lymphatic choriomeningitis viral infection (47) and CMV immunity in bone marrow transplant recipients (48). DNA/poxvirus prime-boost vaccination induced a high frequency and a high avidity of $CD8^+$ cytotoxic T lymphocyte populations (49), with the magnitude of HIV/SIV-specific $CD4^+$ and/or $CD8^+$ T lymphocyte responses in the course of infection inversely correlating with the viral load (50, 51). In addition, MHC class I molecules loading CTL epitopes may help control viral replication (52–56). The exact mechanisms underlying protective immune responses against HIV-1 remain a subject of debate; however, the above studies suggest that the simultaneous induction after vaccination of both Ag-specific $CD8^+$ and $CD4^+$ T cell responses may make it possible to attenuate immunodeficient viral infection. In this study we showed the efficacy of the prime-boost vaccination by monitoring IFN-γ ELISPOT, intracellular IFN-γ, and Ab responses. In the prime-boost group, boosting with rDIsSIV*gag/pol* induced ELISPOT responses (average of 1209 SFC) almost 10-fold higher than those induced by SIV*gag/pol* DNA (average of 154 SFC). In addition, intracellular IFN-γ staining revealed that the prime-boost vaccination generated high levels of Gag-specific intracellular IFN-γ-producing $CD8^+$ T cells (average, 0.82%; range, 0.51–1.22%) as well. However, lower Gag-specific T cell responses were observed in macaques vaccinated with either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone than with the prime-boost regimen. In contrast to the strong Gag-specific T cell responses generated by the prime-boost vaccination, humoral responses specific for the same Ag were apparently low throughout the course of immunization. Although the peak IgG titers in the prime-boost group were observed after the first or second boosting with rDIsSIV*gag/pol*, Ab titers remained low. These results are in line with our previous study using the mouse model (23), suggesting that our prime-boost vaccine immunodominantly generates SIV Gag-specific cellular responses in macaques.

Monitoring ELISPOT and intracellular IFN-γ T cell responses specific for Gag revealed that responses decreased at the time of challenge with pathogenic SHIV, but then rapidly recovered. Gag-specific IFN-γ ELISPOT responses in the prime-boost group averaged 288 SFC on the day of challenge and increased to 1124 SFC on day 3 after challenge. The population of intracellular IFN-γ-producing $CD8^+$ and $CD4^+$ T cells specific for Gag also increased from an average of 0.32 to 0.61% and from an average of 0.11 to 0.38%, respectively, suggesting that our prime-boost vaccine generated a high frequency of very responsive $CD4^+$ and $CD8^+$ memory T cells that immediately reactivated sufficient levels of the Ag-specific immune responses against the SHIV Ag. Furthermore, a kinetic study of plasma viral loads and counts of $CD4^+$ T cells after challenge with SHIV revealed different patterns for each group. Although peak plasma viral loads were observed 2 wk after challenge in all groups, the number of plasma RNA copies peaking at that time in the prime-boost group were ~5-fold lower than in other groups, with numbers remaining depressed during the period extending from 7 to 30 wk after infection. However, high $CD4^+$ T cell counts were maintained in the prime-boost group. These results suggest a correlation between both plasma viral loads and the maintenance of high $CD4^+$ T cell counts and T cell response levels.

With regard to safety of vaccinia DIs as a vaccine vector, its viral replication occurs only in chick embryo fibroblasts, not in any mammalian cell lines tested (22, 24–26, 57). Because a vaccine regimen combining DNA and a defective DIs vector would not run the risk that the virus used as vector might replicate and disseminate, it would pose less of a risk to a severely immunocompromised host. Furthermore, in this study using the macaque model, we demonstrated that the cellular immune responses generated by the prime-boost vaccination were higher than those induced by vaccination with either DNA or rDIs alone and that response levels correlated to plasma viral loads and $CD4^+$ T cell counts after challenge with pathogenic SHIV. In summary, these results demonstrate that the new prime-boost regimen safely and effectively elicits anti-immunodeficiency viral immunity, suggesting its promise as a potential vaccine against HIV-1 infection as well as against HIV-induced disease progression.

Current macaque models of HIV, SIV, and SHIV may fall short of precisely mirroring human HIV infection. In some macaque HIV/AIDS models, SIVmac239 has been targeted as a desirable challenge virus, because it is a typical CCR5-tropic SIVmac and can cause both chronic and progressive disease in macaques (41, 58, 59). However, the virus is very difficult to neutralize and also very difficult to clear even from animals that have been previously immunized with homologous recombinant vector-based vaccines (41, 58, 59). Only live attenuated SIV has been reported to control SIVmac239 (T. Allen, Global HIV Vaccine Enterprise Meeting, Washington, October 21, 2004). Although there may be no macaque model suitable for evaluating the efficacy of an SIV or HIV experimental immunogen, in this study we clearly showed that vaccination with an SIV experimental immunogen consisting of SIV*gag/pol* DNA and replication-defective rDIsSIV*gag/pol* caused a pronounced attenuation of the infection caused by a highly pathogenic variant of SHIV-C2/1 in all five macaques tested. SHIV-C2/1, used as challenge virus, is a variant of SHIV 89.6. Because SHIV89.6 does not induce both a marked decline in $CD4^+$ cells and a high level of plasma viral load in cynomolgus macaques, we passaged serum from virus-infected cynomolgus macaques. The variant was obtained by the serum passages using cynomolgus macaques inoculated with SHIV89.6, and it induced high levels of viremia ($1–10 \times 10^7$ viral RNA copies/ml) and marked $CD4^+$ T cell depletion (<10 cells/μl) within 2 and 3 wk after viral inoculation (30, 31, 39). Furthermore, genomic study revealed 16 mutations of genomic DNA and 15 amino acid mutations in the Env region of parental virus. Thus, the cynomolgus AIDS model challenged with SHIV-C2/1 may represent primary HIV-1 infection in humans. These results should prove useful in determining how potent the new prime-boost vaccine regimen might be at eliciting anti-immunodeficiency virus immunity.

HIV-1 has been reported to preferentially infect $CD45RO^+CD4^+$ T cells in the early stages of infection, with the number of $CD45RA^+CD4^+$ T cells declining in later stages (60–62). Furthermore, the loss of this subpopulation of $CD4^+$ T cells during the early phase of immunodeficiency virus infection correlates to disease progression (63, 64), whereas the low $CD45RA^+CD4^+$ T cell levels in the late stages of infection correlate with an increased risk of death (65–67). The levels of $CD4^+$ T cells expressing the $CD28^+$ molecule have also been demonstrated to correlate with disease progression (68, 69). To confirm the effect of prime-boost immunity after SHIV challenge, we analyzed the kinetics of $CD4^+$ T cells expressing $CD29^+$, $CD45RA^+$, and $CD28^+$ molecules. We observed that the prime-boost group maintained the subpopulations of $CD4^+$ T cells throughout the course of infection, with an average of 8.82% $CD29^+$ cells, 7.59% $CD45\ RA^+$ cells, and 6.75% $CD28^+$ cells. In contrast, $CD4^+$ T cell populations in the other DNA and rDIs groups were reduced to <3%. These results suggest that immunization with the new prime-boost regimen induces protective immunity while maintaining the levels of the various $CD4^+$ T cell subpopulations.

In summary, our study has shown that the vaccine strategy that primes with DNA and then boosts with the replication-defective

vaccinia virus DIs generates both $CD4^+$ and $CD8^+$ T cell responses specific for SIV Gag, resulting in protection of the immunized macaques from pathogenic SHIV. However, it remains to be elucidated whether the *gag/pol*-encoding vaccine may elicit a protective effect against various viral challenges, such as CCR5-tropic viruses and other primary viruses. Nonetheless, this new regimen's twin merits of safety and efficacy position it as a promising vaccine candidate against HIV-1 infection as well as against HIV-induced disease progression.

Acknowledgments

We thank Dr. Naoto Yoshino (Iwate Medical University, Iwate, Japan) for technical support and advice.

Disclosures

The authors have no financial conflict of interest.

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JOURNAL OF VIROLOGY, Feb. 2005, p. 1452–1462
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.3.1452–1462.2005

Vol. 79, No. 3

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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Received 25 June 2004/Accepted 23 September 2004

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 elicitations.

