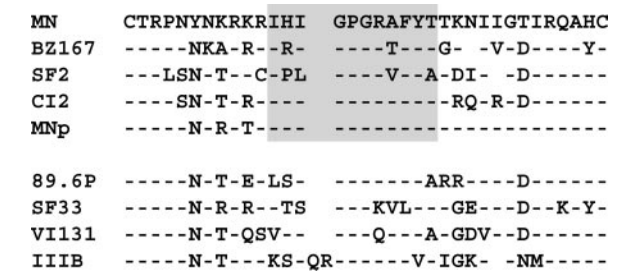


FIG. 5. Alignment of the amino acid sequences of HIV-1 Env V3 from primary and laboratory isolates. The spaces indicate amino acid deletions; dashes indicate homology. The V3 motif of a neutralization-sensitive HIV-1 strain is enclosed in the shaded rectangle (37).

crease in viral load following challenge, and the levels remained high until the animal was sacrificed. These results demonstrate that vaccination with rBCG Env V3 can induce protective immunity in rhesus macaques against a low-dose challenge with SHIV-MN.

Challenge with high-dose SHIV-MN. The second group of eight macaques (R-09 through R-16) was similarly challenged with a higher dose (200 TCID₅₀) of SHIV-MN by intravenous inoculation at 24 weeks p.i. (Fig. 6). Measurements of the viral loads in PBMC and plasma indicated that all the macaques were infected by the high-dose SHIV-MN challenge. However, the level of viremia during the acute phase of viral infection

was reduced by 1 to 2 log units in macaques immunized with rBCG Env V3 compared with controls (from 10⁶ to 10⁷, to <10⁵ to 10⁴ RNA copies/ml) (Fig. 6A). The control macaques developed a transient decrease in CD4⁺-T-cell counts that rebounded to normal levels ~3 weeks postchallenge (Fig. 6B). In contrast, macaques vaccinated with rBCG Env V3 had little or no change in CD4⁺-T-cell numbers.

Despite the low levels of V3 peptide-specific IFN-γ ELISPOT activities noted for animals R-09 and R-10 above (Table 2), these animals exhibited a plasma viral load and a rate of CD4⁺-cell loss after SHIV challenge that was comparable to those seen in the immunized animals designated R-11, -12, and -13. Thus, immunization with rBCG Env V3 generated even low levels of T-cell responses in only 2 animals out of 5 in this group and out of a total of 15 immunized animals. No evidence of higher virus-specific IFN-γ ELISPOT activity was demonstrated in samples obtained 0, 4, or 6 and 24 weeks after vaccination (Table 2), suggesting that few significant cellular anti-SHIV responses were generated and that those few did not affect virus control in this macaque population.

Challenge with pathogenic SHIV-89.6PD. The third group of macaques (R-17 through R-24) was challenged with pathogenic SHIV-89.6PD (20 TCID₅₀) 24 weeks postinoculation. The effects of vaccination with rBCG Env V3 on immune induction against the pathogenic virus were followed for 32 weeks, and the macaques were then autopsied. As shown in

TABLE 2. SHIV-MN-specific serum IgG neutralization titers and Env V3-specific ELISPOT responses^a

