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/5×10^7 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

```
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 gggggggacatcaagcagccatgcaaatgttaaaagagaccatcaatgaggaagctgcagaatgggatagattgcatccagtgcatgcagg
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..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 cattctggacataagacaaggaccaaaggaaccctttagagactatgtagaccgattctataaaactctaagagccgagcaagcttcaca
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.....c..c..g..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..c..g..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

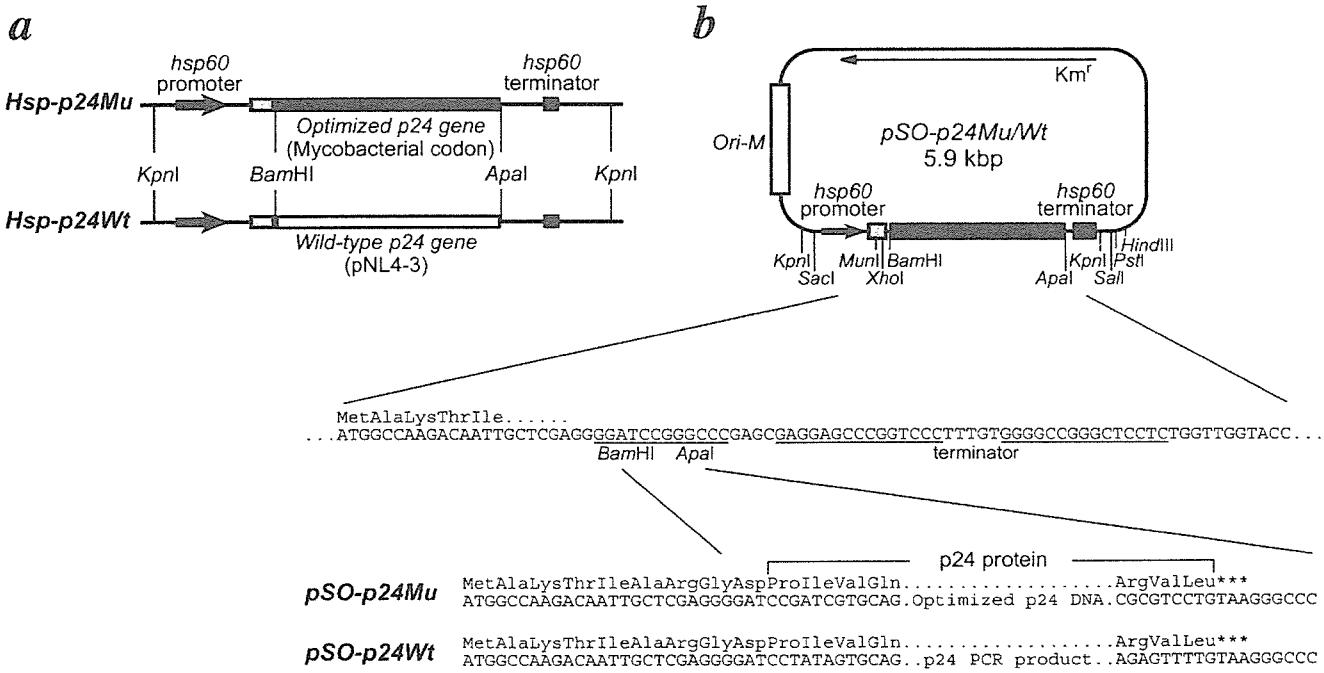

FIG. 2. Structure of expression vectors pSO-p24Mu and -p24Wt. (a) Schematic representation of the expression units of p24. Solid and open bars indicate the synthetic p24 gene and the PCR fragment of the p24 gene, respectively. The arrows and solid squares indicate the transcriptional direction of the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

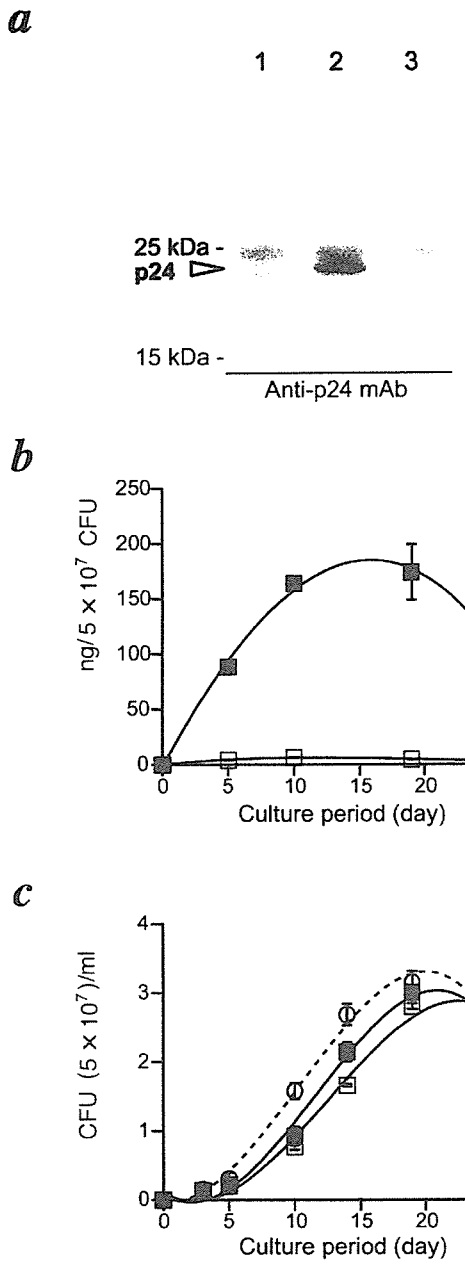

FIG. 3. Comparison of p24 expression levels and growth rates for rBCG-p24Mu and -p24Wt. (a) Anti-p24 monoclonal antibody (V107)-reacted proteins were visualized by Western blotting. Lane 1, lysate of rBCG-p24Wt; lane 2, lysate of rBCG-p24Mu; lane 3, lysate of rBCG-pSO246 (negative control). (b) Comparison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

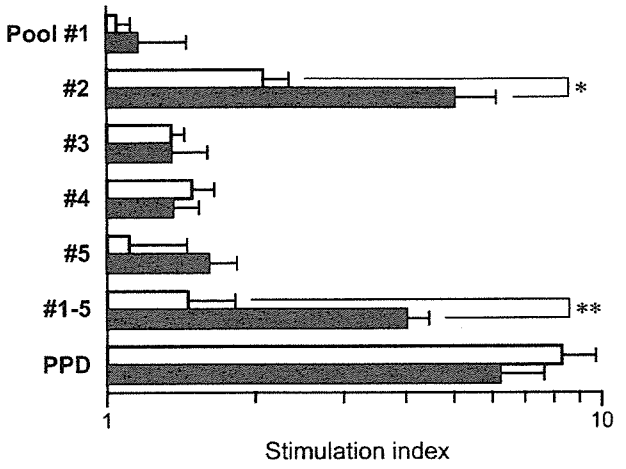