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 effectively 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 (NTRKSIHIGPGRAFYATGS), 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 μg/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 (TCID) 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_{IIIB} (AIDS Research and Reference Reagent Program) were prepared by propagating 100 50% TCID ($TCID_{50}$) 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 μl of peptide MN (DKRIHIGPGRAFYTT)/well in 50 mM carbonate buffer (pH 9.3) at 5 μg/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 μl/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 μl/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 μM 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 $TCID_{50}$ 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 $TCID_{50}$ 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_{IIIB}, 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 ± 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

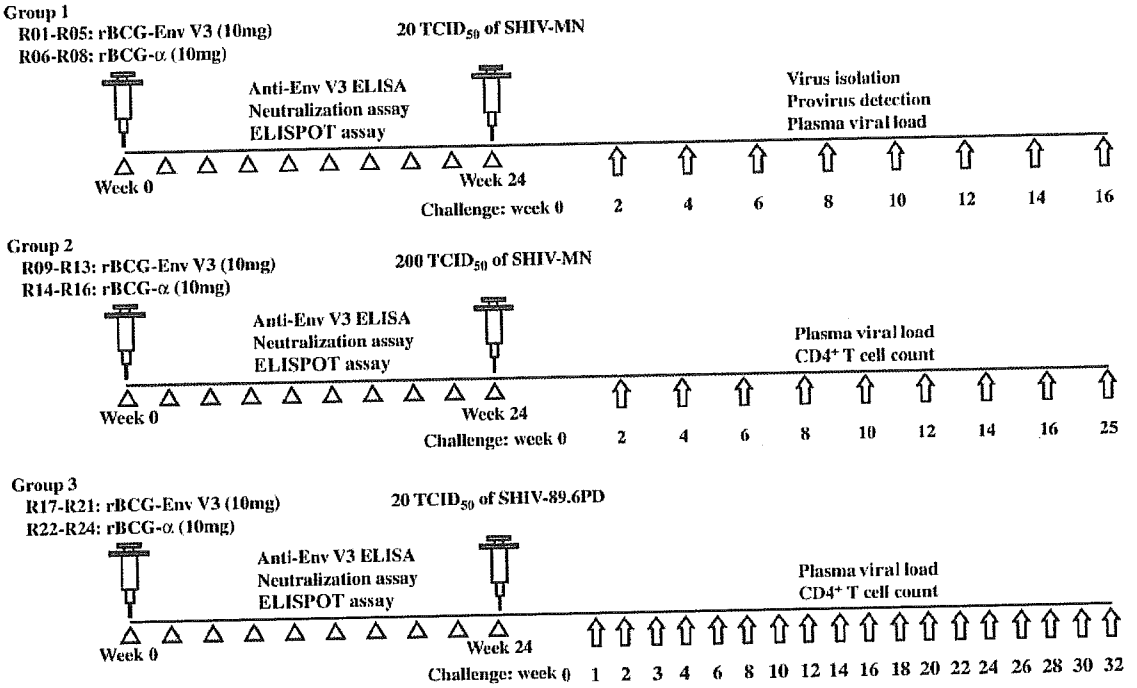

FIG. 1. Schematic representation of the experimental protocol for immunization of rhesus macaques with rBCG Env V3 and challenge with either SHIV-MN or SHIV-89.6PD. A total of 24 macaques were assigned to either the rBCG Env V3 vaccine or rBCG vector control group. The animals each received a single subcutaneous injection and were then split into three groups prior to challenge with either low-dose SHIV-MN, high-dose SHIV-MN, or SHIV-98.6PD.

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 $TCID_{50}$) or SHIV-89.6PD (20 $TCID_{50}$) (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 (IC_{50}) of 0.05 to 0.5 μg of IgG/ml was measured against SHIV-MN, and a mean IC_{90} of ~3.0 μg 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 naive 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 IC_{50} values of 5 to 7, 4 to 7, and 5 to 15 μg/ml, respectively. By comparison, neutralization of HIV-$1_{MNp/X4}$ required ~10-fold more serum IgG, with a mean IC_{50} of 50 μg/ml. Three additional isolates, HIV-$1_{SF33/X4}$, HIV-$1_{SF33/R5}$, and the clade A isolate HIV-$1_{VI313/R5}$,

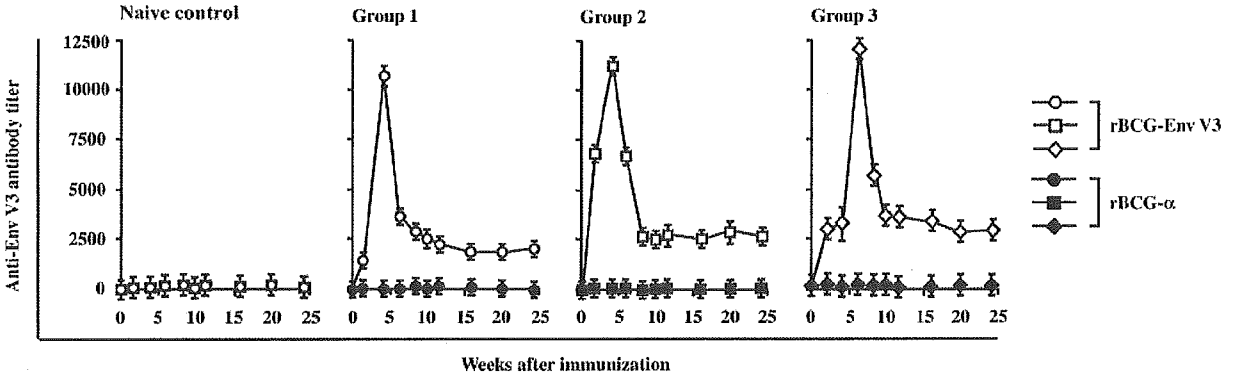

FIG. 2. Serum anti-V3 antibody titers determined by peptide-based ELISA. Preimmune and immune sera from macaques inoculated with rBCG Env V3 were collected and stored at −80°C until they were used. Sera from naïve macaques were used as controls. Data using preimmune sera were within the control levels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

were not neutralized with serum IgG concentrations up to 50 μg/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

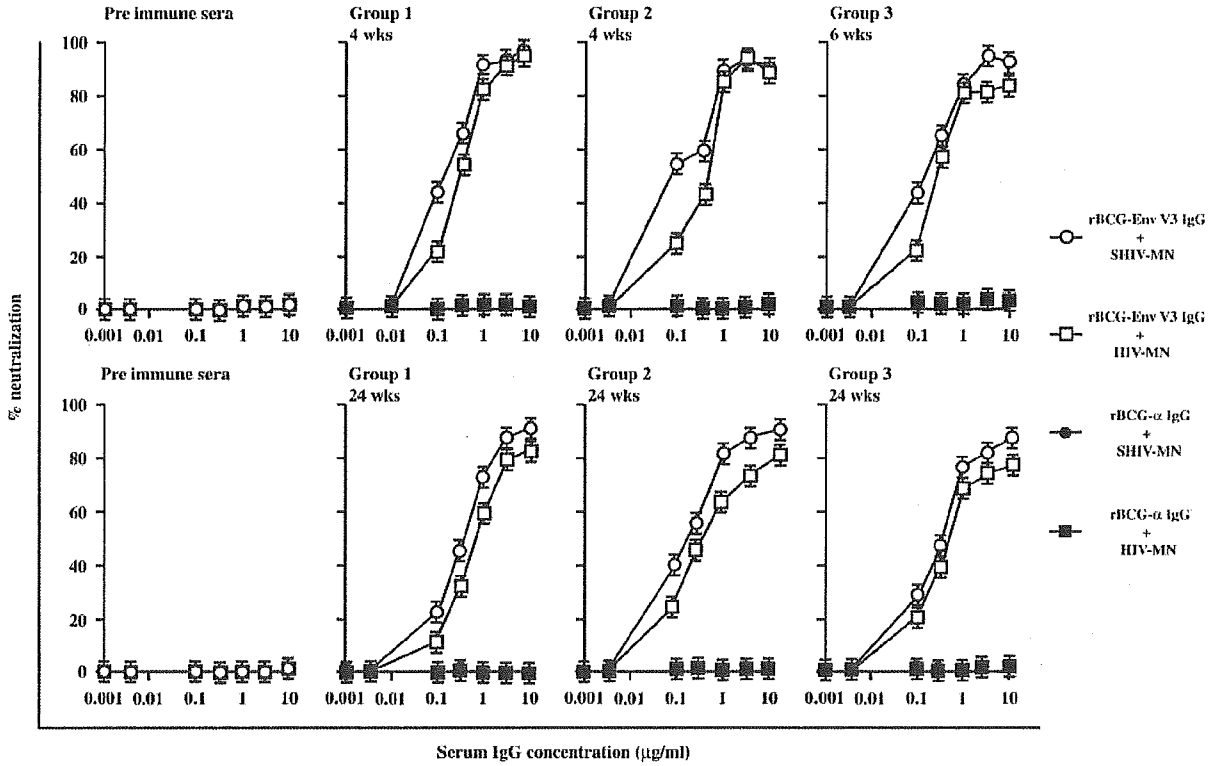

FIG. 3. HIV-1-specific neutralization antibody responses in macaques vaccinated with rBCG Env V3. Analysis of in vitro neutralization of SHIV-MN by anti-rBCG-HIV-1 antibodies using M8166 cell-based virus neutralization assays. Serum IgG was purified from preimmune or immune sera of macaques inoculated with rBCG Env V3 at the indicated times. The results are expressed as the means ± 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	V1313/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. VI313 is an HIV-1 clade A virus.