Monkey no.	Immunogen	IC ₅₀ of neutralization serum IgG (μg/ml) ^b			V3-specific IFN-γ SFCs/10 ⁶ cells ^c		
		0 week	4 or 6 weeks ^d	24 weeks ^e	0 week	4 or 6 weeks	24 weeks
R-01	rBCG Env V3	>50	0.5	0.6	<20	30	20
R-02	rBCG Env V3	>50	0.3	0.4	<20	40	40
R-03	rBCG Env V3	>50	0.5	0.6	<20	40	30
R-04	rBCG-Env V3	>50	0.2	0.3	<20	20	40
R-05	rBCG-Env V3	>50	0.08	0.3	<20	30	80
R-06	rBCG-α	>50	>50	>50	<20	<20	<20
R-07	rBCG-α	>50	>50	>50	<20	<20	<20
R-08	rBCG-α	>50	>50	>50	<20	<20	<20
R-09	rBCG-Env V3	>50	0.04	0.3	<20	180	120
R-10	rBCG-Env V3	>50	0.1	0.2	<20	160	110
R-11	rBCG-Env V3	>50	0.05	0.2	<20	20	30
R-12	rBCG-Env V3	>50	0.03	0.4	<20	60	20
R-13	rBCG-Env V3	>50	0.02	0.4	<20	30	30
R-14	rBCG-α	>50	>50	>50	<20	<20	<20
R-15	rBCG-α	>50	>50	>50	<20	<20	<20
R-16	rBCG-α	>50	>50	>50	<20	<20	<20
R-17	rBCG-Env V3	>50	0.2	0.6	<20	40	90
R-18	rBCG-Env V3	>50	0.3	0.3	<20	50	60
R-19	rBCG-Env V3	>50	0.3	0.4	<20	40	30
R-20	rBCG-Env V3	>50	0.5	0.7	<20	20	50
R-21	rBCG-Env V3	>50	0.4	0.5	<20	20	40
R-22	rBCG-α	>50	>50	>50	<20	<20	<20
R-23	rBCG-α	>50	>50	>50	<20	<20	<20
R-24	rBCG-α	>50	>50	>50	<20	<20	<20

^a Animals were inoculated with either rBCG Env V3 or the vector control. Blood samples were obtained at 0, 4, or 6 and 24 weeks p.i., and antibody inhibitory concentration and the V3-specific IFN-γ ELISPOT activity were compared.

^b The IC₅₀ was derived from the data in Fig. 2 based on neutralization dose-response curves similarly obtained from Fig. 3.

^c Freshly isolated PBMC were assessed for their ability to produce IFN-γ in response to HIV-1_{MN} Env V3 peptide.

^d Mean IC₅₀s: R-01 to R-05, 0.32; R-09 to R-13, 0.05; R-17 to R-21, 0.35.

^e Mean IC₅₀s: R-01 to R-05, 0.44; R-09 to R-13, 0.30; R-17 to R-21, 0.50.

TABLE 3. Comparison of low-dose SHIV-MN infections in macaques vaccinated with either rBCG Env V3 or rBCG- α (control)

Monkey	Immunogen (10 mg)	Efficacy analysis	Results ^a							
			0 ^b	2	4	6	8	10	12	16
R-01	rBCG Env V3	Virus isolation	<1	<1	2	<1	<1	<1	<1	<1
		Provirus by PCR	<500	<500	>500	<500	<500	<500	<500	<500
		Plasma viral load	<500	<500	20,000	<500	<500	<500	<500	<500
R-02	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	<1	<1
		Provirus by PCR	<500	<500	<500	<500	<500	<500	<500	<500
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-03	rBCG Env V3	Virus isolation	<1	32	<1	<1	2	<1	ND	ND
		Provirus by PCR	<500	>500	<500	<500	>500	<500	ND	ND
		Plasma viral load	<500	310,000	<500	<500	20,000	<500	ND	<500
R-04	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	ND	ND
		Provirus by PCR	<500	<500	<500	<500	<500	<500	ND	ND
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-05	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	<1	ND
		Provirus by PCR	<500	<500	<500	<500	<500	<500	<500	ND
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-06	rBCG- α	Virus isolation	<1	32	16	<1	2	2	<1	1<
		Provirus by PCR	<500	>500	>500	>500	>500	>500	>500	500
		Plasma viral load	<500	300,000	50,000	20,000	20,000	20,000	20,000	20,000
R-07	rBCG- α	Virus isolation	<1	2	32	<1	<1	<1	<1	ND
		Provirus by PCR	<500	>500	>500	>500	>500	<500	<500	ND
		Plasma viral load	<500	27,000	310,000	350,000	25,000	<500	<500	<500
R-08	rBCG- α	Virus isolation	<1	32	16	<1	2	2	ND	ND
		Provirus by PCR	<500	>500	>500	>500	>500	>500	ND	ND
		Plasma viral load	<500	300,000	50,000	<500	20,000	20,000	ND	<500

^a Viral loads were determined by either limiting dilution of PBMC or competitive PCR for HIV-1 Env V3 genes, and the results are expressed as the number of infected cells per million PBMC and virus copies per milliliter of blood. Nested PCR for HIV-MN Env V3 was used in all macaques to detect the provirus genome. Naïve macaques were injected intravenously with 20 TCID₅₀ of SHIV-MN and used as controls for SHIV infection. The results are expressed as the mean of three different assays; <1, <500, and <500 were the detection limits of virus isolation, proviral copy number, and plasma viral load, respectively. ND, not determined.