FIG. 4. Lymphocyte proliferation against Gag p24 overlapping peptides and PPD. Splenocytes from immunized animals were cultured with or without antigen for 48 h at 37°C in a humidified 5% CO_2 environment. In the final 6 h before harvesting, [^{3}H]thymidine was added. The level of [^{3}H]thymidine uptake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

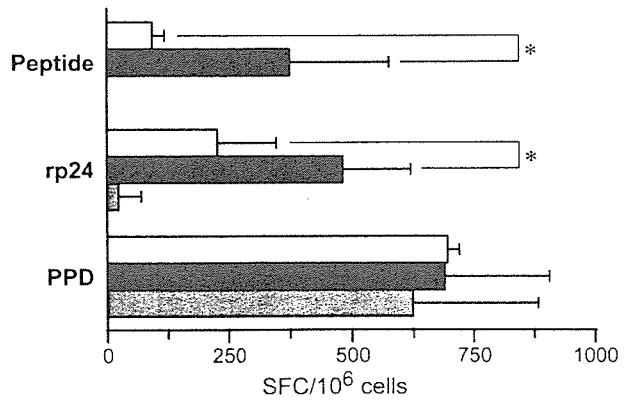

FIG. 5. Determination of antigen-specific IFN-γ-secreting cells by ELISPOT assay. Solid, open, and shaded columns indicate the numbers of SFCs of rBCG-p24Mu-, -p24Wt-, and -pSO246-immunized mice, respectively. The data represent the mean numbers of SFCs/10^6 cells plus one standard deviation. Asterisks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

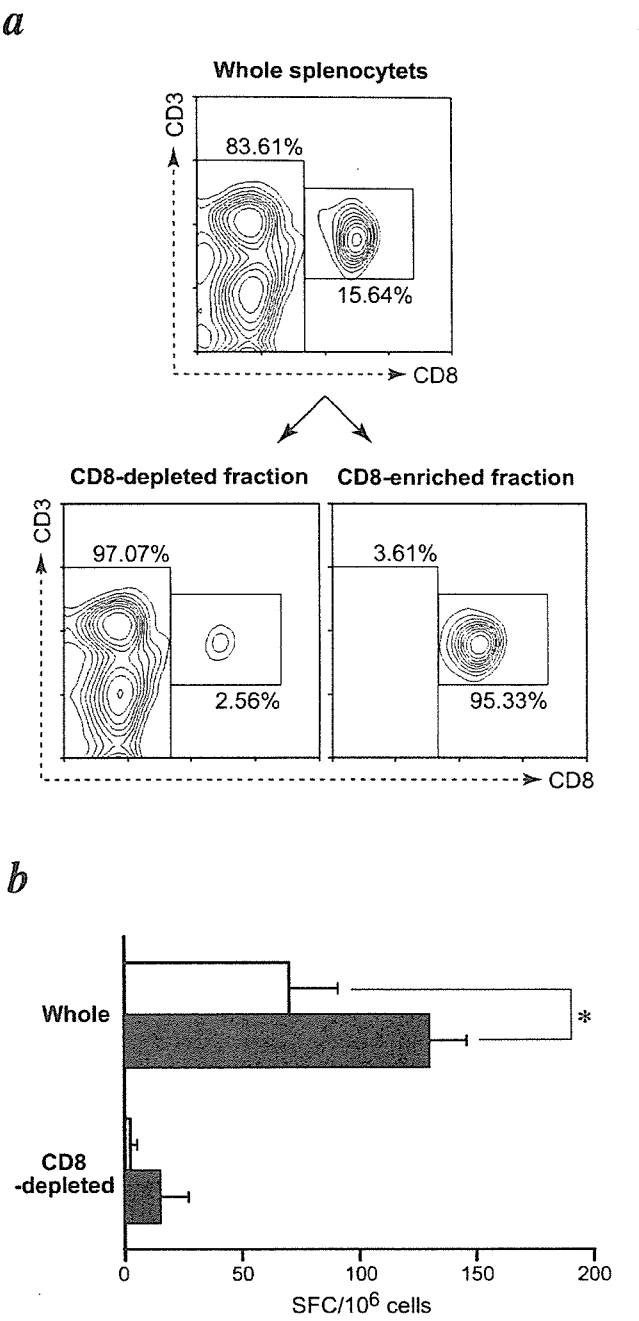

FIG. 6. Decrease of p24-specific cellular immune responses by the depletion of $CD8^+$ T cells. (a) Depletion of $CD8^+$ T cells from splenocytes of immunized animals by magnetic cell sorting of $CD8^+$ T cells by a specific antibody. Splenocytes from mice immunized with rBCGs were sorted and analyzed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

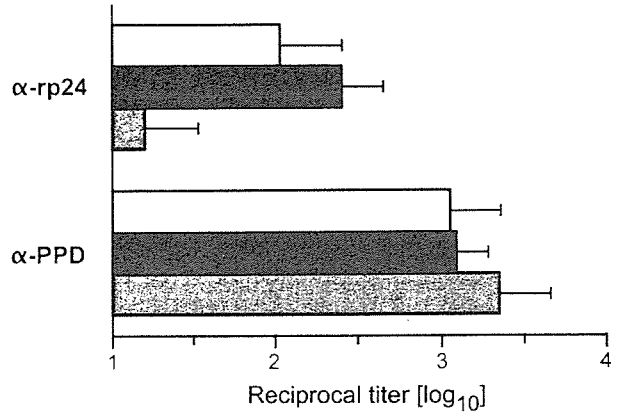

FIG. 7. Anti-p24-specific and anti-PPD-specific IgG antibodies in the plasma of mice immunized with rBCGs. Solid, open, and gray columns indicate reciprocal $\log_{10}$ titers of rBCG-p24Mu, -p24Wt, and -pSO246-immunized mice, respectively. The titers were determined by using an endpoint ELISA. The data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

This work was supported in part by the Panel on AIDS of the U.S.-Japan Cooperative Medical Science Program, the Human Science Foundation of Japan, the Organization of Pharmaceutical Safety and Research, and the Japanese Ministry of Health, Labor and Welfare.