SHIV challenge (120 and 110 SFC/10^6 PBMC at 24 weeks p.i., respectively) (Table 2). In contrast, <100 SFC/10^6 PBMC were observed in other immunized animals, and <20 SFC/10^6 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 $TCID_{50}$) 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 (<10^4 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-

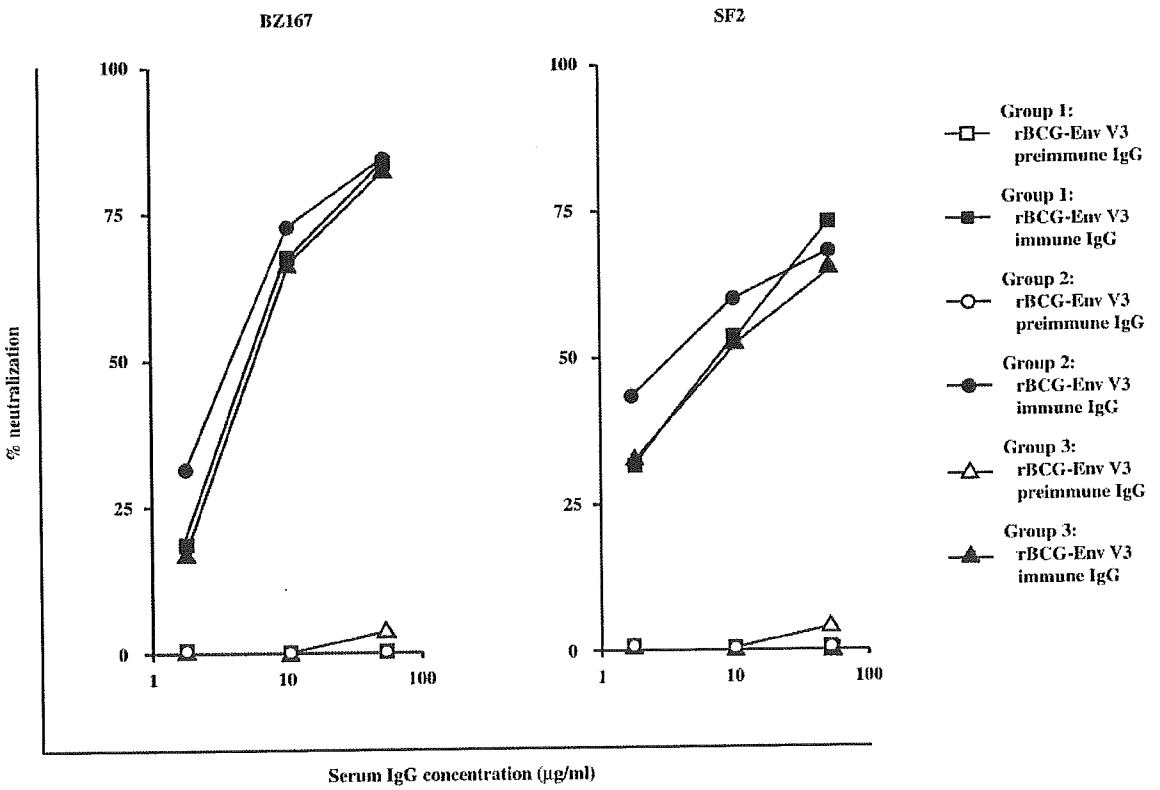

FIG. 4. Neutralization of HIV-1_{BZ167} and HIV-1_{SF2} in GHOST-X4 cells by immune sera from macaques vaccinated with rBCG Env V3. Dilutions of immune sera (closed symbols) and preimmune sera (open symbols) were tested in duplicate, and the percent neutralization was calculated using the mean value.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

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MN      CTRPNYNKRKRIHI  GPGRAFYTTKNIIGTIRQAHC
BZ167   -----NKA-R--R-  ----T---G- -V-D----Y-
SF2     ---LSN-T--C-PL  ----V--A-DI- -D------
CI2     ----SN-T-R----  ---------RQ-R-D------
MNp     -----N-R-T----  --------------------

89.6P   -----N-T-E-LS-  -------ARR----D------
SF33    -----N-R-R--TS  ---KVL---GE---D--K-Y-
VI131   -----N-T-QSV--  ---Q---A-GDV--D------
IIIB    -----N-T---KS-QR------V-IGK- -NM-----
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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 $TCID_{50}$) 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 10^6 to 10^7, to $<10^5$ to 10^4 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 $TCID_{50}$) 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 IC_{50} 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 IC_{50}s: R-01 to R-05, 0.32; R-09 to R-13, 0.05; R-17 to R-21, 0.35.
[e] Mean IC_{50}s: 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 $TCID_{50}$ 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 ~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 IC_{50}s 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 μg/ml, respectively (Table 2). The IC_{50}s of the uncontrolled macaques R-01 and -03 (Table 1) were both 0.6 μg/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 IC_{50} of 0.30 μg/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 $HIV\text{-}1_{MN}$-specific NAb generated by the recombinant $HIV\text{-}1_{MN}$ 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 $TCID_{50}$), 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

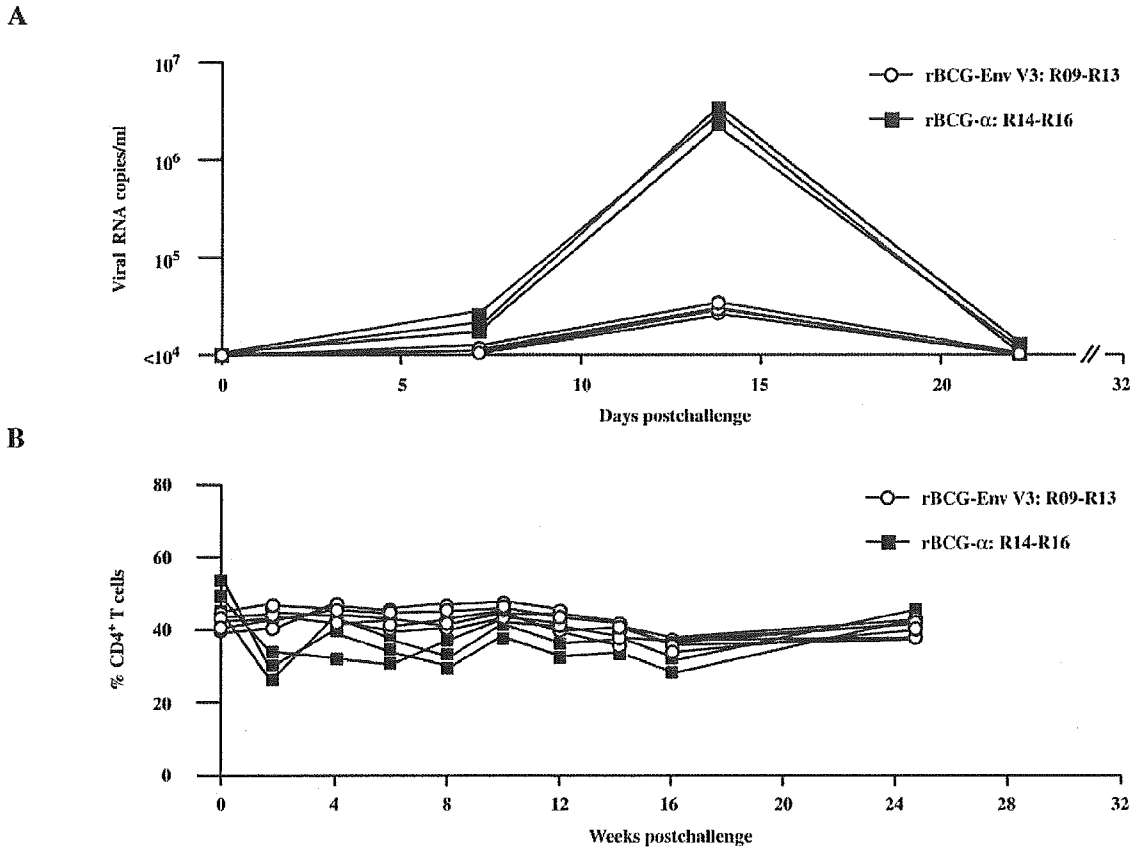

FIG. 6. Comparison of infection kinetics following high-dose (200 $TCID_{50}$) inoculation of SHIV-MN in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Viral RNA copy number per milliliter of serum. (B) $CD4^+$-T-cell count as a percentage of total lymphocytes. The results in 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 IC_{50} and IC_{90} values for neutralization of SHIV-MN were 10^3 to 10^4 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 ~10^3 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 IC_{90} 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-

