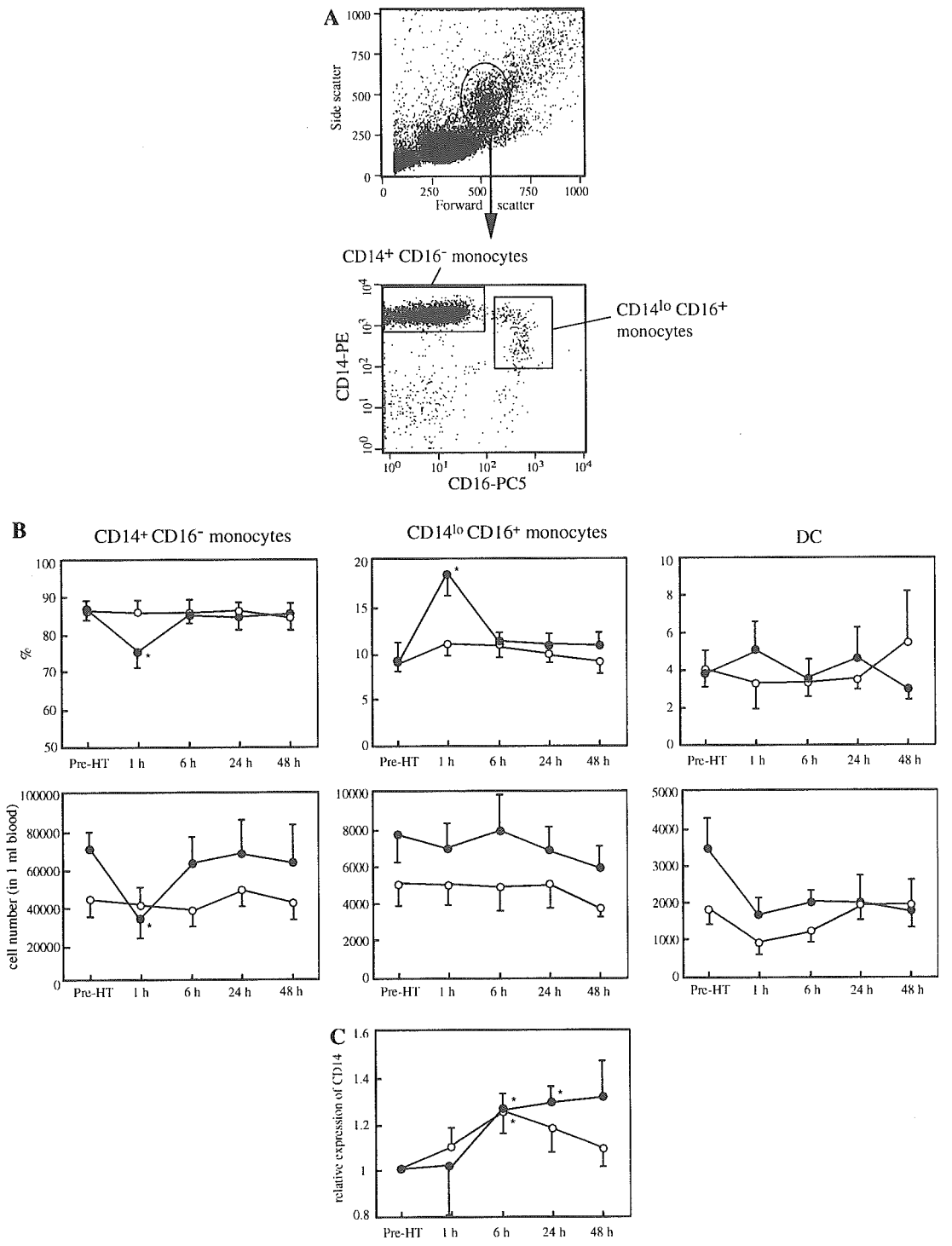

Fig. 4 Effects of the 1 h regional HT on cell numbers of monocyte subsets and their CD14 expression. **a** Monocytes and DCs in PBMC prepared fell into an oval gate in the dot plot of forward and side scatters (*the upper panel*). The monocyte subsets were distinguished by multi-color staining (*lower panels*). **b** Percentages of the above-defined monocyte populations and DCs (*upper panels*) among the whole peripheral monocytes and their absolute numbers (*lower panels*) were analyzed in both groups of volunteers that were exposed to either the liver-targeted (*closed circles*) or lung-targeted (*open circles*) HT treatment. The cell numbers of each subset were calculated by the product of numbers of the entire mononuclear cells counted and the corresponding cell percentages among the mononuclear cells. **c** Cell surface CD14 expression on the $CD14^+$ $CD16^-$ monocytes obtained from the volunteers exposed to either liver-targeted (*closed circles*) or lung-targeted (*open circles*) HT treatment was evaluated by measuring the geometric mean of the fluorescent intensities. The results are represented relative to the expression of CD14 molecules at pre-HT. Each *circle* and *bar* represent mean ± SEM determined from data obtained from five or six volunteers. *Significantly different from the mean value observed before the HT treatment, $P < 0.05$

used as a single treatment modality, we believe that it is currently applicable only in combination with other modalities, as a way to improve the efficacy of chemo-, radio- and, possibly other immunotherapies. The chemo- and radiotherapies often inevitably cause the suppression of host immune responses. Reduced immune responses have also been observed in patients with liver tumors after transcatheter arterial embolization [18]. In contrast, various regimens of HT are known to cause an augmentation in immune responses [4, 12, 24, 25]. This study has provided the evidence that the liver-targeted regional HT treatment induces the full activation of peripheral T cells and suggests the possible enhancement in chemotaxis of monocytes into the liver without severe stress reactions, indicating that this HT treatment may be a promising modality for liver tumors in combination with other anticancer treatments.

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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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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 ($H\text{-}2^d$) 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. $H\text{-}2^d$-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 $\times 10^7$ CFU of bacilli) than that of rBCG-p24Wt (4.7 ± 0.3 ng/5 $\times 10^7$ 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 $\times 10^7$ 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 gaaggctttcagcccagaagtaatacccatgttttcagcattatcagaaggagccaccccacaagatttaaataccatgctaaacacagt
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 ggggggacatcaagcagccatgcaaatgttaaaagagaccatcaatgaggaagctgcagaatgggatagattgcatccagtgcatgcagg
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 cattctggacataagacaaggaccaaaggaacccttagagactatgtagaccgattctataaaactctaagagccgagcaagcttcaca
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..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  *
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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