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Intradermal and oral immunization with recombinant *Mycobacterium bovis* BCG expressing the simian immunodeficiency virus Gag protein induces long-lasting, antigen-specific immune responses in guinea pigs

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Received 10 February 2005; accepted with revision 9 November 2005
Available online 4 January 2006

KEYWORDS
Recombinant BCG;
HIV-1;
SIV;
Gag p27;
Proliferation;
IFNγ;
Human dose;
Guinea pig

Abstract To develop a new recombinant BCG (rBCG) vaccine, we constructed rBCG that expresses the full-length Gag protein of simian immunodeficiency virus (rBCG-SIVGag) at a level of 0.5 ng/mg after 3 weeks of bacterial cell culture. Intradermal (i.d.) inoculation of guinea pigs with 0.1 mg of rBCG-SIVGag resulted in the induction of delayed-type hypersensitivity (DTH) responses to both purified protein derivative (PPD) of tuberculin and SIV Gag p27 protein; responses that were maintained for the duration of the 50-week study. In contrast, guinea pigs orally vaccinated with 160 mg of the same antigen exhibited a long-lasting DTH response to the SIV Gag p27 protein, but mounted no response to PPD. Proliferative responses to SIV Gag p27 and PPD antigens were detected in both i.d. and orally immunized animals; however, the levels of PPD-specific responses were significantly higher in guinea pigs immunized by the i.d. than the oral route. A significant increase in the level of PPD- and SIV Gag p27-specific IFNγ mRNA expression was also detected in both immunization groups receiving rBCG-SIVGag. In addition, both i.d. and oral immunization with rBCG-SIVGag induced PPD- and SIV Gag p27-specific serum IgG responses. Insertion of the SIV *gag* gene into BCG did not appear to change the ability of rBCG-immunized animals to elicit PPD-specific immune responses. These results indicate that rBCG-SIVGag has the ability to effectively induce long-lasting, cell-mediated and humoral immunity against both viral and bacterial antigens in guinea pigs, suggesting that rBCG-Gag has the potential to elicit immunities specific not only for tuberculosis but also for HIV at human doses.

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doi:10.1016/j.clim.2005.11.005

Introduction

The epidemic of human immunodeficiency virus type 1 (HIV-1) infection and AIDS (HIV/AIDS) continues to spread worldwide, particularly in Asia and Africa. Globally, 40 million people are now living with HIV/AIDS [1]. In 2003, there were five million new HIV-1 infections, over 90% of them in developing countries [1] where rapid disease progression is more likely to occur due to co-infection with both HIV-1 and *Mycobacterium tuberculosis*. The best hope for individuals living in these countries is the development of a safe, effective and affordable vaccine to prevent HIV-1 infection. Despite recent advances in medical treatments for HIV-1, including highly active anti-retroviral therapy (HAART), most therapeutic drugs remain prohibitively expensive and inaccessible to people living in countries hardest hit by the epidemic [1].

With this dilemma in mind, our group has developed a recombinant BCG (rBCG)-vector system designed to address both the problem of HIV-1 and *M. tuberculosis* co-infection and the issue of cost facing those in developing countries. The rBCG-vector system has been shown to induce immune responses against both HIV-1 and *M. tuberculosis*, and its use is supported by a number of studies demonstrating efficacy in the induction of antigen-specific immunity. For example, it has been reported that BCG and its cell wall components possess adjuvant properties for enhancing the immunogenicity of an antigen when administered to animals [2–4]. Moreover, rBCG expressing HIV-1 antigens can act simultaneously as both an adjuvant and a vehicle to induce antigen-specific immunity [5]. Our own group has previously demonstrated that rBCG containing a 19-amino-acid insert from the HIV-1 Env V3 region (rBCG Env V3) expressed sufficient V3 antigen to induce HIV-1-specific cell-mediated and humoral immune responses in a small-animal model [6–9]. In addition, several groups have also shown the induction of cellular and/or humoral immune responses by inoculation with rBCG expressing HIV or simian immunodeficiency virus (SIV) proteins [10–14]. However, 10- to 100-fold higher doses than that needed for a common BCG vaccination against tuberculosis in humans, or repeated inoculations, were needed to effectively elicit HIV- or SIV-specific immunity in animal models [6–14]. Moreover, previous studies often used intravenous or subcutaneous routes of inoculation; however, vaccination regimens such as these are not practical for use in humans in terms of safety. Furthermore, these BCG recombinants contained a single epitope from HIV or SIV; however, it was reported that rBCG expressing a SIV gag single epitope failed to protect macaques against intravenous challenge with SIV [15].

One of the strategies to practically use a rBCG-based HIV vaccine is to inoculate 0.1 mg of the vaccine into humans via intradermal (i.d.) route as a priming or boosting immunogen because the dose and route of immunization is commonly used for BCG vaccination in humans. For this purpose, we sought to construct a novel rBCG capable of effectively inducing long-lasting, virus-specific immunity by a single i.d. vaccination with 0.1 mg. To elicit antigen-specific immunity with a multi-epitope rBCG vaccine, we chose to target HIV-1 Gag based on evidence of several cytotoxic T lymphocyte (CTL) epitopes in this region [16], some of which are MHC-linked and known to be immunodominant and relatively conserved among various HIV-1 clades [16–19]. Recently, it was shown that Gag-specific T helper cells and CTL correlate inversely with the level of plasma HIV-1 RNA [20–22]. These findings suggest that the HIV-1 Gag region is strongly immunogenic and may induce effective anti-viral responses.