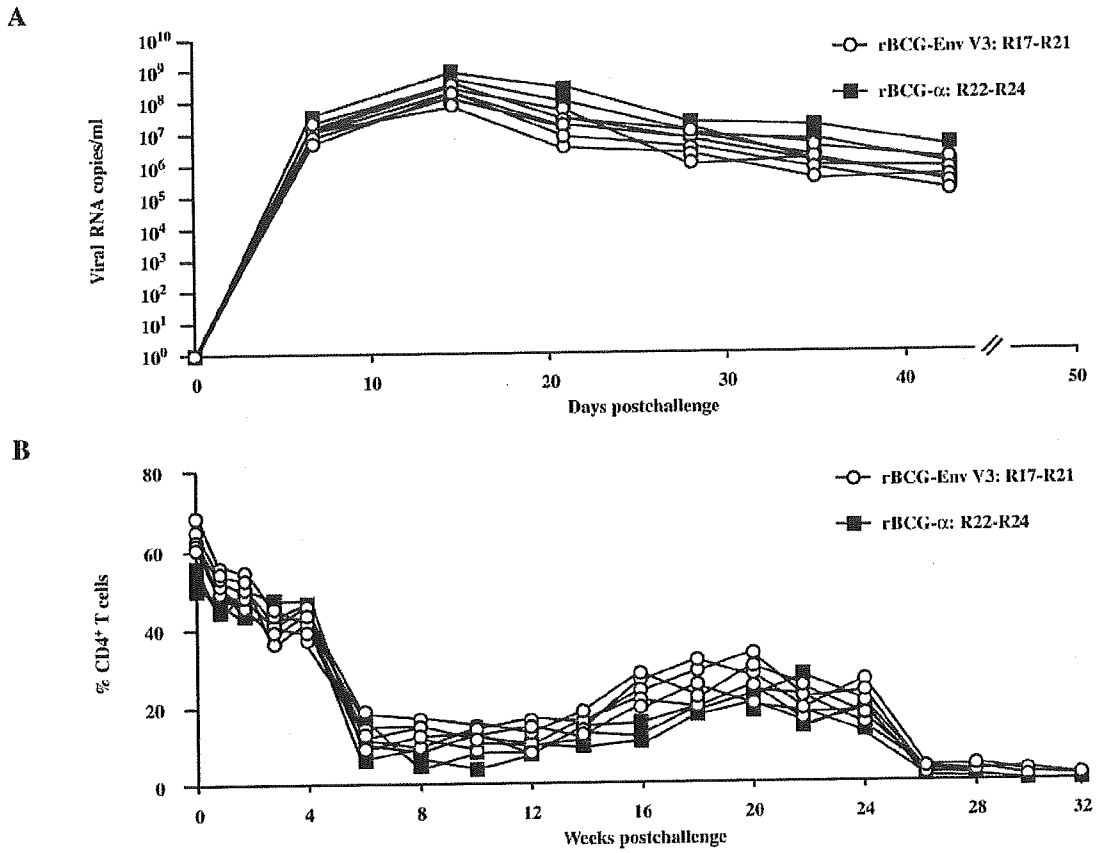

FIG. 7. Comparison of infection kinetics following challenge with pathogenic SHIV-89.6PD in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Plasma viral-RNA copy numbers per milliliter. (B) $CD4^+$-T-cell count as a percentage of total lymphocytes. The results in individual animals 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-$1_{JR\text{-}CSF}$ (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 structure 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 μg 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 elicitations.

ACKNOWLEDGMENTS

We thank L. Yichen, Harvard AIDS Institute, Harvard University, and A. Schultz, NIAID, National Institutes of Health, for providing the SHIV strains and for their helpful discussions. We also thank J. Esparza and S. Osmanov, UNAIDS, Geneva, Switzerland; S. Beddows and J. Weber, Medical Research Council, London, United Kingdom; and Eva-Maria Fenyo, Microbiology and Tumorbiology Center Karolinska, Stockholm, Sweden, for their helpful discussions.

This work was supported by a grant-in-aid from the Ministry of Health and Welfare, Japan, and the Japan Health Sciences Foundation (grants 341-5 and 321-2).

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JOURNAL OF VIROLOGY, July 2005, p. 8716–8723
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.14.8716–8723.2005

Vol. 79, No. 14

Mycobacterial Codon Optimization Enhances Antigen Expression and Virus-Specific Immune Responses in Recombinant *Mycobacterium bovis* Bacille Calmette-Guérin Expressing Human Immunodeficiency Virus Type 1 Gag†

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Received 22 November 2004/Accepted 7 April 2005

Although its potential for vaccine development is already known, the introduction of recombinant human immunodeficiency virus (HIV) genes to *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has thus far elicited only limited responses. In order to improve the expression levels, we optimized the codon usage of the HIV type 1 (HIV-1) p24 antigen gene of *gag* (p24 *gag*) and established a codon-optimized recombinant BCG (rBCG)-p24 Gag which expressed a 40-fold-higher level of p24 Gag than did that of nonoptimized rBCG-p24 Gag. Inoculation of mice with the codon-optimized rBCG-p24 Gag elicited effective immunity, as evidenced by virus-specific lymphocyte proliferation, gamma interferon ELISPOT cell induction, and antibody production. In contrast, inoculation of animals with the nonoptimized rBCG-p24 Gag induced only low levels of immune responses. Furthermore, a dose as small as 0.01 mg of the codon-optimized rBCG per animal proved capable of eliciting immune responses, suggesting that even low doses of a codon-optimized rBCG-based vaccine could effectively elicit HIV-1-specific immune responses.

The *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has been widely used as a live bacterial vaccine against *Mycobacterium tuberculosis* infection. Its recombinant form, rBCG, which has been used successfully to express foreign antigens and to induce immune responses, has been proposed as a vaccine candidate against a number of diseases (26, 32, 33), especially human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) (11, 13, 30). Moreover, mucosal immunization of rBCG has been found to elicit a long-term virus-specific immunity in animals (10, 14, 15), even in Th1- and Th2-deficient conditions (10). In short, an rBCG-based vaccine offers several clear advantages over other types of recombinant vector-based approaches in that it (i) induces cellular immune responses that are maintained for at least 1 to 2 years; (ii) is easy to administer, usually requiring only one or two immunizations; (iii) and is affordable because it can be easily and cheaply produced. These findings suggest that rBCG could be a potent vaccine against HIV-1 infection, one that is likewise capable of inducing safe, virus-specific immunity.

However, the results described above were obtained with high doses of rBCG, doses 10- to 100-fold larger than that needed for a practical BCG vaccination dose against tuberculosis in humans (7, 11). Therefore, the low immunogenicity seen in rBCG-inoculated animals is likely due to their inoculation with only a "normal," not a high, vaccination dose (15). Moreover, high doses of BCG administration in vivo may also act as the driving force for the replication of the immunodeficiency virus and its dissemination by hyperactivating T cells (6, 41).

We sought here to produce an rBCG vaccine that would be efficacious even in the low doses required for human vaccination. Because low-dose immunization of rBCG has been suggested to act as a prophylactic vaccination against HIV-1 (15, 28), we adopted the preferred codon of BCG to enhance the expression of the foreign HIV gene. In recombinant protein production, the potency of codon-optimized gene expression systems was demonstrated in *Escherichia coli* (39) and in mammalian cells (42). These results clearly show that codon-optimized recombinant genes induce vigorous expression by foreign genes in the host. Since 1998, many groups have reported that a sequence-modified DNA vaccine confers high immunogenicity against various foreign antigens, e.g., listeriolysin O of *Listeria monocytogenes* (37), HIV-1 Gag (43), Env (3), tetanus toxin (34), L1 protein of human papillomavirus (18), and merozoite surface protein 1 of *Plasmodium falciparum* (25). Most of these studies focused on demonstrating how mammalian codon usage bias efficiently enhanced the expression and immunogenicity of foreign antigens in DNA vaccination. However, although the effect of codon optimization in mammalian cells has been well documented, its effect in recombinant BCG vector-based vaccines has never been fully elucidated.

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MATERIALS AND METHODS

Animals. Female BALB/c (*H-2*^{d}) mice aged 6 to 8 weeks were purchased from Charles River Japan, Inc. Mice were maintained in the experimental animal facility under pathogen-free conditions and in a manner consistent with the institutional animal care and use guidelines of the National Institute of Infectious Diseases of Japan. The study was conducted in a biosafety level 2 facility with the approval of an institutional committee for biosafety and in accordance with the requirements of the World Health Organization.