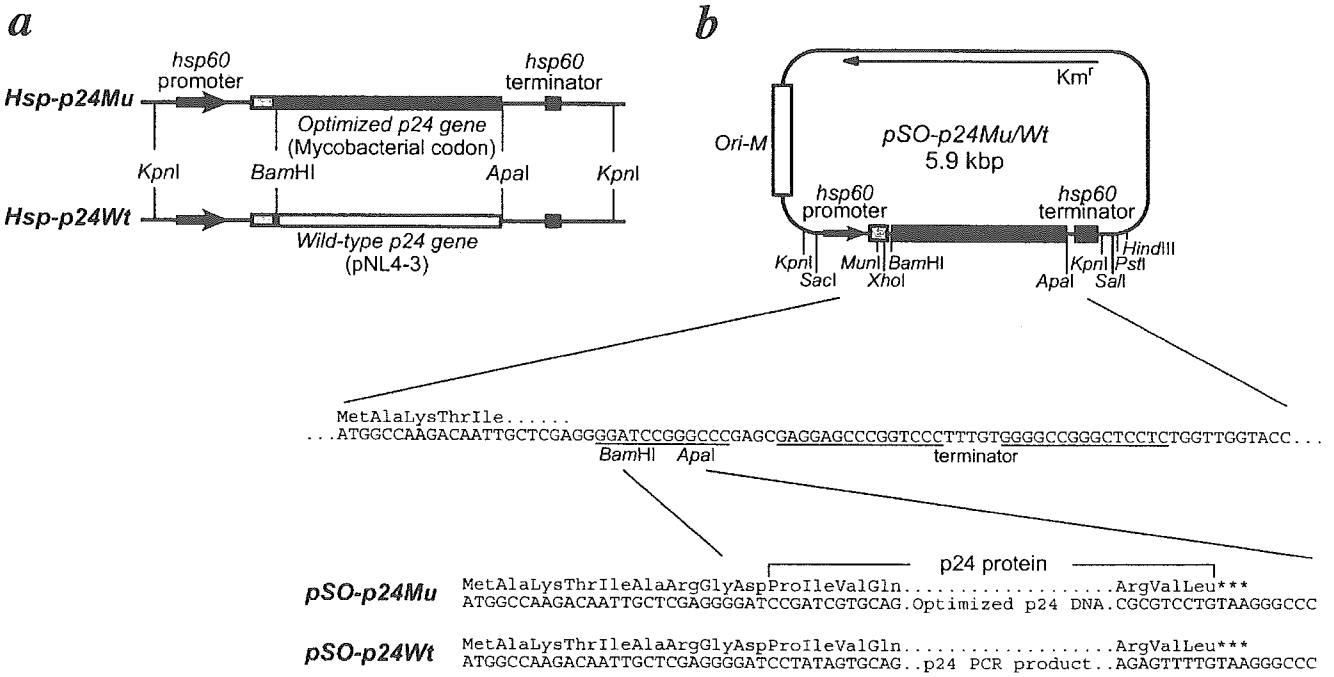

FIG. 2. Structure of expression vectors pSO-p24Mu and -p24Wt. (a) Schematic representation of the expression units of p24. Solid and open bars indicate the synthetic p24 gene and the PCR fragment of the p24 gene, respectively. The arrows and solid squares indicate the transcriptional direction of the *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.

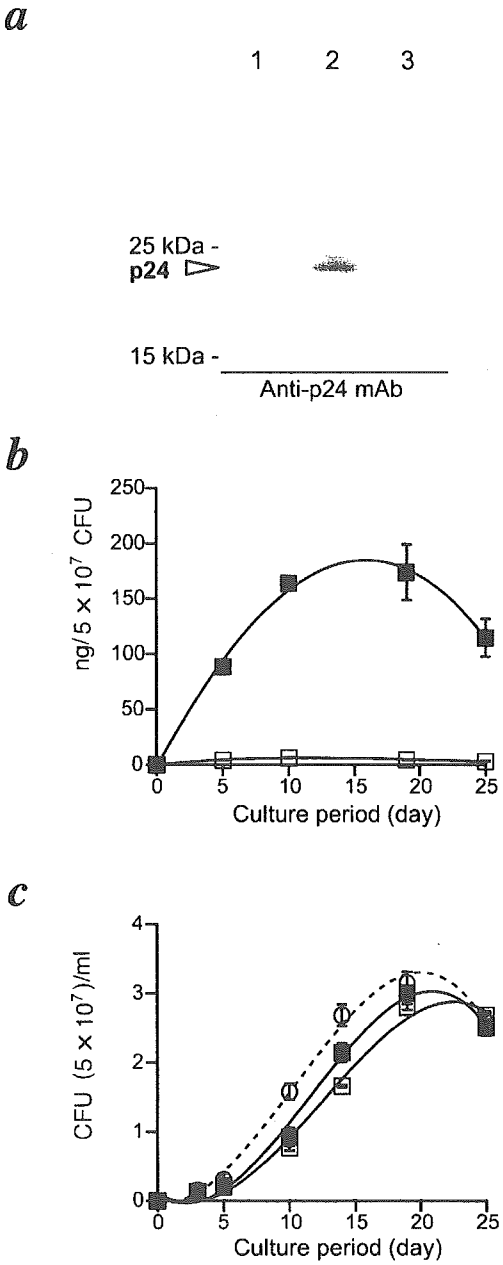

FIG. 3. Comparison of p24 expression levels and growth rates for rBCG-p24Mu and -p24Wt. (a) Anti-p24 monoclonal antibody (V107)-reacted proteins were visualized by Western blotting. Lane 1, lysate of rBCG-p24Wt; lane 2, lysate of rBCG-p24Mu; lane 3, lysate of rBCG-pSO246 (negative control). (b) Comparison 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.

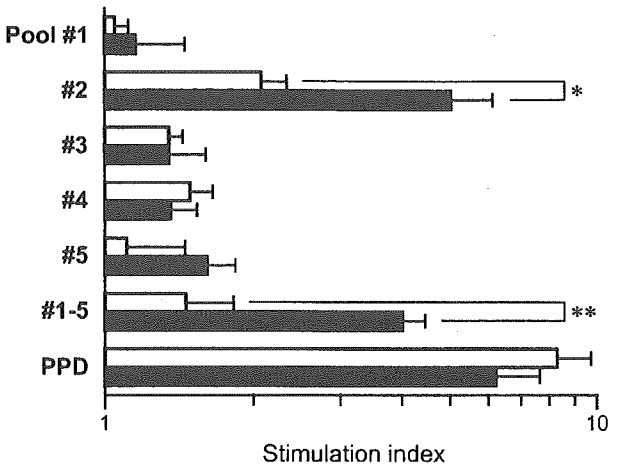

FIG. 4. Lymphocyte proliferation against Gag p24 overlapping peptides and PPD. Splenocytes from immunized animals were cultured with or without antigen for 48 h at 37°C in a humidified 5% CO_2 environment. In the final 6 h before harvesting, [^{3}H]thymidine was added. The level of [^{3}H]thymidine uptake 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-

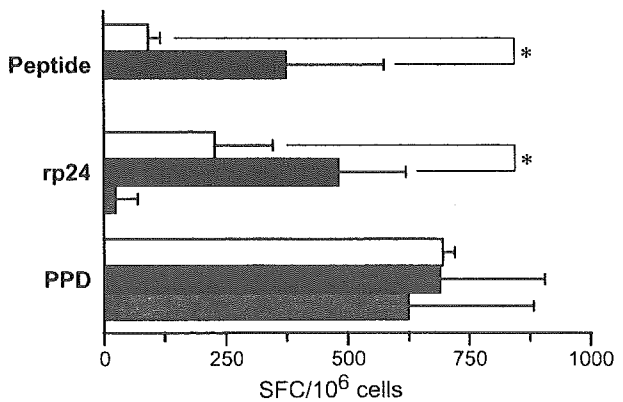

FIG. 5. Determination of antigen-specific IFN-γ-secreting cells by ELISPOT assay. Solid, open, and shaded columns indicate the numbers of SFCs of rBCG-p24Mu-, -p24Wt-, and -pSO246-immunized mice, respectively. The data represent the mean numbers of SFCs/10^6 cells plus one standard deviation. Asterisks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