In the present study, we inserted the full-length *gag* gene of SIV into BCG to create rBCG-SIVGag. We then investigated its ability to elicit antigen-specific immune responses in guinea pigs immunized either intradermally (i.d.) or orally with rBCG-SIVGag at human doses and assessed the possibility of the replacement of common BCG vaccination (0.1 mg by i.d. inoculation) by administration of a rBCG-based vaccine.

Subjects and methods

Animals

Female guinea pigs of the Hartley strain (Shizuoka Laboratory Center, Shizuoka, Japan), weighing 200 to 250 g each, were used in a P2-level animal facility at the National Institute of Infectious Diseases (NIID), Tokyo, Japan. The animals were fed in a specific pathogen-free level 2 facility according to NIID animal care guidelines. The study was conducted in the experimental animal area of a biosafety level 2 NIID facility under the guidance of an institutional committee for biosafety and animal experiments.

Construction of a plasmid containing the full-length SIV *gag* gene

A recombinant *Mycobacterium bovis* BCG substrain Tokyo was produced by transfection of BCG-Tokyo strain cells with either the plasmid pSO246 [23] or pSO246SIVGag. The SIVmac239 *gag* gene [24] was amplified by PCR from simian immunodeficiency virus DNA [25] using primers 5′-CCCGGATCCATGGGCGTGAGAAACTCC-3′ (forward) and 5′-CCGCCCGGGCTACTGGTCTCCTCCAAAGAG-3′ (reverse). The resulting PCR product was inserted into the multi-cloning site of pSO246 under control of the *hsp60* promoter of BCG [26]. BCG was transformed with the recombinant plasmid by electroporation and selected on Middlebrook 7H10 agar (BBL Microbiology Systems, Cockeyville, MD) containing 10% OADC enrichment (BBL Microbiology Systems) and 20 μg/ml kanamycin. The resulting recombinant clones containing either pSO246SIVGag or pSO246 were designated rBCG-SIVGag and rBCG-pSO246, respectively.

Western blot and ELISA detection of expressed SIV Gag

Expression of the SIV Gag protein by rBCG-SIVGag was determined by both Western blot and ELISA. rBCG-SIVGag was harvested from Middlebrook 7H9 broth containing ADC (BBL Microbiology Systems) 3 weeks after initiation of the culture, when the growth curve of the transformant had reached its peak. The harvested rBCG-SIVGag was sonicated completely and centrifuged, and the supernatant was heated at 95°C for 5 min in sample buffer (10% 2-mercaptoethanol, 20% glycerol, 123.9 mM Trizma base, 138.7 mM SDS, 3.0 mM bromphenol blue). SDS-polyacryl-

amide gel electrophoresis (PAGE) was performed with a 4–20% gradient polyacrylamide gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). The separated proteins were transferred to nitrocellulose membranes at 144 mA for 30 min, after which the membranes were probed with mouse anti-SIV p27 (kindly supplied by Dr. Sata of NIID) followed by anti-mouse IgG-alkaline phosphatase conjugate (New England BioLabs, Inc., Beverly, MA). Proteins were visualized by reactivity with NBT/BCIP (Roche Diagnostics Co., Indianapolis, IN). The concentration of SIV Gag p27 antigen in the cell extract was determined by a commercial antigen ELISA (SIV Core Antigen Assay kit, Coulter Corporation, Miami, Florida) as per the manufacturer's instructions.

Immunization of guinea pigs with rBCG-SIVGag

Guinea pigs were inoculated with either rBCG-SIVGag or BCG by the i.d. or oral route. For i.d. immunization, six guinea pigs were given a single inoculation of 0.1 mg of rBCG-SIVGag. Three separate control groups consisting of three animals each received i.d. inoculation with either (1) rBCG-pSO246 as a plasmid vector control, (2) BCG-Tokyo as a BCG vector control or (3) saline alone. Prior to oral immunization, fifteen guinea pigs were deprived of food and water overnight. The following day, 1 ml of 3% sodium bicarbonate was administered orally to each animal via a micropipette to neutralize stomach fluid, after which 500 μl of saline containing either 80 mg of rBCG-SIVGag (n = 6), rBCG-pSO246 (n = 3), BCG-Tokyo (n = 3) or saline alone (n = 3) was administrated by the same route. To flush the remaining antigens in their mouths and esophagi, saline (500 μl) was given orally to the animals after antigen ingestion. These procedures were performed on the animals under non-anesthetic conditions. Oral immunization was performed once a week for 2 consecutive weeks, providing a total dose of 160 mg of either rBCG-SIVGag, rBCG-pSO246 or BCG-Tokyo per animal.

Induction of a delayed type hypersensitivity (DTH) skin reaction

To investigate antigen-specific T cell immunity, DTH skin tests were performed at 8 and 50 weeks after immunization with either rBCG-SIVGag, rBCG-pSO246 or BCG-Tokyo. To evaluate SIV Gag-specific DTH responses, 10 μg of SIV Gag p27 protein (Advanced Biotechnologies Inc., Columbia, MD) per 100 μl of saline was injected i.d. into both immunized and non-immunized guinea pigs. To evaluate tuberculosis-specific DTH responses, 0.5 μg of PPD was administered by the same procedure. Saline (100 μl) was used as a negative control. After 24, 48 and 72 h, the diameter of each area of induration was measured.

Isolation of peripheral blood mononuclear cells (PBMC), spleen cells, intestinal intraepithelial lymphocytes (i-IEL) and lamina propria lymphocytes (LPL)

PBMC were separated from heparinized blood using Lympho-separ according to the manufacturer's instructions (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). To isolate spleen cells, guinea pigs were sacrificed while under anesthesia with ketamine hydrochloride (Sankyo Co., Ltd., Tokyo, Japan), and their spleens were harvested. Spleen cells were prepared by gentle dispersion through a 70-μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ). The preparations were treated with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) for 1 min at room temperature to remove red blood cells, and the remaining cells were washed three times in PBS. Preparation of i-IEL was performed as previously described [27–29]. Briefly, large or small intestines were opened longitudinally and washed with PBS containing 1 mM DTT. The tissues were then placed in 20 ml of RPMI 1640 containing 1 mM EDTA in a 50-ml centrifuge tube and incubated for 20 min at 37°C with shaking. After incubation, the tube was shaken vigorously for 15 s, and the cell-containing medium was removed and saved. This process was repeated three times. To isolate LPL, the remaining intestinal tissues were treated with 0.5 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO) and 1.0 mg/ml hyaluronidase (Sigma) for 30 min at 37°C with shaking, and the cell-containing medium was removed and saved. This process was repeated twice, and the harvested cells were then purified through a discontinuous 40/75% percoll gradient (Pharmacia, Uppsala, Sweden).