Construction of an HIV antigen expression vector and transformation of BCG. We used *E. coli* HB101-competent cells (Takara Bio, Inc.) for gene manipulation and the $BCG_{Tokyo172}$ as a mycobacterial strain which does not accelerate disease progression in HIV-infected children (9). Middlebrook 7H9 broth containing albumin-dextrose complex (7H9-ADC; BBL Microbiology Systems) was used as the culture medium for rBCG. A DNA fragment encoding the *hsp60* gene of BCG (36) was cloned into SmaI-SalI sites of pUC18 (pUC-hsp60). A synthetic DNA fragment corresponding to the multicloning site and terminator region of the *hsp60* gene was cloned into the MunI-KpnI sites of pUC-hsp60. A KpnI linker was then inserted at the EcoRI site, giving rise to the pUC-hspK vector. The *gag* p24 gene of the subtype B NL4-3 virus was amplified by PCR from pNL4-3 plasmid using the primers AATggatccTATAGTGCAGAACCTC (forward, with lowercase letters indicating the BamHI site) and AATgggcccTT ACAAAACTCTTGCTTTATGG (reverse, with lowercase letters indicating the ApaI site). The PCR product was cloned into BamHI-ApaI sites of pUC-hspK in frame (pUC-hspK-p24Wt). The whole p24 gene was also chemically synthesized with the preferred codons in BCG and then cloned into the same sites of the pUC-hspK vector (pUC-hspK-p24Mu). These vectors were digested with KpnI, and then small fragments containing p24 expression units were subcloned into a KpnI site of the stable *E. coli*-mycobacteria shuttle vector pSO246 (pSO-p24Wt and -p24Mu) (19). These plasmids and pSO246 were transformed into BCG by using a Gene-Pulser (Bio-Rad Laboratories, Inc.), and transformants were selected on Middlebrook 7H10 agar containing 20 μg of kanamycin/ml and supplemented with an OADC enrichment (BBL Microbiology Systems).

Western blot analysis. Transformants of rBCG were grown in 7H9-ADC broth for 2 weeks. A portion of the culture medium was periodically collected, sonicated, and subjected to immunoblot analysis with V107 monoclonal antibody (20) as described previously (11).

Lymphocyte proliferative assays. Single-cell suspensions from spleens of immunized animals were cultured with or without 25 μg of HIV-HXB2 Gag-overlapping peptide (NIH AIDS Research and Reference Reagent Program)/ml or 2.5 μg of tuberculin purified protein derivative (PPD)/ml. In the present study, the overlapping peptides p11 (LERFAVNPGLLETSE) through p35 (NIQGQ MVHQAISPRT) covering the Gag p24 region were used for stimulation, either as a whole or in pools of 5. Proliferation was measured by determining the level of [^{3}H]thymidine uptake (31).

Antigen-specific IFN-γ ELISPOT assay. P24- and PPD-specific IFN-γ-secreting cells were assessed by using the mouse gamma interferon (IFN-γ) development module and the enzyme-linked immunospot assay (ELISPOT) blue color module (R&D Systems, Inc.). Briefly, single-cell suspensions were cultured in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 55 μM β-mercaptoethanol, 50 U of penicillin/ml, and 50 μg of streptomycin/ml) with or without 25 μg of pooled Gag-overlapping peptide (p11-35)/ml, 5 μg of recombinant p24 protein (rp24; $HIV\text{-}1_{IIIB}$ p24; ImmunoDiagnostics, Inc.)/ml, or 2.5 μg of PPD/ml for 48 h at 37°C in a humidified 5% CO_2 environment. After incubation, cells were transferred to anti-IFN-γ antibody-coated 96-well nitrocellulose plates (Millititer HA; Millipore Co.) at various concentrations and incubated for 16 h at 37°C in a humidified 5% CO_2 environment before being developed according to manufacturer's instructions. Spot-forming cells (SFCs) were then quantified by using the KS ELISPOT compact system (Carl Zeiss) (23).

Assay for assessment on major histocompatibility complex class I-restricted CD8$^+$-T-cell response. *H-2*^{d}-restricted CD8$^+$-T-cell responses were measured by ELISPOT assay using A9I (AMQMLKETI) peptide (27, 38). Single-cell suspensions were labeled with microbead-conjugated anti-CD8a monoclonal antibody (53-6.7; Miltenyi Biotec GmbH) and depleted labeled cells by using Auto MACS (Miltenyi Biotec GmbH). Whole splenocytes and CD8-depleted splenocytes from each mouse were used in an A9I-specific IFN-γ ELISPOT assay. The cells were incubated with or without A9I peptide at 50 μM for 24 h at 37°C in a humidified 5% CO_2 environment, and the subsequent steps were as described above.

Serum antibody titration by HIV-1 Gag p24- and PPD-based ELISA. P24- and PPD-specific immunoglobulin G titers in plasma were determined by an end-point enzyme-linked immunosorbent assay (ELISA) (10).

Statistical analyses. Statistical analyses were carried out by using the StatView program (version 3.0; SAS Institute). The lymphocyte proliferative activities and IFN-γ SFC counts of each group were compared by using the two-sided Student *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Mycobacterial codon usage optimization of HIV-1 *gag* p24 gene and construction of an rBCG encoding the codon-optimized gene. In order to determine whether mycobacterial codon optimization could enhance the expression of the HIV gene in vitro, we first targeted the HIV-1 subtype B NL4-3 *gag* p24 gene for our research. Once we had designed the mycobacterial codon-optimized p24 gene, aligned it with the wild-type gene, and deduced the amino acid sequence (Fig. 1), we determined that the total G+C content of the coding region in the synthetic p24 gene was higher (67.4%) than that of the wild-type p24 gene from pNL4-3 (43.4%). (A translation table showing all 20 amino acids used in the present study is available [Table S1 in the supplemental material]). These two genes were initially cloned into the pUC-hspK vector (Fig. 2a) and subcloned into the pSO246 vector (Fig. 2b). Once these expression vectors were transformed into the $BCG_{Tokyo172}$ strain, rBCG-p24Mu (with optimal codon usage of the p24 gene) and rBCG-p24Wt (with wild-type codon usage) were selected for further experimentation.

Effects of codon usage modification on the expression levels of HIV-1 Gag p24 in vitro. We next sought to compare the expression levels of the p24 gene in the two types of BCG-HIV recombinants by studying the kinetics of the growth curve of the cultured rBCG cells and by measuring the levels of p24 protein to assess the production ability of the HIV antigen (Fig. 3). Using Western blot analysis at 2-week intervals, we observed that recombinant p24 protein in each of the lysates of rBCG-p24Wt and -p24Mu consistently appeared as a single band measuring ca. 24 kDa (lanes 1 and 2 of Fig. 3a, respectively). The p24 antigen expression level of rBCG-p24Mu was 37-fold higher (175.0 ± 25.1 ng/5 × 10^7 CFU of bacilli) than that of rBCG-p24Wt (4.7 ± 0.3 ng/5 × 10^7 CFU of bacilli) (Fig. 3b). Both rBCG-p24Mu and -p24Wt showed a more normal BCG growth curve than did the rBCG-pSO246 control transformant, and both peaked 21 days after cell culture (Fig. 3c), suggesting a correlation between p24 antigen generation and the growth rate of cultured rBCG-p24Mu. Thus, the codon-optimized BCG recombinant was successfully generated and found to express remarkable levels of p24 antigen, i.e., almost 200 ng of p24 antigen/5 × 10^7 CFU or 1 mg of bacilli.

Codon optimization of the HIV-1 Gag p24 antigen in rBCG generates strong HIV-specific immune responses in mice after intradermal immunization. We then analyzed how the modification of codon usage affected the immunogenicity of BCG vector-based vaccines encoding the HIV-1 *gag* p24 antigen gene. 35 BALB/c mice were divided into three experimental groups of 10 mice each, with the remaining five mice administered saline alone and used as normal healthy controls. Five mice from each experimental group were intradermally immunized with 0.01 mg, and five mice from each group were immunized with 0.1 mg of rBCG-p24Mu, -p24Wt, and -pSO246. At 10 weeks postinoculation (p.i.), we examined lymphocyte proliferation, IFN-γ ELISPOT cell generation, and antibody

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pNL4-3[a]      1 GGATCCtatagtgcagaacctccaggggcaaatggtacatcaggccatatcacctagaactttaaatgcatgggtaaaagtagtagaaga
optimized      1 ....g.c.c.....g...c.g....c.c.....c.g.gc.c..cc.g..c..c....c.g..c..c.g.
A.A. seq.        P I V Q N L Q G Q M V H Q A I S P R T L N A W V K V V E E

pNL4-3        91 gaaggctttcagcccagaagtaataccatgttttcagcattatcagaaggagccaccccacaagatttaaataccatgctaaacacagt
optimized     91 .....c...tcg..g..g..c..c..g....c..g..cc.g..g..g..c.....g..g..cc.g..c.....g....c..
A.A. seq.        K A F S P E V I P M F S A L S E G A T P Q D L N T M L N T V

pNL4-3       181 gggggacatcaagcagccatgcaaatgttaaaagagaccatcaatgaggaagctgcagaatgggatagattgcatccagtgcatgcagg
optimized    181 c..c..c..c..g..c.....g...c.g..g.....c....g..c..c..g.....cc.cc....c..g..c..c..c..
A.A. seq.        G G H Q A A M Q M L K E T I N E E A A E W D R L H P V H A G

pNL4-3       271 gcctattgcaccaggccagatgagagaaccaaggggaagtgacatagcaggaactactagtacccttcaggaacaaataggatggatgac
optimized    271 c..c..c....g.....c.c..g..tc.c..ctcg....c..c..c..c.ctcg....c....g..g..c..c.....
A.A. seq.        P I A P G Q M R E P R G S D I A G T T S T L Q E Q I G W M T