^b Weeks after challenge.

Fig. 7, high levels of plasma viremia were detected in the control macaques, with a viral set point of $\sim 10^6$ RNA copies/ml, accompanied by an abrupt decline in CD4⁺-T-cell counts. Prior vaccination with rBCG Env V3 appeared to have no positive effect on the viral load and CD4⁺-T-cell counts compared with the control animals.

Association of in vitro neutralization antibody responses following rBCG Env V3 immunization with control of viremia after SHIV challenge. Of the macaques challenged with low doses of homologous SHIV-MN (group 1), the three virus-controlled macaques R-02, -04, and -05 (Table 1) had higher IC₅₀s of SHIV-MN-specific neutralizing antibodies as measured in M8166 cells at 24 weeks p.i. or on the day of challenge, with serum IgG concentrations of 0.4, 0.3, and 0.3 μ g/ml, respectively (Table 2). The IC₅₀s of the uncontrolled macaques R-01 and -03 (Table 1) were both 0.6 μ g/ml (Table 2).

When the challenge dose was increased 10-fold (Fig. 1), all five animals in group 2 had high neutralizing antibody titers with a mean IC₅₀ of 0.30 μ g/ml on the day of challenge (Table 2). These animals in group 2 showed partial protection against the same homologous virus challenge (Fig. 6). In contrast, no animals similarly immunized with rBCG elicited any in vivo protection against a low-dose, heterologous viral challenge with SHIV-89.6PD (Table 2 and Fig. 7).

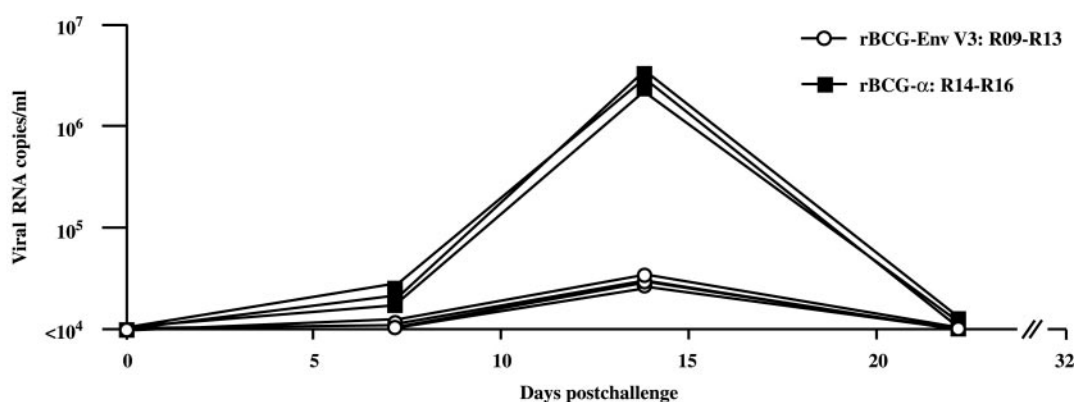
In summary, the rBCG Env V3-elicited NAb response afforded some degree of protection against a homologous viral challenge. However, infection by the heterologous virus SHIV-89.6PD was not controlled by heterologous virus SHIV-MN- or HIV-1_{MN}-specific NAb generated by the recombinant HIV-1_{MN} Env V3-expressed BCG immunization.