Antigen-specific T cell proliferative responses

PBMC were re-suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 10 μg/ml gentamicin. The cells were cultured in triplicate wells at a density of 2×10^6 cells/ml in the presence or absence of antigen (20 μg/ml of either PPD or SIV Gag p27) in 96-well U-bottomed plates (Costar, Cambridge, MA) for 3 days at 37°C under 5% CO_2. The cells were then pulsed with 0.5 μCi [^{3}H] thymidine (Amersham, Arlington Height, IL) for the last 6 h of incubation and harvested onto filter paper disks. Uptake of [^{3}H] thymidine was determined by scintillation counting, and the results were expressed as the stimulation index (S.I.), which was calculated as a ratio of the counts per minute in the presence or absence of antigen.

RNA extraction from PBMC, spleen cells, i-IEL and LPL

Isolated PBMC, splenocytes, i-IEL and LPL were adjusted to a concentration of 0.5×10^7 to 1.0×10^7/ml in RPMI 1640 supplemented with 10% FCS, 50 μg of streptomycin, 50 U of penicillin and 10 μg of gentamicin/ml, and then cultured with either 20 μg/ml of PPD or SIV Gag p27 at 37°C for 4 days. Non-stimulated cells were used as controls. Following culture, total cellular RNA was extracted according to the instructions provided with the RNeasy Mini Kit (QIAGEN, Valencia, CA) and stored at −80°C.

To investigate Gag-specific IFNγ responses in T cell subsets, $CD4^+$ and $CD8^+$ T cell populations from the immunized guinea pigs were obtained from PBMC and spleen cells using magnetic cell sorting (autoMACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, after in vitro stimulation of total PBMC and spleen cells with 20 μg/ml of SIV Gag p27 at 37°C for 4 days, the cells were incubated with FITC-conjugated anti-guinea pig CD4 (Serotec Ltd., Oxford UK) or CD8 antibodies (Serotec Ltd.) followed by anti-FITC MicroBeads (Miltenyi Biotec). Non-stimulated cells were used as controls. $CD4^+$ T cell or $CD8^+$ T cell subpopulations

were negatively selected, and then total RNA was extracted with the RNeasy Mini Kit (QIAGEN).

Amplification of guinea pig IFN-γ by reverse transcription (RT) and fluorogenic PCR

To examine antigen-specific IFN-γ mRNA expression in PBMC, spleen cells, i-IEL and LPL, RT-PCR was performed using a TaqMan EZ RT-PCR kit according to the instructions provided (Applied Biosystems, Foster City, California). The reaction mixture consisted of 3 μl of appropriately diluted RNA sample; 5 μl of 5× TaqMan EZ buffer; 3 μl of 25 mM manganese acetate; 0.75 μl each of dATP, dCTP, dGTP and dUTP; 0.25 μl of primer for IFN-γ at 100 μM; 1 μl of fluorogenic probe; 2.5 U of recombinant *Tth* DNA polymerase; 0.25 U of AmpErase uracil-*N*-glycosylase; and 8.25 μl of RNase-free water in a final volume of 25 μl. Thermal cycling conditions consisted of 2 min at 50°C, 30 min at 60°C and 5 min at 95°C, followed by 50 cycles of 10 s at 95°C and 45 s at 62°C. The ABI Prism 7700 sequence detection system (Perkin-Elmer, Applied Biosystems, Inc.) was employed for PCR cycling, real-time data collection and analysis.

Ribosomal RNA (rRNA) was used as an internal control, and its expression level was quantitatively determined using the TaqMan rRNA control reagent (Applied Biosystems) under the same conditions as described above.

The level of cytokine expression was determined in three independent samples for each animal. Quantification was normalized by dividing the amount of IFN-γ mRNA in the target sample by the amount of rRNA in the same sample. Data are shown as fold induction of mRNA and expressed as the ratio of values obtained for antigen-stimulated cells to non-stimulated cells; ratios <1 indicate down-regulation, ratios >1 indicate up-regulation. Oligonucleotide primers (5′-CATGAACACCATCAAGGAACAAAT-3′, 5′-TTTGAATCAGGTTT-TTGAAAGCC-3′) and a fluorogenic-labeled probe (5′-6-carboxyfluorescein-TTCAAAGACAACAGCAGCAACAAGGTGC-6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine-3′) specific for guinea pig IFN-γ mRNA were used for detection and quantification [30]. The RNA standard template used for quantitative determination of guinea pig-specific IFN-γ mRNA was prepared as described by us elsewhere [30].

Antigen-specific serum antibody titration by ELISA

Sera were collected from each guinea pig at 50 weeks after immunization and stored at −80°C. Antigen-specific antibody titers were determined by ELISA. Maxisorp plates (Nunc A/S, Roskilde, Denmark) were coated with either PPD (0.5 μg/well) or SIV Gag p27 (0.1 μg/well) and incubated overnight at 4°C. Serially diluted sera were added to the wells and incubated for 2 h at 37°C. After three washes, rabbit anti-guinea pig IgG-horseradish peroxidase (HRP) conjugate (Zymed Laboratories, Inc., San Francisco, CA) was added to the wells and incubated for 2 h at 37°C, and the plates were then washed and visualized by adding TMB substrate (Moss, Inc., Pasadena, MD). After 30 min at room temperature, rates of absorbance were measured at 450 nm with an ELISA reader. Endpoint titers for antigen-specific IgG were calculated using Microsoft Office Excel and expressed as the last dilution giving an OD_{450} of 0.1 U above pre-immunization serum samples.

Statistical analysis

Data analysis was carried out with the Statistica program (StatSoft, Tulsa, OK). Data are expressed as the mean ± standard deviation (SD), and P values <0.05 were considered significant. DTH responses for the i.d. and orally immunized groups were compared using the unpaired t test, and the responses at week 8 and week 50 for each group were compared using the Student's paired t test. Levels of antigen-specific proliferative responses and IFN-γ mRNA expression for the two groups were compared using the Mann–Whitney U test because of variability in values among animals in each group.