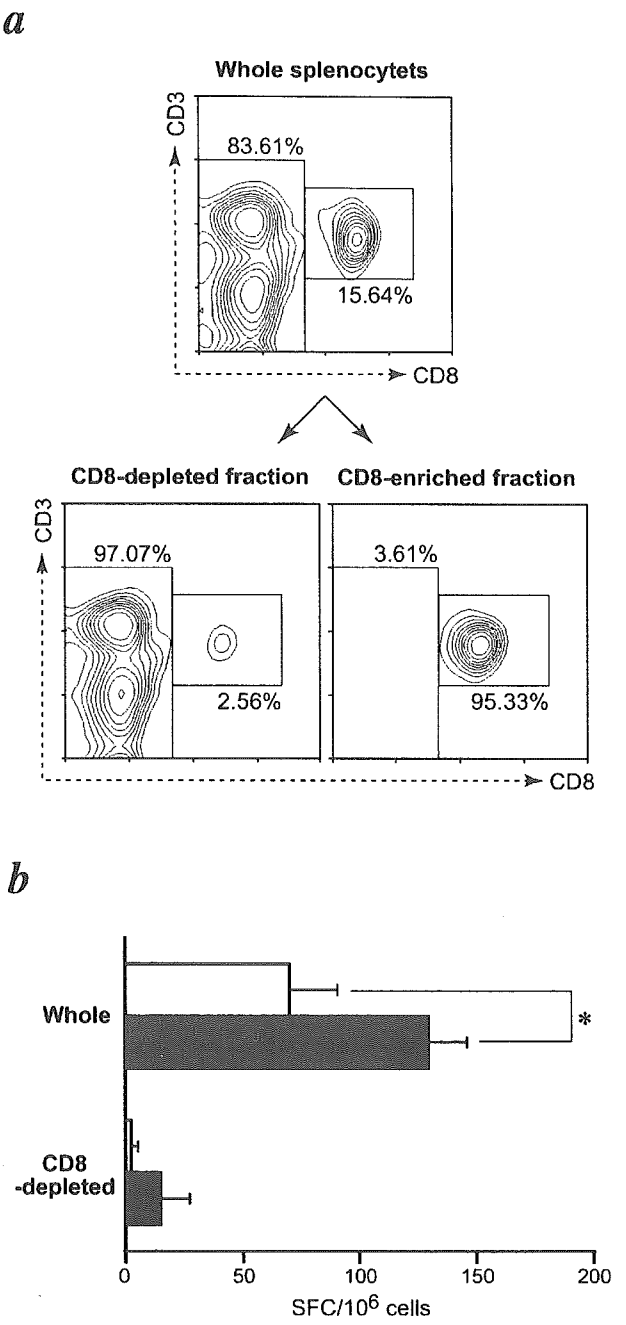

FIG. 6. Decrease of p24-specific cellular immune responses by the depletion of $CD8^+$ T cells. (a) Depletion of $CD8^+$ T cells from splenocytes of immunized animals by magnetic cell sorting of $CD8^+$ T cells by a specific antibody. Splenocytes from mice immunized with rBCGs were sorted and analyzed 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).

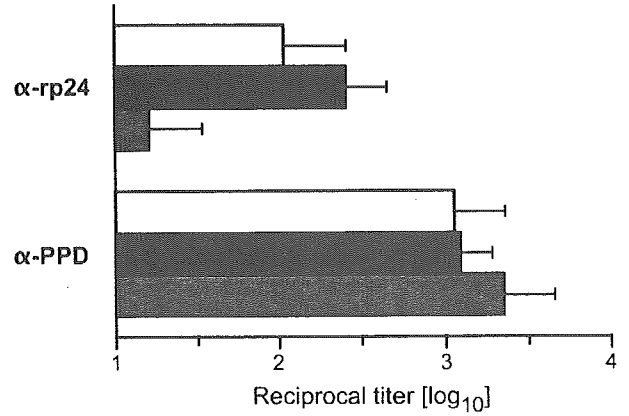

FIG. 7. Anti-p24-specific and anti-PPD-specific IgG antibodies in the plasma of mice immunized with rBCGs. Solid, open, and gray columns indicate reciprocal $\log_{10}$ titers of rBCG-p24Mu, -p24Wt, and -pSO246-immunized mice, respectively. The titers were determined by using an endpoint ELISA. The data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

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Priming-Boosting Vaccination with Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin and a Nonreplicating Vaccinia Virus Recombinant Leads to Long-Lasting and Effective Immunity

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Virus-specific T-cell responses can limit immunodeficiency virus type 1 (HIV-1) transmission and prevent disease progression and so could serve as the basis for an affordable, safe, and effective vaccine in humans. To assess their potential for a vaccine, we used *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-Tokyo and a replication-deficient vaccinia virus strain (DIs) as vectors to express full-length *gag* from simian immunodeficiency viruses (SIVs) (rBCG-SIVgag and rDIsSIVgag). Cynomolgus macaques were vaccinated with either rBCG-SIVgag dermally as a single modality or in combination with rDIsSIVgag intravenously. When cynomologus macaques were primed with rBCG-SIVgag and then boosted with rDIsSIVgag, high levels of gamma interferon (IFN-γ) spot-forming cells specific for SIV Gag were induced. This combination regimen elicited effective protective immunity against mucosal challenge with pathogenic simian-human immunodeficiency virus for the 1 year the macaques were under observation. Antigen-specific intracellular IFN-γ activity was similarly induced in each of the macaques with the priming-boosting regimen. Other groups receiving the opposite combination or the single-modality vaccines were not effectively protected. These results suggest that a recombinant *M. bovis* BCG-based vector may have potential as an HIV/AIDS vaccine when administered in combination with a replication-deficient vaccinia virus DIs vector in a priming-boosting strategy.

As the rate of new infections with human immunodeficiency virus type 1 (HIV-1) continues to increase globally, an effective preventive vaccine is urgently needed to stem further spread of the virus (24). Because long-term survival in humans has been observed when HIV-1 replication is controlled by protective immunity (12, 29), targeted experimental immunogens have been designed to closely mimic the long-lasting protective immunity induced in long-term human survivors by the natural infection (8, 25). Recently, various vaccine modalities, including live viral vectors and DNA, have been used to elicit protective immunity in nonhuman primate models (9). However, before an HIV-1 vaccine regimen can be considered promising, it must be shown to be not only effective at inducing protective immunity, but also safe, affordable, and compatible with other vaccines (2, 32).

When it comes to safety, traditional live vaccines, which have been administered safely to both the healthy and the infected, may be the vectors of choice for HIV-1 vaccines. In order to fully take advantage of the potential benefits of traditional live vectors in HIV-1 vaccine development, we studied the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) substrain Tokyo 172 (6) and the replication-deficient vaccinia virus vaccine strain DIs (22, 50), both of which have been shown to be nonpathogenic when inoculated into immunodeficient animals (41, 51, 53) as live recombinant vaccine vehicles (1, 17–19, 46–48). As further evidence of the potential of the live vectors for use in HIV/AIDS vaccines, we noted that a recombinant *M. bovis* BCG vector candidate vaccine for HIV-1-induced positive immune responses in animals (17, 46). Moreover, we found that recombinant vaccinia virus DIs encoding the simian immunodeficiency virus (SIV) gene was effective at eliciting anti-SIV immunity in mice when administered as a booster antigen after priming with SIV DNA (47). In this study, we have developed a new combination regimen, priming with recombinant *M. bovis* BCG-SIV Gag followed by boosting with rDIsSIVgag. This immunization regimen elicited effective positive immunity against an immune deficiency virus in macaques for the 1 year they were under study.

MATERIALS AND METHODS

Animals and virus challenge stocks. All animals used in this study were captive bred and obtained from the Philippines. They were mature, cycling, male cynomologus macaques (*Macaca fascicularis*) from the Tsukuba Primate Center, the National Institute of Infectious Diseases, Japan. Animals used in these studies were free of known simian retroviruses, herpes viruses, bacteria, and parasites. They were housed in accordance with the Guidelines for Animal Experimenta-

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tion of the Japanese Association for Laboratory Animal Science, 1987, under the Japanese Law Concerning the Protection and Management of Animals (46) and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases, Japan. Once approved by an institutional committee for biosafety level 3 experiments, these studies were conducted at the Tsukuba Primate Center, National Institute of Infectious Diseases, Japan, in accordance with the requirements specifically stated in the laboratory biosafety manual of the World Health Organization. The animals' condition was monitored by analyzing a hemogram parameter as well as absolute $CD4^+$ and $CD8^+$ T-lymphocyte counts with an automated blood analyzer Celltac (Nihon Koden, Tokyo Japan), as described below.

Two thousand 50% tissue culture infectious doses ($TCID_{50}$) of SHIV KS661c, a pathogenic molecular clone, were intrarectally administered as a challenge virus (39). The parent virus, SHIV-C2/1, is an SHIV-89.6 variant isolated by in vivo passage in cynomolgus macaques (40, 42) and the original SHIV-89.6 strain was kindly provided by Y. Lu at the Harvard AIDS Institute (Boston, MA) (26, 37). SHIV-C2/1 and SHIV KS661c were shown to infect cynomologus macaques by both the intravenous and intrarectal routes (39). Both viruses induced high levels of viremia and marked $CD4^+$ T-cell depletion within 2 and 3 weeks after inoculation, respectively (39, 40, 42). Virus stocks were stored at −125°C and thawed just prior to use.