pNL4-3       361 acataatccacctatcccagtaggagaaatctataaaagatggataatcctgggattaaataaaatagtaagaatgtatagccctaccag
optimized    361 c..c..c..g..g....g..c..c..g....c..gc.c.....c.....cc.g..c..g..c..cc.c.....ctcg..g...tc
A.A. seq.        H N P P I P V G E I Y K R W I I L G L N K I V R M Y S P T S

pNL4-3       451 cattctggacataagacaaggaccaaaggaacccttttagagactatgtagaccgattctataaaactctaagagccgagcaagcttcaca
optimized    451 g..c.....cc.c..g..t..g....g..g..cc.c.....c..c.....c....c..g..c..cc.c.....g..g..g..
A.A. seq.        I L D I R Q G P K E P F R D Y V D R F Y K T L R A E Q A S Q

pNL4-3       541 agaggtaaaaaattggatgacagaaaccttgttggtccaaaatgcgaacccagattgtaagactattttaaaagcattgggaccaggagc
optimized    541 g.....c..g..c.....c..g....c..c.....g..c..c....g..c..c.....c..cc.g..g..cc....t..g..c..
A.A. seq.        E V K N W M T E T L L V Q N A N P D C K T I L K A L G P G A

pNL4-3       631 gacactagaagaaatgatgacagcatgtcagggagtgggggacccggccataaagcaagagttttgtaaGGGCCC
optimized    631 c..c..g..g..g.....c..c..c.....c..c..g....c..g..gc.c..cc.....
A.A. seq.        T L E E M M T A C Q G V G G P G H K A R V L *
```

FIG. 1. Nucleotide sequences and deduced amino acid sequence alignments of the p24 gene from pNL4-3 and the synthetic p24 gene with mycobacterial optimal codons. For cloning to the pUC-hspK vector, the BamHI and ApaI restriction sites were attached at both the 5′ terminus and the 3′ terminus of each DNA fragment (shown as boldface uppercase letters). Dots indicate sequences identical to those of the pNL4-3 p24 gene. The asterisk indicates the termination codon. The superscript *a* indicates sequence data that are available from GenBank under accession no. AF324493.

production in immunized animals. The same study was repeated three times, and all three results were summarized.

Significant lymphocyte proliferative responses (stimulation indices of 5.04 ± 1.09 and 4.02 ± 0.44) were obtained with pooled peptides p16-20 (pool 2) and pooled total p24 peptides p11-35 (pool 1-5) in mice immunized with 0.01 mg of rBCG-p24Mu. When this dosage was increased to 0.1 mg, the lymphocyte proliferative responses to pool 2 and pool 1-5 increased to 10.08 ± 2.40 and 8.05 ± 1.16, respectively (data not shown). In contrast, we could not detect any significant virus-specific proliferation in mice immunized with 0.01 or 0.1 mg of rBCG-p24Wt (Fig. 4). These in vivo differences in proliferative

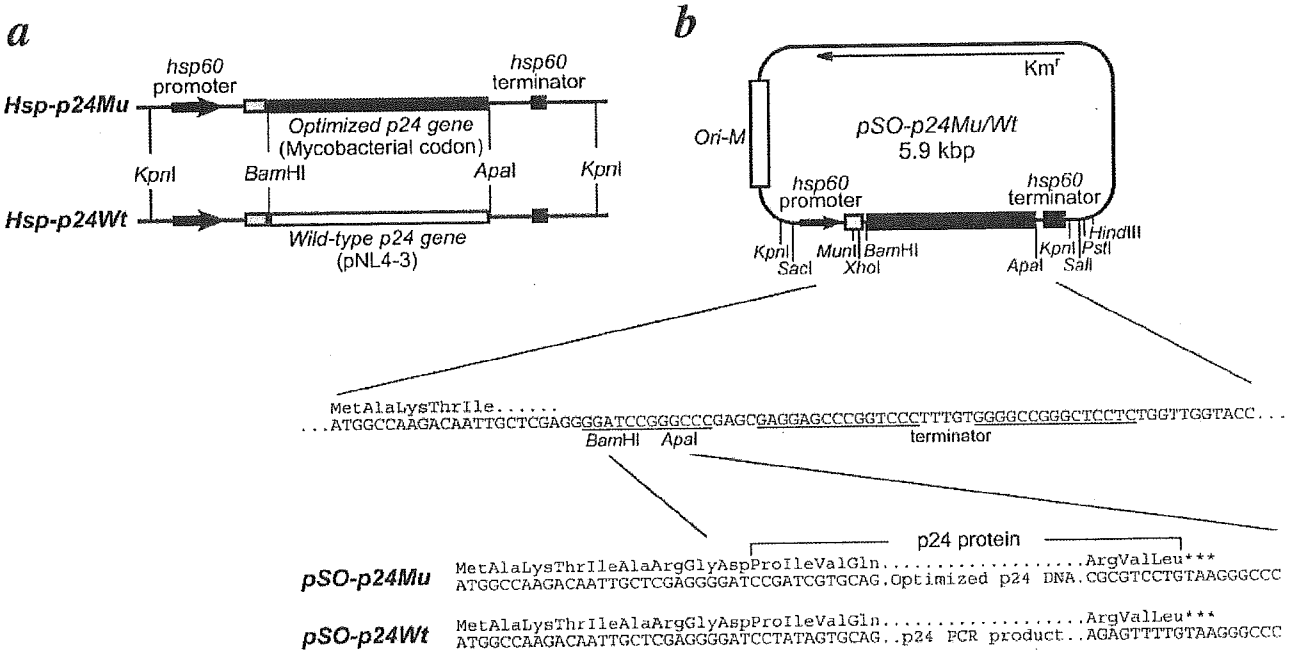

FIG. 2. Structure of expression vectors pSO-p24Mu and -p24Wt. (a) Schematic representation of the expression units of p24. Solid and open bars indicate the synthetic p24 gene and the PCR fragment of the p24 gene, respectively. The arrows and solid squares indicate the transcriptional direction of the *hsp60* promoters and terminators. Gray bars show the DNA fragment of the mycobacteria. (b) Details of expression vectors pSO-p24Mu and -p24Wt. *Ori-M* indicates the origin of mycobacterial replication, and Km^r denotes the kanamycin resistance gene. Asterisks indicate the termination codon for each gene.

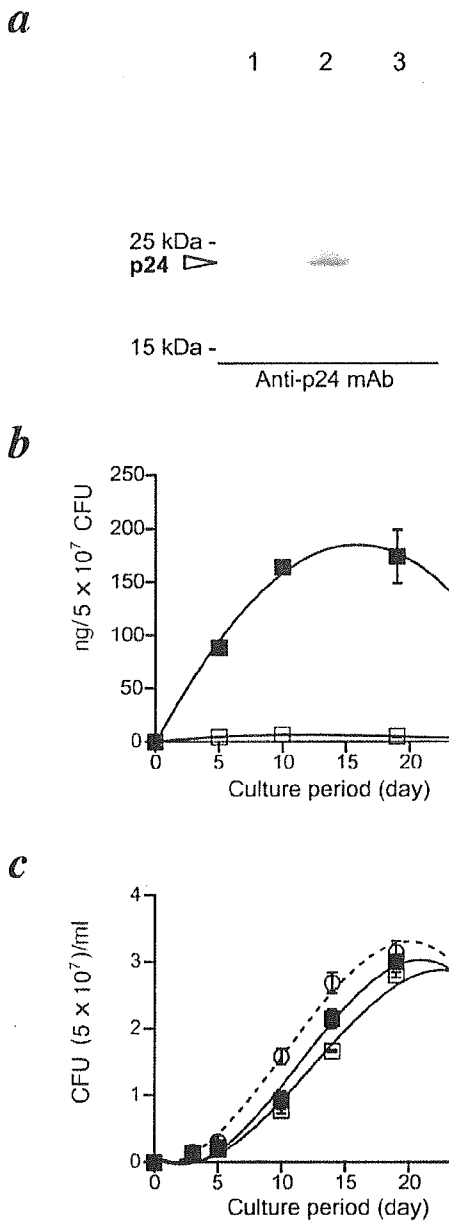

FIG. 3. Comparison of p24 expression levels and growth rates for rBCG-p24Mu and -p24Wt. (a) Anti-p24 monoclonal antibody (V107)-reacted proteins were visualized by Western blotting. Lane 1, lysate of rBCG-p24Wt; lane 2, lysate of rBCG-p24Mu; lane 3, lysate of rBCG-pSO246 (negative control). (b) Comparison of p24 concentration in whole-cell lysates of rBCG-p24Mu and -p24Wt. rBCG cells were harvested from each culture periodically, sonicated, and subjected to a commercial p24 antigen enzyme immunoassay (HIVAG-1MC; Abbott Laboratories). Expression of the p24 protein is represented as p24 concentrations (in nanograms per milligram) or 5×10^7 CFU of bacilli. Solid and open squares indicate rBCG-p24Mu and -p24Wt, respectively. Data are presented as means ± the standard deviations. (c) Kinetics of growth rates in recombinant clones. After periodic collection of each culture, the optical density at 470 nm was measured. The cell densities were calculated based upon the rate of absorbance, using the following formula: density (μg/ml) = absorbance at 470 nm × 1,412.3 + 73.063. The CFU were translated from densities and plotted. The ■, □, and ○ symbols indicate rBCG-p24Mu, -p24Wt, and -pSO246, respectively. The data represent means ± the standard deviations.

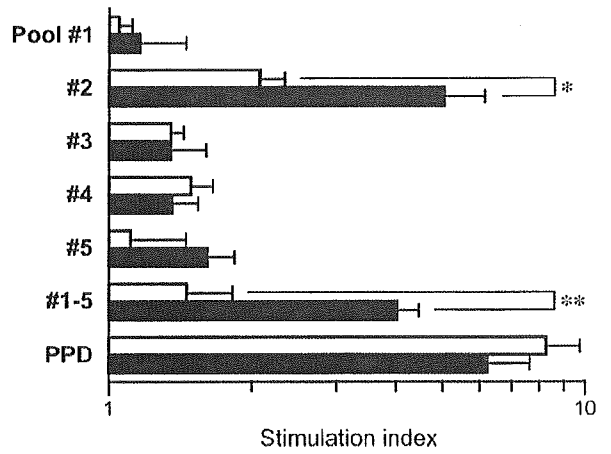

FIG. 4. Lymphocyte proliferation against Gag p24 overlapping peptides and PPD. Splenocytes from immunized animals were cultured with or without antigen for 48 h at 37°C in a humidified 5% CO_2 environment. In the final 6 h before harvesting, [^{3}H]thymidine was added. The level of [^{3}H]thymidine uptake was then measured. Proliferative activity is measured by using the stimulation index. Solid and open columns indicate stimulation index values of rBCG-p24Mu- and -p24Wt-immunized mice, respectively. The data represent the mean stimulation index plus one standard deviation. Asterisks indicate statistical significance (*, $P < 0.02$; **, $P < 0.002$).

responses between rBCG-p24Mu and -p24Wt were statistically significant comparing pool 2 ($P = 0.010$) and pool 1-5 ($P = 0.001$). No p24-specific proliferation was detected in either rBCG-pSO246-immunized mice or normal healthy controls (data not shown). PPD-specific lymphocyte proliferations were obtained in all immunized animals similarly (stimulation indices were ca. 7).