Results

Construction of a rBCG-SIVGag vector expressing full-length SIV Gag

To achieve expression of the complete SIV Gag protein, we inserted the full-length *gag* DNA fragment of SIVmac239 into the plasmid pSO246, yielding a rBCG clone. The resulting recombinant clones (pSO246SIVGag and pSO246) were designated as rBCG-SIVGag and rBCG-pSO246, respectively. Transformation of cells with rBCG-SIVGag and analysis of the cell lysates by Western blot revealed a single band corresponding to 55 kDa consistent with the expected molecular weight of the SIV Gag protein (Fig. 1). The concentration of SIV Gag in the cell lysates was determined by SIV Gag p27 antigen ELISA and found to be 0.5 ng/1 mg of rBCG-SIVGag.

DTH skin responses to PPD and SIV Gag p27 antigen

For each vaccine, DTH skin tests for PPD and SIV Gag p27 antigens were performed at 8 and 50 weeks after immunization. DTH responses to PPD and Gag p27 antigens peaked 24 h after antigen injection. At week 8, DTH responses to PPD were detected in all six guinea pigs immunized i.d. with rBCG-SIVGag with a mean area of induration of 15.0 mm (Fig. 2A). The magnitude of induration in this group was similar to that seen in both the rBCG-pSO246- and BCG-Tokyo-inoculation groups (mean indurations = 15.5 and 15.0 mm, respectively). In contrast, only three of six animals orally immunized with rBCG-SIVGag exhibited a PPD-specific DTH response, and that response of six animals had a mean induration of 4.1 mm (Fig. 2A). Thus, it appeared that immunization with rBCG-SIVGag induced stronger DTH responses via the i.d. than the oral route (Fig. 2A). The three guinea pigs that showed no PPD-specific DTH responses were also included in further analyses of proliferation, levels of IFN-γ mRNA expression and antibody production. Evaluation of animals receiving rBCG-pSO246 and BCG-Tokyo by the oral route also showed similar levels of PPD-specific DTH reactions (4.6 and 4.3 mm, respectively). At week 50, PPD-specific DTH responses were again detected in all six animals immunized i.d. with rBCG-SIVGag. The mean area of induration of these responses was 12.4 mm (Fig. 2A), equivalent to that seen in animals inoculated i.d. with either rBCG-pSO246 (11.0 mm) or BCG-Tokyo (13.5 mm). However, no DTH responses were seen in

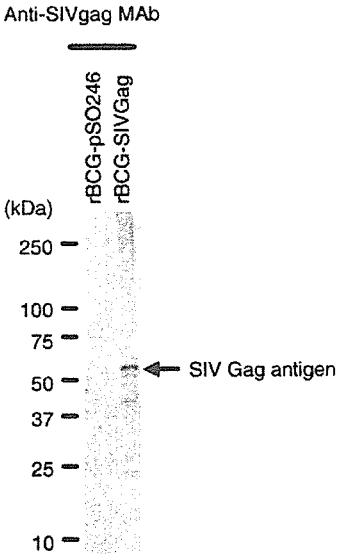

Figure 1 Western blot detection of SIV Gag in rBCG cell lysates. A 55-kDa molecule was identified, corresponding in size to the expected molecular weight of SIV Gag. Insertion of the empty plasmid 246 into rBCG (rBCG-pSO246) was used as a negative control.

any of the animals immunized orally with either rBCG-SIVGag, rBCG-pSO246 or BCG-Tokyo (Fig. 2A). With respect to DTH responses against SIV Gag p27, similar responses were seen at 8 weeks in groups i.d. and orally immunized with rBCG-SIVGag, with mean indurations of 15.8 and 16.1 mm, respectively (Fig. 2B).

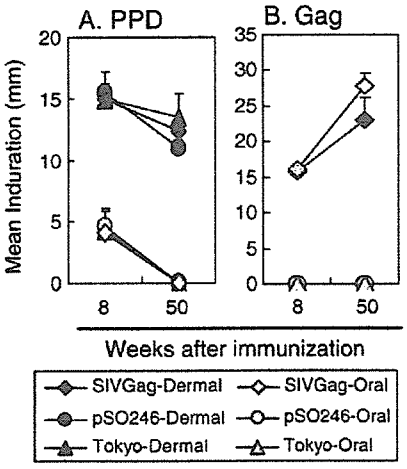

Figure 2 Induction of DTH skin reactions against (A) PPD and (B) SIV Gag p27 in guinea pigs intradermally or orally immunized with rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. DTH induction was performed at 8 and 50 weeks post-immunization. PPD (0.5 μg) and SIV Gag p27 (10 μg) were intradermally injected into the immunized animals, and the diameter of each area of induration was measured 24 h later. Only 3 of 6 animals orally immunized with rBCG-SIVGag exhibited PPD-specific DTH responses. Shown are the mean values ± standard deviation of all animals per group.