Production and preparation of recombinant *M. bovis* BCG (rBCG) and vaccinia virus DIs expressing full-length SIV Gag. Detailed methods for plasmid construction were described previously (7, 17, 18, 21). Briefly, a DNA fragment encoding the full-length *gag* sequence of SIVmac239 was cloned downstream of the *hsp60* promoter (52) and then inserted into the multicloning site of the plasmid pSO246 (28). Recombinant *Mycobacterium bovis* BCG substrain Tokyo 172 that stably expressed the inserted DNA fragment (designated rBCG-SIVgag) was then selected and used for all rBCG inoculations. For the Western blot analysis, the transformant of rBCG was grown in 7H9-ADC broth for 2 weeks and a portion of the culture medium was periodically collected, sonicated and blotted using the monoclonal antibody IB6, as described previously (47). Since the recombinant DIs virus (rDIs) encoding the SIVmac239 *gag-pol* open reading frame elicited remarkably high SIV Gag-specific T-cell responses but low polymerase responses in mice (47), confirming the findings of a previous report (20), we named it rDIsSIVgag. The rDIsSIVgag and rDIs encoding β-galactosidase (rDIsLacZ) were prepared with chicken embryo fibroblast (CEF) cells (18, 47). Virus preparations were purified by sucrose density gradient ultracentrifugation and were adjusted to 10^7 PFU/ml. P27 antigen generation in cells was measured by antigen-specific enzyme-linked immunosorbent assay (42).

Virus-specific IFN-γ ELISPOT assays. ELISPOT assays were performed using the method developed by and following the direct instructions of Mothe and Watkins, Wisconsin University Primate Center (31, 46). In brief, 96-well flat-bottomed plates (U-CyTech-BV, Utrecht, Netherlands) were coated with anti-gamma interferon (IFN-γ) monoclonal antibody MD-1 (U-CyTech-BV). Freshly isolated peripheral blood mononuclear cells (PBMC) were added with either concanavalin A or pooled Gag peptides (AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD). The cells were then incubated in anti-IFN-γ-coated plates before lysing with ice-cold deionized water. After plates had been washed, rabbit anti-IFN-γ polyclonal biotinylated detector antibody (1 μg per well; U-CyTech-BV) was added. The plates were reacted with gold-labeled antibiotin immunoglobulin G solution by adding 30 μl of the activator mix (U-CyTech-BV) to each of the wells and allowing them to develop for 15 min.

Wells were imaged and spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Carl Zeiss, Germany) (31, 46). An SFC was defined as a large black spot with a fuzzy border. To determine significance levels, we established a baseline for each peptide using the average and standard deviation of the number of SFC for each peptide. A threshold significance value corresponding to this average and two standard deviations were then determined. A response was considered positive if the number of SFC exceeded the threshold significance level of the sample with no added peptide.

Detection of intracellular IFN-γ by flow cytometry. Intracellular macaque IFN-γ was detected by intracellular IFN-γ cytokine staining as previously described (30). Briefly, freshly isolated PBMC was incubated with antigen for 16 h at 37°C with 5% CO_2. During the final 6 to 8 h, brefeldin A (Sigma Chemical Co., St. Louis, MO) was added at 10 μg/ml. Antibody to CD28 (1 μg/ml, BD Pharmingen, San Diego, CA) was also added during the incubation as a costimulator molecule. After stimulation, the cells were stained with fluorescein isothiocyanate-conjugated anti-CD3 (FN18; Biosource, Camarillo, CA) and peridinin chlorophyll protein-conjugated anti-CD8 antibodies (Leu-2a; Becton Dickinson Biosciences, San Jose, CA). The cells were then sequentially incubated with fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson) for 10 min and FACS permeabilizing solution (Becton Dickinson) for another 10 min. The cells were washed, stained with phycoerythrin-conjugated anti-human IFN-γ antibody (4S.B3; BD Pharmingen), and fixed with 2% paraformaldehyde. Samples were analyzed with a FACSCalibur using Cell Quest software (Becton Dickinson).

Lymphocyte proliferative responses. SIV-specific proliferative responses were measured in freshly isolated PBMC as described by Gauduin et al. and Hel et al. (14, 15). PBMC were cultured in flat-bottomed 96-well plates with either concanavalin A or purified SIVmac251 p27 protein (Advanced BioScience Laboratories, Rockville, MD) (15) for three days before the addition of [^{3}H]thymidine. Cells were harvested 16 h later to determine uptake.

Absolute $CD4^+$ and $CD8^+$ T-lymphocyte counts. An absolute cell count of peripheral blood was measured as previously described (55). Briefly, 50 μl of whole blood was placed in a polypropylene tube and incubated with FITC-conjugated monoclonal anti-CD3 (FN18; Biosource), phycoerythrin-conjugated anti-CD4 (Leu-3a; Becton Dickinson), and peridinin chlorophyll protein-conjugated anti-CD8 (Leu-2a; Becton Dickinson) antibodies at 4°C. After incubation with FACS lysing solution (Becton Dickinson), the cells were analyzed with a FACSCalibur using Cell Quest software (Becton Dickinson).

Plasma viral RNA copy numbers. Plasma viral RNA copy numbers were measured using a real-time quantification assay based on the TaqMan system (Applied Biosystems, Foster City, CA) and the Prism 7700 sequence detection system (Applied Biosystems), as reported previously (30, 46). Briefly, viral RNA was extracted and purified from macaque plasma samples using a QIAamp viral RNA mini kit (QIAGEN, Valencia, CA). The RNA was subjected to reverse-transcription and amplification using a TaqMan EZ RT-PCR Kit (Applied Biosystems) with SIV Gag consensus primers SIVmac239-1224F and SIVmac239-1326R, and the SIV Gag consensus Taqman probe FAM-SIV-1272T. To obtain control RNA for quantification, SIVmac239 *gag* RNA was synthesized using T7 RNA polymerase and pKS460, a template plasmid that contains SIVmac239 *gag* under control of the T7 promoter.

To measure the RNA recovery rate, 10^5 copies of SHIV KS661c, in which the viral RNA copy number was previously determined by branched DNA assay (Bayer), were extracted and purified using the same kit as for the sample. Plasma viral load was calculated based on the standard curve of control RNA and the RNA recovery rate. All assays were carried out in duplicate.

Statistical analysis. Data analysis was carried out using the Stat View program (SAS Institute, Cary, NC) and data are expressed as the mean ± standard deviation. A P value of <0.05 was considered significant.

RESULTS

Construction and preparation of recombinant *M. bovis* BCG Tokyo 172 and vaccinia virus DIs expressing whole SIV Gag. In initial studies, we cloned DNA encoding SIVmac239 *gag* downstream of the *hsp60* promoter and the expression unit was inserted into the KpnI restriction site of plasmid pSO246. We also constructed a recombinant *M. bovis* BCG vaccine based on the Tokyo 172 strain expressing the full-length *gag* gene of SIVmac239 (rBCG-SIVgag) (Fig. 1A). The presence of SIV Gag-specific DNA was confirmed in recombinant bacteria by DNA-PCR (42). To determine the in vitro expression of the SIV Gag protein in the cells, we analyzed cell extracts of rBCG-SIVgag bacteria after 2 weeks of culture by Western blot using anti-SIV Gag monoclonal antibody IB6. The rBCG clone produced an SIV Gag recombinant protein that strongly reacted as a single band with the specific monoclonal antibody (Fig. 1B). The concentration of SIV Gag^{P27} protein in transformed bacteria was 28.56 ± 8.30 ng/10^8 CFU of bacilli. In contrast, neither SIV Gag protein nor *gag* DNA was detected in bacteria transformed with rBCG-pSO246, a control construct lacking the SIV *gag* insert and used as a vector control (Fig. 1B).

rDIsSIV*gag* and a control vaccinia virus, rDIsLacZ, were propagated in CEF and adjusted to 10^7 PFU/ml. Using Western blot, we confirmed the expression of each foreign gene in