DISCUSSION

First, our study demonstrates the potential of anti-Env V3 NAb induced by immunization of rhesus macaques with rBCG Env V3 to afford protection against homologous challenge with SHIV-MN but not against the heterologous SHIV-89.6PD. With the low-dose homologous SHIV-MN challenge (20 TCID₅₀), sterile protection was achieved in three of five immunized animals. These findings correlate well with our in vitro neutralization data for these animals. Protected animals showed higher levels of potent neutralization antibodies than did unprotected animals. Macaques serving as vector and naïve controls experienced high levels of replication of the SHIV-MN challenge virus. With a high-dose challenge, rBCG Env V3 vaccination was effective at reducing viremia during acute infection by ~ 100 -fold. The vaccine consisted of an rBCG vector that expresses a chimeric HIV-1 Env V3 region peptide and the α -antigen of *M. bovis*. The kinetics and magnitude of the HIV-1 Env V3-specific antibody responses elicited in macaques were comparable to those observed in our previous studies using guinea pigs vaccinated with rBCG Env V3 (9, 16).

Secondly, the levels of neutralizing antibodies generated after injection with a recombinant BCG vector-based vaccine expressing a chimeric protein of HIV-1 Env V3 peptide and α -antigen protein were maintained for at least 24 weeks p.i. with no diminishment in titer. A plausible explanation for the longevity of the neutralizing antibody titers after rBCG immunization is that the carrier protein, α -antigen (also known as MPT59 or antigen 85B), is derived from mycobacteria and has

A



B

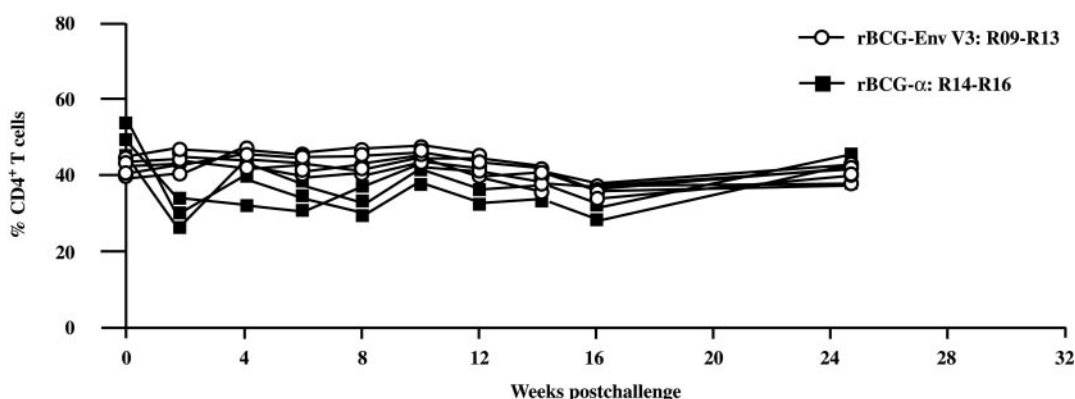


FIG. 6. Comparison of infection kinetics following high-dose (200 TCID₅₀) inoculation of SHIV-MN in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Viral RNA copy number per milliliter of serum. (B) CD4⁺-T-cell count as a percentage of total lymphocytes. The results in individual animals are expressed.

the ability to elicit potent Th1-type immune responses (24, 43). Our result is consistent with those of other groups, which have shown that BCG immunity is maintained for at least a few years and that the BCG bacillus is effective at increasing NAb responses (40). These characteristics might help to explain the long-lasting enhanced levels of NAb elicited by vaccination with rBCG Env V3.

The concentration of purified macaque IgG in serum was determined to be ~10 mg/ml. By this estimation, 0.5 mg corresponds to a serum dilution of 1:1 in virus neutralization assays. The IC₅₀ and IC₉₀ values for neutralization of SHIV-MN were 10³ to 10⁴ and 166, respectively (similar values were obtained for neutralization of HIV-1_{MN}). These neutralization titers suggest that antibody responses generated *de novo* may contribute to a degree of protection against SHIV-MN. The observed relationship of the NAb titer and viral protection is consistent with results obtained by repeated immunization with SHIV-89.6 C4-V3 peptides in guinea pigs and rhesus macaques (6, 27). In this case, NAb titers to homologous SHIV-89.6 were ~10³ greater than those against heterologous HIV-1_{MN}, while responses to HIV-1 R5 viruses were weak or absent. This suggests that the protection mediated by a C4-V3 peptide vaccine against SHIV-89.6 may be type (or strain) specific. Thus, we assume that the NAb generated by

SHIV-89.6 C4-V3 peptide immunization (6) would not mediate protection against a heterologous SHIV-MN challenge.