In addition, p24-specific IFN-γ-secreting cells were determined by ELISPOT assay. Both pooled p24 peptides (pool 1-5) and rp24-specific SFCs were detected in mice immunized with 0.1 mg of rBCG-p24Mu and -p24Wt but not in those immunized with the same dosage of rBCG-pSO246 (Fig. 5). In rBCG-

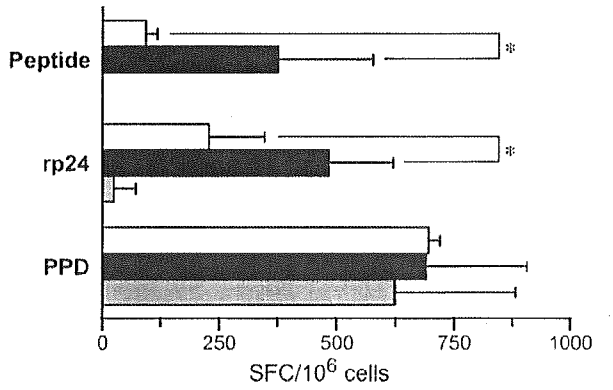

FIG. 5. Determination of antigen-specific IFN-γ-secreting cells by ELISPOT assay. Solid, open, and shaded columns indicate the numbers of SFCs of rBCG-p24Mu-, -p24Wt-, and -pSO246-immunized mice, respectively. The data represent the mean numbers of SFCs/10^6 cells plus one standard deviation. Asterisks indicate statistical significance ($P < 0.05$ against rBCG-p24Wt-immunized mice).

p24Mu-immunized mice, stimulation with peptides resulted in 375 ± 202 SFC/10^6 splenocytes and stimulation with rp24 resulted in 483 ± 138 SFC/10^6 splenocytes—rates much higher than those observed for rBCG-p24Wt (93 ± 25 and 227 ± 120 SFC/10^6 splenocytes, respectively). These differences in response by groups immunized with rBCG-p24Mu and -p24Wt to peptides and to rp24 were also statistically significant (peptides, $P = 0.033$; rp24, $P = 0.031$). PPD-specific SFCs were strongly expressed in all mice receiving rBCGs (670 ± 180 SFC/10^6 splenocytes). Furthermore, similar levels of rp24-specific IFN-γ SFC activity were observed in splenocytes of rBCG-p24Mu-immunized animals even 6 months p.i. (402 ± 198 SFC/10^6 splenocytes, data not shown).

Furthermore, we studied whether these IFN-γ ELISPOT activities were attributed to major histocompatibility complex class I-restricted $CD8^+$-T-cell response with 12 BALB/c mice immunized with 0.1 mg of rBCG-p24Mu ($n = 4$), -p24Wt ($n = 4$), or -pSO246 ($n = 4$). After 2 weeks p.i., the mice were sacrificed, and their spleens were used for the study. By peptide-antigen-specific IFN-γ ELISPOT assay, $H\text{-}2^d$-restricted $CD8^+$-T-cell responses specific for the $CD8^+$-T-cell epitope A9I were detected in the two animal groups immunized with rBCG-p24Mu and -p24Wt (Fig. 6b). In rBCG-p24Mu-immunized mice, stimulation with 50 μM A9I resulted in 130 ± 16 SFC/10^6 splenocytes, activities significantly higher than that obtained by immunization with rBCG-p24Wt (70 ± 21 SFC/10^6 splenocytes, $P = 0.011$). Furthermore, by using magnetic cell sorting, the $CD8^+$-T-cell-depleted cell fractions were purified to be >97% and >99% viable (Fig. 6a). The $CD8^+$-T-cell response of immunized animal groups decreased significantly upon stimulation with A9I peptide compared to nonseparated splenocytes (rBCG-p24Mu immunized, 15 ± 12 SFC/10^6 cells, $P = 0.001$; rBCG-p24Wt immunized, 3 ± 3 SFC/10^6 cells, $P = 0.006$) ("CD8-depleted" in Fig. 6b). No A9I-specific IFN-γ responses were detected in rBCG-pSO246-immunized mice either whole or CD8-depleted splenocytes were used (data not shown).

Finally, sera from all animals immunized with 0.1 mg of rBCG-p24Mu, -p24Wt, and -pSO246 were assessed for specific antibody generation at 10 weeks p.i. by endpoint antibody-ELISA against rp24 and PPD (Fig. 7). Again, only low levels of antibodies against rp24 were generally elicited in animals immunized with rBCG-p24Mu and -p24Wt (antibody titers in sera of $10^{2.41}$ and $10^{2.03}$, respectively). Moreover, PPD-specific antibodies were similarly detected in all immunized animals at titers of ca. 10^3. In summary, virus-specific cell-mediated immunity was significantly induced during the initial immune response, but its antibody response was low.

DISCUSSION

In this study, we have clearly demonstrated that codon optimization is a useful strategy for enhancing foreign antigen expression in rBCG and for obtaining significant levels of foreign antigen-specific immune responses. This strategy is key to rBCG-HIV vaccine development, since low-dose immunization and/or intradermal immunization with 0.1 mg of codon-optimized rBCG has proven effective for induction of HIV-specific cellular immunity by (i) allowing for a smaller dosage of rBCG, one that is far more practicable for use in human tuberculosis vaccination than the 1 to 10-mg dose otherwise

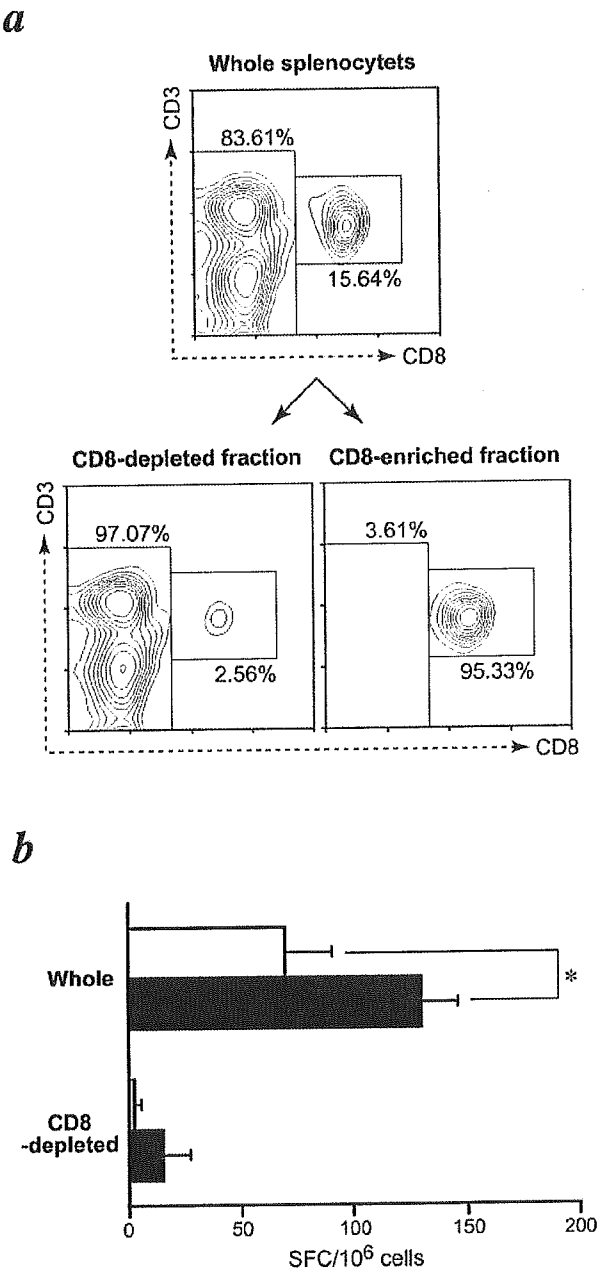

FIG. 6. Decrease of p24-specific cellular immune responses by the depletion of $CD8^+$ T cells. (a) Depletion of $CD8^+$ T cells from splenocytes of immunized animals by magnetic cell sorting of $CD8^+$ T cells by a specific antibody. Splenocytes from mice immunized with rBCGs were sorted and analyzed by flow cytometry. Whole splenocytes (upper row) and CD8-depleted fraction (lower left panel) were used for subsequent study. (b) Assessment of A9I-specific $CD8^+$-T-cell responses by peptide-specific IFN-γ ELISPOT assay. Effect of $CD8^+$-T-cell depletion from splenocytes from immunized animals was studied by measuring the A9I peptide-specific IFN-γ ELISPOT response of whole or CD8-depleted splenocytes. Solid and open columns indicate the numbers of SFCs of rBCG-p24Mu and -p24Wt-immunized mice, respectively. The data represent the mean numbers of SFCs/10^6 cells plus one standard deviation. The asterisk indicates statistical significance ($P < 0.02$ against rBCG-p24Wt-immunized mice).

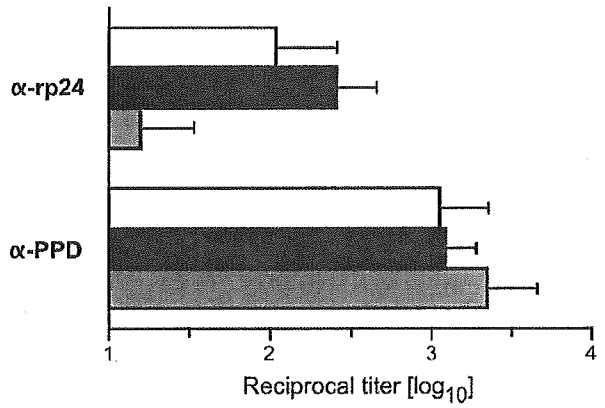

FIG. 7. Anti-p24-specific and anti-PPD-specific IgG antibodies in the plasma of mice immunized with rBCGs. Solid, open, and gray columns indicate reciprocal $\log_{10}$ titers of rBCG-p24Mu, -p24Wt, and -pSO246-immunized mice, respectively. The titers were determined by using an endpoint ELISA. The data represent mean titers plus one standard deviation.