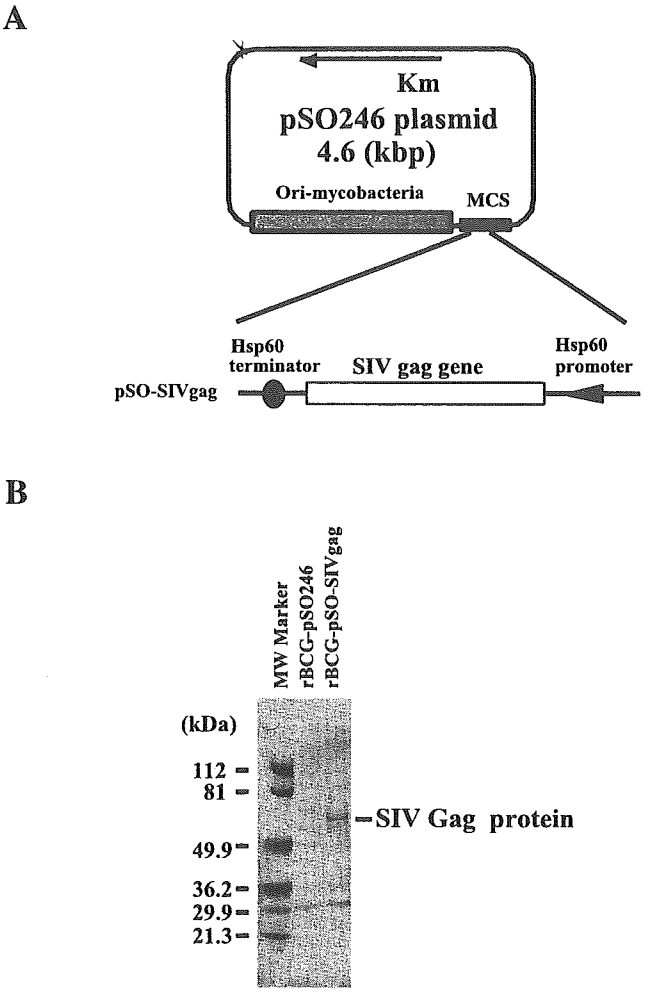

FIG. 1. Vector construction and expression of rBCG-SIVgag. (A) Construction of the expression vector pSO-SIVgag. Full-length DNA of SIVmac239 *gag* was inserted into the multicloning site of pSO246 and expressed in the vaccine strain *M. bovis* BCG Tokyo 172. (B) Detection of SIV Gag protein by Western blot with anti-p27 Gag monoclonal antibody IB6.

the cell extract, and purified virions used as immunogens in this study.

Immune induction after single-modality or combined immunization regimens with vaccine candidates. We examined whether rBCG expressing the full-length *gag* gene of SIVmac239 would be suitable for use in combined prime-boost protocols with the replication-deficient vaccinia virus strain DIs recombinant. The rBCG was intradermally delivered to the inner region of the thigh and rDIs was intravenously administered into the small saphenous vein on the back of the leg. Of the 15 macaques registered in this study, 13 were divided into five groups and immunized using either a single-modality regimen plus vector controls or with a priming-boosting regimen consisting of *M. bovis* BCG and vaccinia virus DIs recombinants (Table 1). The remaining two macaques were inoculated with phosphate-buffered saline and served as naïve controls throughout the experiment.

Group 1 (control group, $n = 3$) served as a vector control group that received rBCG-pSO246 intradermally followed by two inoculations of rDIsLacZ intravenously; group 2 (rBCG group, $n = 2$) received rBCG-SIVgag intradermally followed by two inoculations of rDIsLacZ intravenous, while group 3 (rBCG/rDIs group, $n = 3$) received rBCG-SIVgag intradermally followed by two inoculations of rDIsSIVgag intravenously. Finally, group 4 (rDIs group, $n = 2$) received two inoculations of rDIsSIVgag intravenously followed by rBCG-pSO246 intradermally, while group 5 (rDIs/rBCG group, $n = 3$) received two inoculations of rDIsSIVgag intravenously followed by rBCG-SIVgag intradermally. The 13 immunized and two naive animals were studied for immune induction for 64 weeks before being mucosally challenged with virulent SHIV for a period of 1 year (Table 1).

Antigen-specific T-cell responses in all 15 animals were monitored by SIV Gag peptide-specific IFN-γ-ELISPOT assays (Fig. 2). Fifty weeks postinfection, the rBCG/rDIs group showed the highest SIV Gag-specific IFN-γ-ELISPOT responses; that group's responses peaked at 1,020 ± 360 SFC/10^6 PBMC at 56 weeks postinfection or 2 weeks after the second booster inoculation (Fig. 2A). At 56 weeks postinfection, the ELISPOT responses of the rDIs/rBCG group (380 ± 35 spots per million PBMC, Fig. 2B) were significantly lower than those of the rBCG/rDIs group ($P < 0.05$), as were the ELISPOT

TABLE 1. Immunization and challenge schedule[a]

Group no. (regimen)	Macaque no.	Priming immunization, route, and schedule	Boost immunization, route, and schedule	Mucosal challenge[b]
1 (control)	06, 90, and 91	rBCG-pSO246, 10 mg, i.d., wk 0	rDIsLacZ, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
2 (rBCG)	29 and 93	rBCG-SIV *gag*, 10 mg, i.d., wk 0	rDIsLacZ, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
3 (rBCG/rDIs)	08, 10, and 46	rBCG-SIVgag, 10 mg, i.d., wk 0	rDIsSIVgag, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
4 (rDIs)	01 and 42	rDIsSIVgag, 10^6 PFU, i.v., wk 0 and 8	rBCG-pSO246, 10 mg, i.d., wk 54	2,000 $TCID_{50}$, i.r., wk 64
5 (rDIs/rBCG)	85, 36, and 40	rDIsSIVgag, 10^6 PFU, i.v., wk 0 and 8	rBCG-SIVgag, 10 mg, i.d., wk 54	2,000 $TCID_{50}$, i.r., wk 64

[a] Vaccines, immunization, and challenge studies for all the macaques are described in the text. Animal studies were simultaneously conducted using cynomolgus macaques. i.d., intradermal inoculation; i.v., intravenous inoculation; i.r., intrarectal inoculation.

[b] All of the animals were mucosally challenged with virulent SHIV KS661c at 64 weeks postimmunization and were observed for at least 1 year or, if they did not survive for a year, until the time of their death.

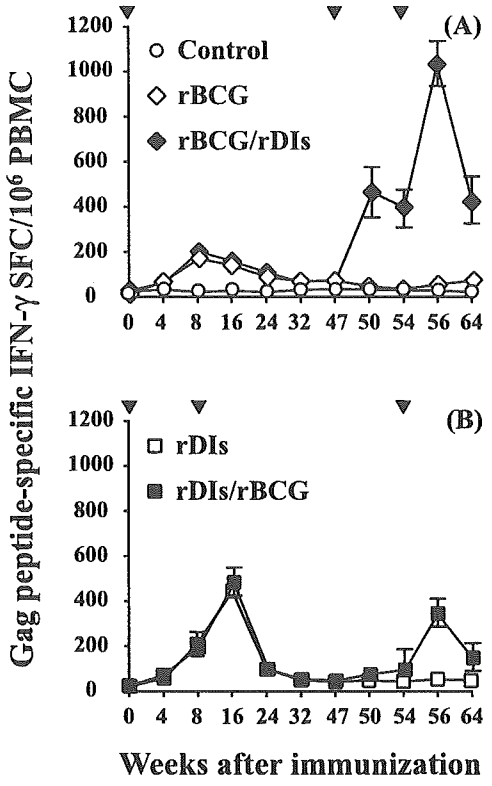

FIG. 2. Kinetics of SIV Gag peptide-specific IFN-γ spot-forming cell responses. PBMC freshly isolated from macaques immunized with either rBCG-SIVgag or rDIsSIVgag alone or with the two in combination were assessed for their ability to produce IFN-γ in response to stimulation by overlapping peptides that span the SIV Gag protein. Arrows indicate inoculation dates of the rBCG/rDIs, rBCG,and control groups, and error bars represent mean ± standard deviation.