The present study suggests that the vaccine-elicited antibodies directed against the HIV-1 Env V3 peptide can in some cases confer a degree of neutralization against primary isolates of HIV-1 (26). Following vaccination of rhesus macaques with rBCG Env V3, both binding and NAb responses against this novel construct were clearly evident. At the time of SHIV challenge, immune sera from the vaccinated macaques efficiently neutralized a homologous, type-specific TCLA HIV-1 strain (HIV-1_{MN}) and a related SHIV strain (SHIV-MN) with IC₉₀ values of <5 µg/ml. Controls, including preimmune sera and sera from macaques vaccinated with rBCG vector alone, had no neutralizing activity in assays using GHOST cells expressing either CCR5 or CXCR4 or in M8166 cells. Immune sera from macaques vaccinated with rBCG Env V3 were able to neutralize several primary HIV-1 X4 isolates (HIV-1_{BZ167}, HIV-1_{SF2}, and HIV-1_{CI2}); however, neutralization of an X4-R5 dual-tropic strain (HIV-1_{MNP}) was weak. No neutralization of HIV-1 R5 isolates and primary HIV-1 isolates from different clades was observed. These findings were confirmed in an independent international neutralization trial (conducted by Simon Beddows and Jonathan Weber, Imperial College School of Medicine, Medical Research Council, London, En-