required, and by (ii) thereby reducing the risks associated with high-dosage cutaneous administration, including adverse local skin reactions, possible association with Th2-type immune responses, or exacerbation of retroviral infections. Given these results, rBCG is clearly poised to play a key role in the development of an HIV/AIDS vaccine.

When the mycobacterial codon usage of the p24 antigen gene of the HIV-1 *gag* was optimized, the codon-optimized rBCG expressed nearly 40-fold more antigen than did the wild-type rBCG. This enhancement of the Gag p24 expression level in rBCG is on a par with the 10- to 50-fold increase seen when DNA vaccine is codon optimized (3). Why was the mycobacterial codon optimization so effective? BCG is a high G+C gram-positive bacteria, with a genomic G+C content ca. 64.8%, and so has a strong bias toward C- and G-ending codons for every amino acid. Overall, the G+C content at the third position of codons is 81.0% (2). From the accumulated information on BCG genes (24), it should be noted that the AGA codon for Arg and the TTA codon for Leu make up only 0.9 and 1.6% of the total codons for Arg and Leu, respectively. In contrast, HIV-1 prefers the adenine or the thymidine at the third position of the codon (60.9%). In the coding sequence of the p24 gene of HIV-1 *gag*, 9 out of 11 Arg codons used AGA and 6 out of 18 Leu codons used TTA. Because it is generally accepted that codon preference correlates with the amount of aminoacyl tRNA in unicellular organisms (12), only low levels of aminoacyl tRNA for AGA and TTA codons would be expected in the BCG cell. These low levels of aminoacyl tRNA for AGA and TTA codons might help explain why the codon-optimized p24 gene was highly expressed in BCG.

Recombinant HIV-1 Gag p24 antigen expression in codon-optimized rBCG is 175 ng/mg of bacilli of $BCG_{Tokyo172}$ or ca. 5.3% of the total cytoplasmic rBCG protein, when calculated using the method of Langermann et al. (17). The previously reported production levels of recombinant HIV protein were all for non-codon-optimized BCG using a different expression system and a different BCG strain. The levels varied from 1% of cellular protein (HIV-1 Nef [40] and SIV_{mac251} Gag [22]) to 0.1% of the HIV-1 Gag protein (1), suggesting that codon-optimized recombinant HIV-1 protein induced responses 5- to 50-fold higher than those previously reported for non-codon-optimized rBCG. The codon-optimization of HIV Gag p24 is also effective in elicitation of antigen-specific $CD8^+$-T-cell responses in animals. Since there is no difference in the growth/persistence in the various BCG (S. Yamamoto et al., unpublished data), the enhanced expression of the HIV protein by the recombinant construct suggests that it is responsible for the enhanced immunogenicity of the codon-optimized rBCG vaccine.

A successful preventive HIV vaccine must not only effectively protects against HIV-1 or SIV, a goal already achieved in nonhuman primate AIDS models using different vaccine modalities, but also will prove safe for use in humans. Instead of seeking to elicit sterilizing protection from the HIV infection, current vaccine research on HIV/AIDS is focused mainly on the induction of efficient cellular immune responses that may play a critical role in protective immunity.

One of the prospective measures is to evoke host immunity by delivering recombinant vector-based vaccines expressing recombinant antigens, e.g., modified vaccinia virus Ankara (4, 21), adenovirus type 5 (29), fowlpox virus (16), canarypox virus (8), and NYVAC (5). In combination with boosting or priming antigens, most of these recombinant vector-based vaccines effectively induce antiviral immunity. We also showed that rBCG could induce long-lasting anti-HIV-1 or -SIV specific immunity in small animals (14). In the present study, we have demonstrated the promise of a codon-optimized rBCG-HIV vaccine, one which could, even at low doses, elicit long-lasting cell-mediated immune responses without triggering humoral immunity.

Previous reports have demonstrated that a high-dose intravenous inoculation of BCG can induce disease progression, as it did, for example, with BCG-specific $CD4^+$-T-cell activation in monkeys infected with SIV (6). Others have reported a correlation between the magnitude of T-cell activation of CDR3-restricted cells and the disease progression to AIDS in monkeys (41). These results suggest that these $CD4^+$ T cells, once activated by a high dose of any live vaccine, may become infectious and even lead to the replication of the immunodeficiency virus at the coinfection stage. In this regard, our previous study indicated that high doses of BCG did indeed induce a remarkable expansion of I^a-positive activated T cells in guinea pigs but that intradermal inoculation with 0.1 mg of BCG, the common dose and route of BCG vaccination in humans, did not (35).

In showing that a low-dose vaccination with rBCG-HIV is both possible and practicable with the mycobacterial codon optimization of the foreign HIV gene, we offer here a way around this problem. Collectively, these results suggest that a novel vaccination strategy using a low dose of codon-optimized rBCG-HIV, one comparable to the common dosage used for BCG vaccination in humans, might promote stable cell-mediated immune responses and thereby help establish positive immunity against subsequent immunodeficiency virus infection.

ACKNOWLEDGMENTS

We thank Vijai Mehra and Patricia Fast of the International AIDS Vaccine Initiative and William Jacobs, Jr., of the Albert Einstein College of Medicine for helpful comments.