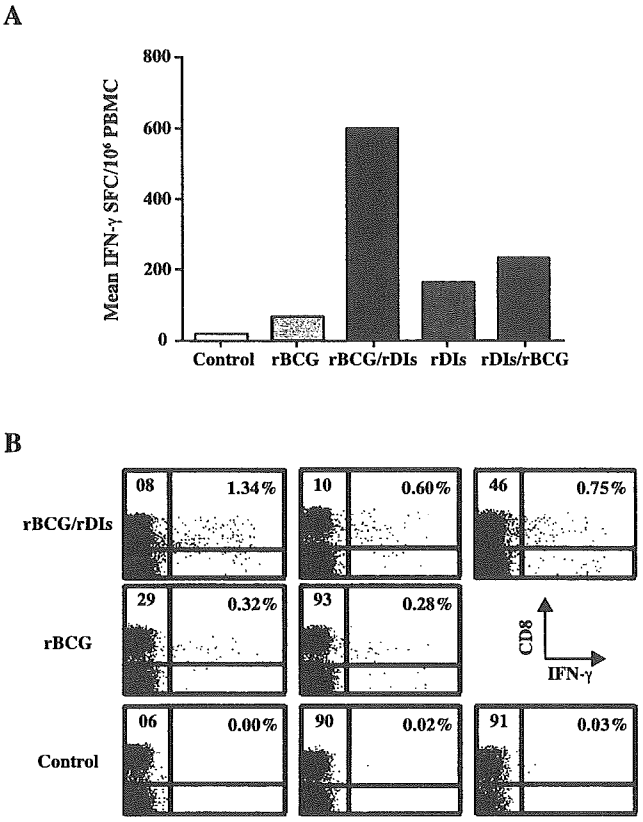

FIG. 3. SIV Gag-specific IFN-γ production in both $CD8^+$ and non-$CD8^+$ T cells in animals immunized with the rBCG/rDIs priming-boosting regimen. (A) SIV Gag protein-specific IFN-γ Elispot responses in immunized monkeys. Monkey PBMC were prepared 2 weeks after final boosting, and 2×10^5 cells were stimulated with 2 μg of recombinant SIV Gag p27 antigen protein. The bars indicate mean values of antigen-specific IFN-γ spot-forming cells per 10^6 PBMC. (B) Flow cytometric analysis of IFN-γ-producing T cells specific for SIV Gag. PBMC from macaques were cultured in vitro with overlapping peptides and stained for intracellular IFN-γ. The percentage of IFN-γ-producing $CD8^+$ T cells in each macaque's PBMC was determined by flow cytometry 2 weeks after final boosting.

responses in other rBCG and rDIs groups, both at 56 weeks postinfection and before mucosal challenge with pathogenic SHIV ($P < 0.05$). Furthermore, the number of SFC in the control and in the two naïve macaque groups did not exceed twenty during the 64-wk immunization period. Thus, the two booster inoculations of rDIs in rBCG-immunized animals effectively induced Gag peptide-specific IFN-γ-ELISPOT responses in peripheral blood, with the booster effect of DIs somewhat resembling that observed in our previous report on DNA/DIs prime-boost immunization in mice (47).

We further studied the induction of SIV Gag-specific IFN-γ ELISPOT by stimulating PBMC with SIV Gag^{p27} protein 56 weeks postinfection (Fig. 3A). The rBCG/rDIs group expressed whole-protein-specific IFN-γ responses of 615 ± 49 cells per million PBMC and the highest peptide-specific ELISPOT responses at 56 weeks postinfection (Fig. 2) of all five groups, with the peptide-specific responses being higher than the protein-specific responses (Fig. 2 and 3A). Other groups exhibited fewer than 200 cells per million PBMC.

To characterize the cellular immune responses in the rBCG/rDIs group, PBMC from the rBCG/rDIs-immunized macaques were compared with those of the rBCG and control groups by staining the surface for CD8 and intracellular SIV Gag-specific IFN-γ expression ($CD8^+$IFN-γ^+ cells) and then performing flow cytometric analysis (Fig. 3B). In vitro stimulation of PBMC with SIV Gag peptides in macaques 08, 10, and 46 of the rBCG/rDIs group generated a higher percentage of $CD8^+$ IFN-γ^+ T cells (1.34, 0.60, and 0.75%, respectively) than it did in animals of the rBCG group. Furthermore, non-$CD8^+$ T cells in PBMC from each animal of the rBCG/rDIs group expressed higher levels of SIV Gag-specific IFN-γ activities (macaque 8: 0.42%; macaque 10: 0.29%; macaque 46: 0.55%) than did those of the other two animal groups. The vector control animals had fewer than 0.03% of both $CD8^+$ IFN-γ^+ and non-$CD8^+$ IFN-γ^+ double-positive cells in PBMC. These findings show that the rBCG/rDIs prime-boost immunization augmented numbers of both IFN-γ-specific intracellular staining-positive cells and ELISPOT in the immunized animals, and that antigen-specific IFN-γ activities were highly induced in $CD8^+$ as well as in non-$CD8^+$ T cells, the latter most likely being $CD4^+$ T cells.

Mucosal challenge study with virulent SHIV KS661c for vaccine efficacy. Ten weeks after the second booster immunization or 64 weeks postinfection, the macaques were chal-

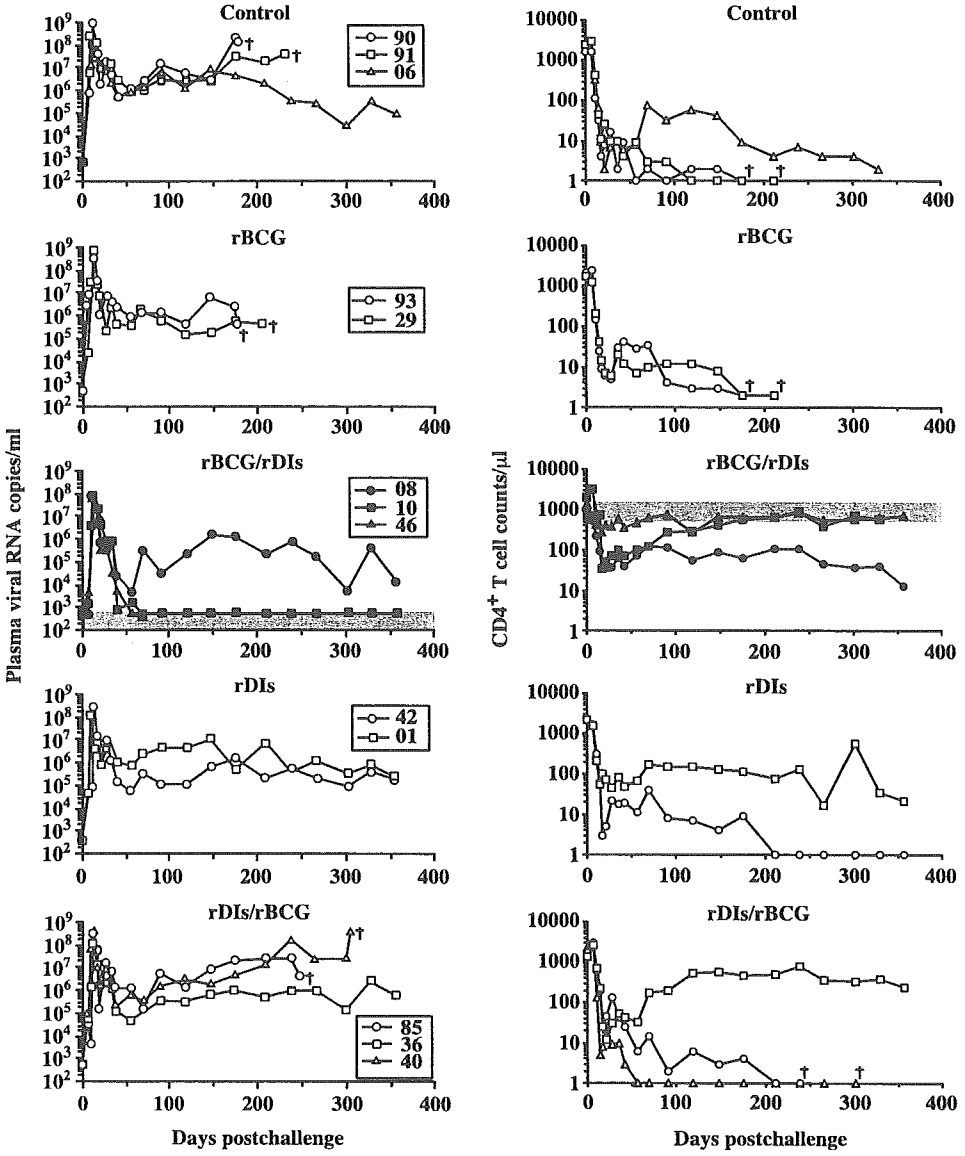

FIG. 4. Plasma viral loads and $CD4^+$ T-cell counts after viral challenge. Postchallenge plasma viral RNA copies and absolute $CD4^+$ T-cell counts in peripheral blood were detected in macaques in each of five groups immunized with a consecutive prime-boost regimen consisting of rBCG-SIVgag and rDIsSIVgag. In the study, 13 macaques were divided into five groups following the experimental designs described in Table 1.