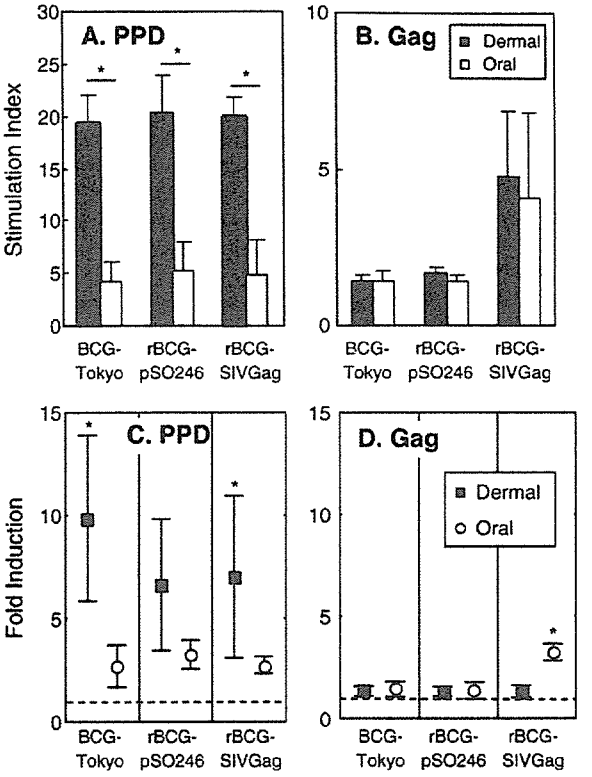

Figure 3 Induction of PPD- or SIV Gag-specific T cell proliferative responses (A, B) and IFNγ mRNA expression (C, D) in PBMC from guinea pigs intradermally or orally immunized with either rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. PBMC were isolated at week 20 after immunization and were cultured with or without antigen (20 μg/ml of either PPD or SIV Gag p27) for 3 days. During the final 6 h of incubation, 0.5 μCi [^{3}H] thymidine was added to each well. The cells were harvested, and the levels of [^{3}H] thymidine incorporation were determined by scintillation counting. Data are expressed as the stimulation index, as described in Subjects and methods. Shown are the means (solid bars, intradermal immunization; open bars, oral immunization) ± standard deviations. To investigate antigen-specific IFNγ responses, PBMC harvested 20 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of either PPD or SIV Gag p27) for 4 days. Total RNA was extracted, and IFNγ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction, as described in Subjects and methods. Shown are the mean values (symbols; ■, intradermal immunization; ○, oral immunization) ± standard deviations. *$P < 0.05$ (i.d. versus oral groups for each vaccine strain).

Interestingly, animals immunized with rBCG-SIVGag via either the i.d. or oral route showed strong, statistically identical SIV Gag p27-specific DTH responses (23.1 and 27.8 mm, respectively) at week 50 (Fig. 2B). The magnitude of the DTH to Gag at week 50 was significantly higher than that at week 8 in the group orally immunized with rBCG-SIVGag ($P = 0.004$), while it did not reach statistical significance in the i.d. immunized group. No significant levels of Gag p27 antigen-specific DTH responses were detected in animals inoculated with either rBCG-pSO246 or BCG-Tokyo via either the i.d. or oral routes (Fig. 2B). In addition, no significant

DTH responses to PPD and Gag p27 antigens were found in control animals inoculated with saline alone (data not shown).

Antigen-specific proliferative responses and IFNγ mRNA expression in PBMC

To investigate the T cell responses specific for SIV Gag p27 and PPD antigens, proliferation assays were performed at week 20 after immunization (Figs. 3A and B). In the groups immunized with rBCG-SIVGag, PPD-specific proliferative responses were evaluated in PBMC from the immunized animals. The levels of these responses were found to be significantly higher in the i.d. immunized group than in the orally immunized group (19.9 ± 2.0 and 4.9 ± 3.2, respectively, $P = 0.02$) (Fig. 3A). Conversely, similar levels of proliferative responses to SIV Gag p27 were elicited by either i.d. or oral immunization with rBCG-SIVGag (4.8 ± 2.1 and 4.1 ± 2.8, respectively) (Fig. 3B). PPD-specific proliferative responses to BCG-Tokyo or rBCG-pSO246 were 19.3 ± 2.8 and 20.2 ± 3.9, respectively, in the i.d. group, while those inoculated by the oral route had proliferative responses of 4.2 ± 1.9 and 5.3 ± 2.6, respectively.

To assess Th1-type helper T cell functions specific for PPD and SIV Gag, IFNγ mRNA expression in PBMC was measured quantitatively at week 20 using real-time, fluorogenic RT-PCR (Figs. 3C and D). In animals receiving rBCG-SIVGag i.d. or orally, the level of IFNγ mRNA expression was elevated following stimulation with PPD over baseline values obtained from non-stimulated PBMC. Mean values specific for PPD in the i.d. and orally immunized groups were 7.0 ± 3.9 and 2.8 ± 0.4, respectively ($P = 0.04$, Fig. 3C). With respect to IFNγ mRNA expression specific for SIV Gag p27, the level was enhanced in animals immunized orally with rBCG-SIVGag, whereas not activated in animals vaccinated i.d. with the vaccine (3.3 ± 0.4 and 1.3 ± 0.3, respectively, $P = 0.02$, Fig. 3D). By comparison, the levels of PPD-specific IFNγ mRNA expression were 9.9 ± 4.0 and 6.8 ± 3.2, respectively, in animals receiving either BCG-Tokyo or rBCG-pSO246 by the i.d. route, and 2.7 ± 1.0 and 3.2 ± 0.7, respectively, for animals inoculated by the oral route.

Long-term antigen-specific IFNγ mRNA expression in PBMC, spleen cells, i-IEL and LPL

To assess whether Th1-type T cells persist in systemic and mucosal compartments, the level of IFNγ mRNA expression was determined at week 50 using PBMC, splenocytes, i-IEL and LPL from guinea pigs immunized either i.d. or orally with rBCG-SIVGag (Fig. 4). Higher levels of IFNγ mRNA induced by PPD were clearly detected in PBMC from guinea pigs immunized with rBCG-SIVGag (Fig. 4A). However, the mean levels of PPD-specific IFNγ mRNA expression were significantly higher in animals immunized by the i.d. route compared to the oral route (205.0 ± 51.2 and 15.5 ± 19.4, respectively, $P = 0.02$) (Fig. 4A). Similarly, the levels of Gag p27-specific IFNγ mRNA expression in the rBCG-SIVGag immunized animals were higher in the i.d. group compared with those in the orally immunized group, but these differences did not reach significance (53.3 ± 50.9 and 6.4 ± 3.2, respectively, $P = 0.15$, Fig. 4B). PBMC from control animals inoculated with

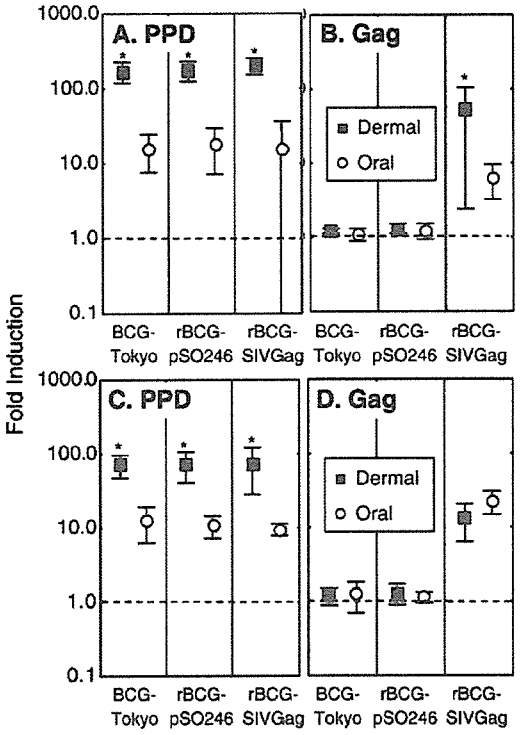

Figure 4 Profile of IFNγ responses at 50 weeks against PPD and SIV Gag p27 antigens in PBMC (A, B) and spleen cells (C, D) from guinea pigs intradermally or orally immunized with rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. Cells harvested 50 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of either PPD or SIV Gag p27) for 4 days. Total RNA was extracted, and IFNγ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction. Shown are the mean values (symbols; ■, intradermal immunization; ○, oral immunization) ± standard deviations. *$P < 0.05$ (i.d. versus oral groups for each vaccine strain).