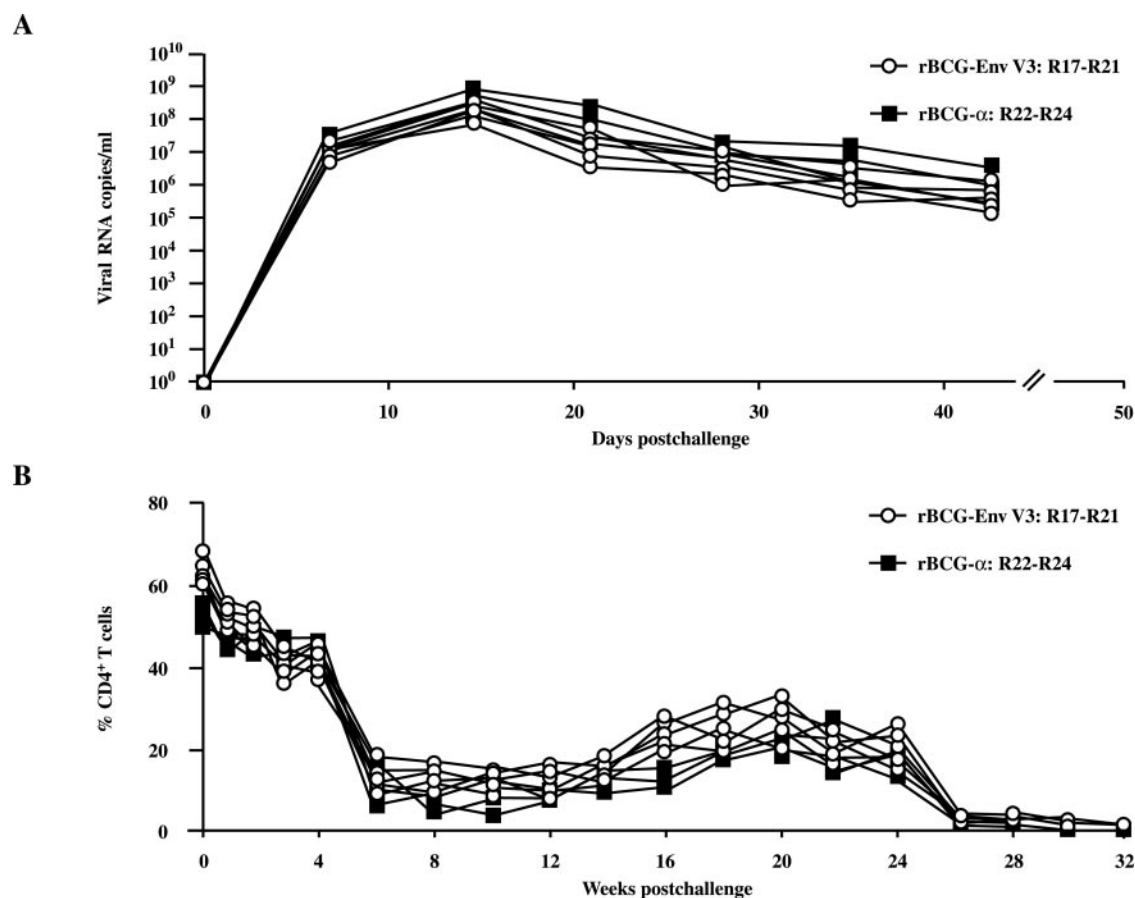


FIG. 7. Comparison of infection kinetics following challenge with pathogenic SHIV-89.6PD in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Plasma viral-RNA copy numbers per milliliter. (B) CD4⁺-T-cell count as a percentage of total lymphocytes. The results in individual animals are expressed.

gland, and Pia Scott and Eva-Maria Fenyo at Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden). Preliminary results from this study have had been summarized and reported (11). Despite similarities in the V3 sequence motif, neutralization of the TCLA strain HIV-1_{MN} was found to be 10- to 50-fold more sensitive than neutralization of primary HIV-1 isolates, such as HIV-1_{CI2}, HIV-1_{MNP}, or HIV-1_{JR-CSF} (11). A reasonable explanation for the relative insensitivity of primary HIV-1 isolates—particularly primary HIV-1 R5 isolates—to neutralization is the presence of cryptic or occluded sites within the virus-associated V3 region (13, 53).

In the Japanese consensus HIV-1 Env V3 expressed in the rBCG construct, the core V3 motif of the neutralization epitope is IHIGPGRAF (39). Although the consensus sequence of the V3 loop differs from the MN-V3 sequence in five amino acid positions, the neutralization epitope of the tip V3 region in the Japanese consensus is identical to that of MN-V3. Some substitutions of amino acids at certain positions within this motif (for example, H to R and A to T in the core motif in BZ167) are tolerated, suggesting that NAbS generated by immunization with rBCG Env V3 are not strictly type specific. Immune sera from macaques vaccinated with rBCG Env V3 were able to neutralize primary HIV-1 X4 and some HIV-1 X4-R5 dual-tropic isolates, suggesting that the antigenic struc-

ture of the chimeric V3 peptide mimics to some extent that of the virus-associated V3 region. Indeed, the chimeric V3- α -antigen protein is estimated to be 38 kDa and contains four cysteine residues, suggesting the possible formation of a new loop structure in the V3 portion of the protein. With regard to the heterologous SHIV-89.6PD challenge in macaques vaccinated with rBCG Env V3, NAbS specific for SHIV-89.6PD were not generated efficiently (IC_{50} , >50 μ g of immune serum IgG/ml) and did not provide any protection against the SHIV-89.6PD challenge. The V3 neutralization site of SHIV-89.6PD may differ in sequence or structure or both from that of SHIV-MN or other viral strains, including some of the HIV-1 isolates, making it unrecognizable to antibodies. Such a difference could account for the poor cross-neutralization activity against SHIV-89.6PD.

Thus, our data from the SHIV-macaque models show that the in vitro neutralization titers generated in rBCG-immunized animals correlate with protection. Although a present goal of HIV-1 vaccine development is to reduce the viral set point by eliciting high levels of virus-specific cellular immune responses, induction of cross-reactive NAbS may also contribute to control virus replication in the course of HIV-1 infection and may therefore be useful in the context of a preventive vaccine. Furthermore, although the choice of HIV Env V3 and the

autologous challenge virus SHIV-MN are unlikely to provide information that predicts efficacy in humans, the results presented here demonstrate that recombinant BCG vectors have the potential to deliver a more appropriate immunogen for desirable immune elicitation.

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