lenged by intrarectal inoculation with 2×10^3 $TCID_{50}$ or 50 50% monkey infectious doses (MID_{50}) of SHIV KS661c, a molecular clone derived from an SHIV-89.6 variant. As shown in Fig. 4, only those macaques in the rBCG/rDIs group first primed with rBCG-SIVgag and then boosted with two inoculations of rDIsSIVgag showed evidence of protective immune responses (rBCG/rDIs). For two animals in this group (macaques 10 and 46), plasma viremia levels remained undetectable (<500 RNA copies/ml, shadow in left panel of rBCG/rDIs in Fig. 4) and $CD4^+$ T-cell counts stayed above 500 cells/μl (shadow in right panel of rBCG/rDIs in Fig. 4) for the entire year of testing. The third animal in this group (macaque 08) had fluctuating levels of viremia that were still significantly lower than those of animals in the other immunization groups. Coincidentally, this animal also had significantly decreased $CD4^+$ T-cell counts.

All macaques in the rBCG/rDIs group remained clinically healthy during the one-year observation period. Those in the rDIs/rBCG group maintained antigen-specific immune responses (Fig. 6), but showed no protective immunity against viral challenge, except for macaque 36 who showed fluctuation in the number of $CD4^+$ T cells, with numbers dipping at times below 500 cells/μl (rDIs/rBCG in Fig. 4). macaques in the other three groups all showed high levels of plasma viremia and a loss of $CD4^+$ T cells, suggesting that vaccination with rBCG and rDIs, either alone or as a priming agent, may not be suitable to induce effective, long-term positive immunity against mucosal challenge by virulent virus. By day 170 after

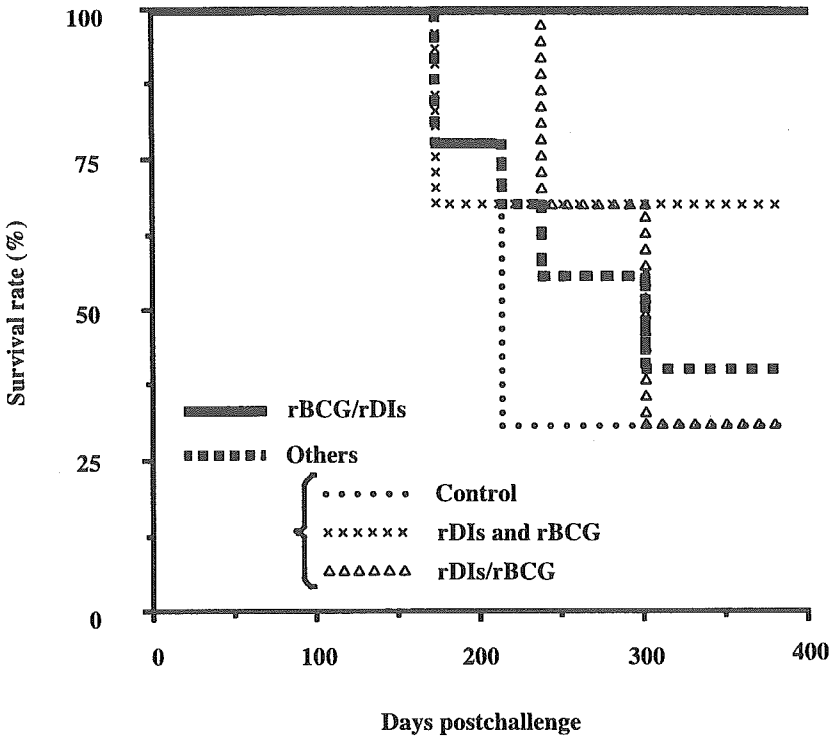

FIG. 5. Survival rates of immunized and control macaques in each of the five immunization groups. A Kaplan-Meier plot of cumulative survival rates at 1 year postchallenge with pathogenic SHIV is shown. The bold line represents group 3 immunized with the rBCG/rDIs priming-boosting regimen; the rectangular broken line represents the mean value for the total number of animals in groups 1, 2, 4, and 5.

challenge, six of the 13 macaques had died with symptoms consistent with simian AIDS: four with interstitial pneumonia, one with neurological disturbances, and one with acquired hemorrhagic diathesis. Analysis of the cumulative survival rate using the Kaplan-Meier plot showed that the rBCG/rDIs group vaccinated with the priming-boosting regimen had a superior survival rate ($P = 0.012$) to the other groups receiving vaccine protocols ($P = 0.548$) and to the control group (Fig. 5). These findings demonstrate that a prime-boost immunization with rBCG-SIVgag/rDIsSIVgag controlled virulent immunodeficiency virus infection in macaques for at least 1 year and more significantly improved survival rates than did other vaccine protocols.

Immune correlates of protection after viral challenge. In order to study virus-specific immune enhancement by SHIV challenge, we followed the postchallenge expansion of the virus-specific IFN-γ-positive cells in each animal by comparing the virus-specific IFN-γ-positive cell numbers pre- and postchallenge (Fig. 6). In all of the challenged animals of the rBCG/rDIs group, the mean number of IFN-γ-positive cells expanded from 369 ± 73 at the time of viral challenge to 629 ± 41 cells per 10^6 PBMC at 7 days after viral challenge, the sharpest increase noted with any of the animal groups. The animals of the rDIs/rBCG group showed much less enhancement, from a mean of 108 ± 46 cells per 10^6 PBMC before challenge to 224 ± 64 postchallenge, demonstrating that cellular immune responses are enhanced by viral challenge in the initial viral infection period in animals. Although in the rBCG/rDIs group high levels of IFN-γ production were observed in both $CD8^+$ and non-$CD8^+$ T cells in all three monkeys, macaque 10 and macaque 46 maintained undetectable setpoint levels of plasma viral load and normal numbers of $CD4^+$ lymphocytes, while macaque 08 did not. The macaques showed no clinical sign of weight loss, lymphoadenopathy, splenomegaly, anemia, or thrombocytopenia in the 1-year observation period. Furthermore, macaques in the rDIs group survived under low

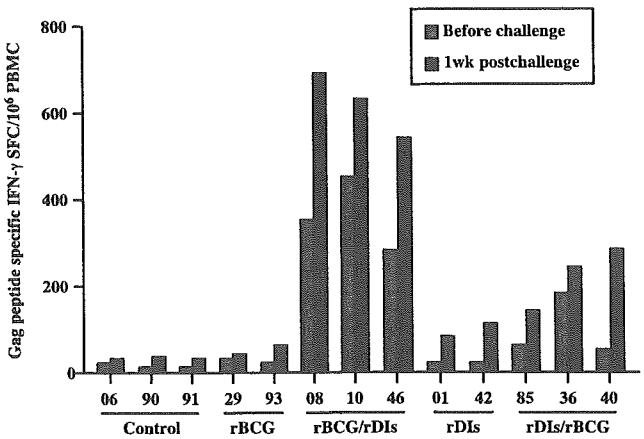

FIG. 6. Virus challenge enhances the SIV Gag peptide-specific IFN-γ ELISPOTs in PBMC from immunized macaques. PBMC from immunized animals were tested with pools of peptides spanning all the proteins from SIVmac239. Results show the production of IFN-γ to pooled peptides in $CD8^+$ and non-$CD8^+$ T lymphocytes.

immune induction but exhibited $CD4^+$ T-cell loss and plasma viremia. Notably, all macaques in the control group exhibited very low levels of immune induction by viral challenge and showed no viral control.

DISCUSSION

In the current study, we initially produced rBCG expressing SIV whole Gag. Second, by introducing a priming-boosting regimen combining rBCG-SIVgag with a nonreplicating rDIsSIVgag, we found that the rBCG/rDIs vaccination induced a long-lasting and effective immunity that was able to control a highly pathogenic virus after mucosal challenge in macaques. Third, elicitation of virus-specific immunity was observed to be important in exerting viral control in the animals immunized with the prime-boost vaccine regimen. Further investigation using larger groups of animals will be needed to determine whether high levels of immune induction correlate with increased efficacy. In this study, the macaques in the rBCG/rDIs group developed high levels of cellular immunity and were protected against the loss of $CD4^+$ T lymphocytes and the increase of viral RNA levels induced by viral challenge. Furthermore, the rBCG/rDIs group showed no evidence of clinical diseases or mortality after viral challenge during the 1-year period of observation.

The rBCG/rDIs prime-boost vaccine controlled the infection efficiently for the duration of the one-year observation period, reducing viral loads to below the threshold level for RNA copies in peripheral blood and maintaining the $CD4^+$ cell numbers above 500 cells per microliter of peripheral blood in two of the three animals in group 3. The remaining animal in the group showed fluctuations in the two parameters. Viral loads and $CD4^+$ cell numbers were not significantly affected in animal groups following the other vaccine regimens. The level of vaccine efficacy for the rBCG/rDIs group seems to be comparable to that observed in previous studies with DNA/fusion protein of interleukin-2 and immunoglobulin G (5), DNA/MVA (3), DNA/recombinant adenovirus type 5 (Ad5) (45), and MVA/recombinant vesicular stomatitis virus (36); that is, effective control of pathogenic SHIV 89.6P infection was achieved in macaques for 6 to 8 months.

SHIV KS661c, which was used as a mucosal challenge virus in this study, is a highly pathogenic molecular clone of a variant of SHIV-89.6 possessing a tropism of CXCR-4. In our preliminary study, the SHIV virus infected GHOST-X4 cells in vitro and the virus challenge eliminated the naïve $CD4^+$ T-cell population in the peripheral blood in macaques, findings which confirmed those by Nishimura et al. (33, 34). In conjunction with CCR5-tropic pathogenic SIVsmE660, Ourmanov et al. obtained similar results with the partial control of homologous viremia by the recombinant MVA vaccination (35). Furthermore, the potential of the DNA vaccination to induce a broad spectrum of mucosal protection against heterologous SIV/DeltaB670 has been demonstrated (13).

Although the virus-specific immune elicitation by DNA/Ad5 vaccination was extremely high in immunized animals (45), the efficacy results for a DNA/Ad5 study with an SIVmac239 were not comparable to those for SHIV 89.6 (43). These discrepancies in vaccine efficacy by challenge viruses suggest that SIVmac239 might be a difficult virus to control by the active immunization of various vaccine candidates. Since DNA/Ad5 is expected to elicit higher levels of immunity than either MVA or DNA alone (43–45), it might be possible to obtain vaccine efficacy in conjunction with different CCR5-tropic SIV or SHIV from SIVmac239. Alternatively, a multicomponent DNA/Ad5 might elicit broad-spectrum immunity as well as protection against SIV or CCR5-SHIV. Recently, a DNA/Sendai virus vaccination (27) proved to be as effective at controlling SIVmac239 as an attenuated live SIV vaccine (10, 11), opening the possibility for studies comparing the protective immunity elicited by ordinary vaccination to that induced by attenuated live SIV vaccination.

Because the lack of an exact HIV-1 macaque model significantly limits our ability to study and calibrate vaccine efficacy, we may need to rely on parameters such as the control of viremia, the loss of $CD4^+$ cells, and the absence of mortality to establish the efficacy of a tested vaccine against an immunodeficiency virus. Certainly, such parameters would represent a more realistic goal for the development of a preventive vaccine in the macaque model. They may also play a key role in the evaluation of vaccine efficacy in human trials I/II using the vaccine modalities developed in the macaque model.

It was recently reported that the AIDS vaccine failed in rhesus macaques approximately six months post-virus challenge, with viral avoidance of cytotoxic T-lymphocyte recognition posing a major limitation to cytotoxic T-lymphocyte-based AIDS vaccines (4). In contrast, the rBCG/rDIs prime-boost vaccine was shown in this study to control viral load throughout the 1-year observation period, suggesting that it may improve the prospects for a vaccine regimen capable of providing long-term protection against HIV-1 replication and disease progression (38, 49). Work is under way to determine whether this rBCG/rDIs vaccine will fail to control the plasma viral load in the macaque model, a failure associated with the viral escape of antigen-specific cytotoxic T lymphocytes.

The route of recombinant DIs administration will be key to effectively inducing immunity in humans. In the preliminary study to determine cellular immune induction, hundred times more rDIs was needed to achieve SIV Gag antigen-specific immunity in macaques by the intradermal (10^8 PFU/ml) than by the intravenous (10^6 PFU/ml) route (K. Someya et al., unpublished data). These findings may suggest that replication-defective vaccinia virus DIs is effective at eliciting antigen-specific immunity by intravenous administration. In addition, they suggest that the intravenous inoculation of rDIs may more effectively induce specific immunity than intradermal inoculation, although intravenous inoculation is not practical for use in human.

This study did not show a clear correlation between levels of virus-specific cellular immunity induced by booster inoculations with rDIs to rBCG-primed animals and protection against a highly virulent immunodeficiency virus after mucosal challenge. The levels of both virus-specific IFN-γ ELISPOT and gamma interferon cytokine staining responses in peripheral blood from animals in the rBCG/rDIs group were the highest of the five groups studied. Why did the prime-boost vaccination of animals of the rBCG/rDIs group prove more effective than the vaccine protocols used with the other groups? We speculate that rBCG priming, which occurs at the skin region of the thigh near the inguinal and iliac lymph nodes

draining the genitorectal mucosa, may elicit mucosal immunity in the region (23). Furthermore, we showed that the two booster intravenous inoculations with rDIs help induce a level of protective immunity sufficient to control a mucosal viral challenge in the immunized animals. Although the two intravenous inoculations with rDIsSIVgag alone proved capable of inducing some virus-specific immunity in peripheral blood after the homologous booster immunization in the immunized animals (DIs group), they appeared to provide no protection against the mucosal viral challenge.

The *M. bovis* BCG/DIs prime-boost vaccination might thus provide the opportunity to study the relationship between protection against mucosal viral challenge and elicitation of systemic or mucosal immunity. Our findings regarding the efficacy of the *M. bovis* BCG/DIs prime-boost vaccine regimen confirm those by Lehner et al. (23) and they further demonstrated a significant association between protection from mucosal rectal infection with SIV and an increase in the levels of CD8 suppressor factor and beta-chemokine. Although we cannot fully explain the differences in vaccine efficacy at this moment, it is likely that the routes of immunization and of challenge, the character of the vaccine vectors and the immunization schedule all play profound roles in eliciting vaccine efficacy in macaques.

Recently, considerable progress has been made in understanding *M. bovis* BCG as a HIV vaccine vector. Our own group demonstrated that recombinant *M. bovis* BCG vectors have the potential to deliver an HIV immunogen for desirable immune elicitation in macaques (46). Furthermore, *M. bovis* BCG vaccine substrain Tokyo 172 was revealed to be avirulent in HIV-infected children (16). The insertion of a full-length SIVmac239 *gag* into the *M. bovis* BCG substrain Tokyo 172 does not affect its toxicity, stability, or efficacy against *Mycobacterium tuberculosis* (54). Furthermore, rBCG has been shown to be nonvirulent in immunodeficient mice (54). These findings highlight the utility of rBCG as a vector for HIV-1 vaccine development.

In summary, our results demonstrate that a prime-boost vaccine regimen using rBCG as the prime and vaccinia virus rDIs as the boost can induce effective immunity against a mucosal infection with a highly virulent immunodeficiency virus for at least a year. Both of the vectors are safe for humans, making them attractive candidates for use in a preventive prime-boost vaccine against HIV-1.

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