either BCG-Tokyo or rBCG-pSO246 had levels of PPD-specific IFNγ mRNA comparable to animals vaccinated with rBCG-SIVGag via same immunization route (Fig. 4A). As expected, no Gag-specific IFNγ mRNA responses were found in animals inoculated with either BCG-Tokyo or rBCG-pSO246 (Fig. 4B).

Splenocytes from guinea pigs receiving rBCG-SIVGag expressed considerably higher levels of IFNγ mRNA in response to PPD and SIV Gag p27 compared to baseline values obtained from non-stimulated splenocytes (Figs. 4C and D). PPD-specific IFNγ responses were significantly higher in animals immunized with rBCG-SIVGag by the i.d. route (75.0 ± 46.5) compared with those immunized by the oral route (9.7 ± 1.7, $P = 0.02$, Fig. 4C). Marked increases in the response to SIV Gag p27 were found in both i.d. and orally immunized animals receiving rBCG-SIVGag (13.6 ± 7.2 and 22.8 ± 7.8, respectively, Fig. 4D). Splenocytes from animals inoculated with either BCG-Tokyo or rBCG-pSO246 had levels of PPD-specific IFNγ responses similar to those from animals vaccinated with rBCG-SIVGag via the same immunization route (Fig. 4C), while no Gag-specific IFNγ responses were observed in these animals (Fig. 4D).

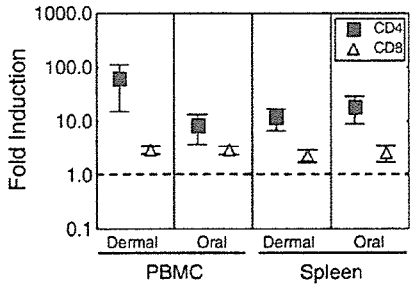

Figure 5 Profile of IFN-γ responses at 50 weeks against SIV Gag p27 antigen in PBMC and spleen cells from guinea pigs intradermally or orally immunized with rBCG-SIVGag. Cells harvested 50 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of SIV Gag p27) for 4 days. After separation of $CD4^+$ and $CD8^-$ T cell subsets, total RNA was extracted, and IFN-γ mRNA levels were measured quantitatively by real-time RT-PCR. The results are expressed as the fold induction. Shown are the mean values (symbols; ■, $CD4^-$ T cell subsets; △, $CD8^-$ T cell subsets) ± standard deviations.

To further investigate Gag-specific IFN-γ responses in T cells, PBMC and splenocytes from guinea pigs vaccinated with rBCG-SIVGag were separated at week 50 into $CD4^+$ and $CD8^+$ T cell subsets. Higher levels of IFN-γ mRNA expression were detected in $CD4^+$ T cells in comparison to $CD8^+$ T cells from PBMC and splenocytes from guinea pigs in both the i.d. and oral immunization groups (Fig. 5). The mean values of Gag-specific IFN-γ responses were 63.5 ± 48.4 and 8.4 ± 4.8 for PBMC $CD4^+$ T cells, and 11.7 ± 5.2 and 18.8 ± 10.0 for spleen-derived $CD4^+$ T cells, in the i.d. and orally immunized groups, respectively. Comparatively, the magnitude of Gag-specific IFN-γ responses in $CD8^+$ T cell subsets from PBMC and splenocytes ranged from 2.3 to 3.0 in the i.d. and orally immunized animals (Fig. 5). These results indicate that long-lasting, Gag-specific IFN-γ responses are induced by immunization with rBCG-SIVGag, and these responses are mediated to a large extent by $CD4^+$ T cells at 50 weeks after vaccination.

PPD- and Gag-specific IFN-γ responses were also determined for i-IEL from the large intestines of guinea pigs receiving rBCG-SIVGag. The mean values of PPD-specific IFN-γ responses were 1.1 ± 0.4 and 0.7 ± 0.1 for the i.d. and oral groups respectively, while those for Gag-specific IFN-γ responses were 1.7 ± 0.7 and 0.8 ± 0.1, respectively. Similarly, LPL from the small intestines of the i.d. and orally immunized animals had mean values for PPD-specific IFN-γ responses of 1.0 ± 0.2 and 1.1 ± 1.0, respectively, while the means for Gag-specific IFN-γ responses were 1.4 ± 0.2 and 1.3 ± 0.7, respectively. LPL from the large intestines exhibited no significant increase in antigen-specific IFN-γ mRNA expression. i-IEL from the small intestines were not isolated in sufficient quantity for analysis due to the enormous quantity of mucus, which is copious in the small intestine. Thus, our results indicate that no significant increases occurred in levels of IFN-γ mRNA expression upon in vitro stimulation with PPD or SIV Gag p27 in i-IEL and LPL from guinea pigs immunized with rBCG-SIVGag by either route. Previous studies have suggested that i-IEL and LPL have different activation requirements than do PBMC [31,32]. Further study is needed on the antigen-specific mucosal immunity induced by rBCG-SIVGag.

Induction of antigen-specific serum antibody responses

To investigate the induction of humoral immune responses to PPD and SIV Gag p27 in guinea pigs immunized with rBCG-SIVGag, antigen-specific serum IgG titers were determined by ELISA (Fig. 6). Even at 50 weeks after immunization,

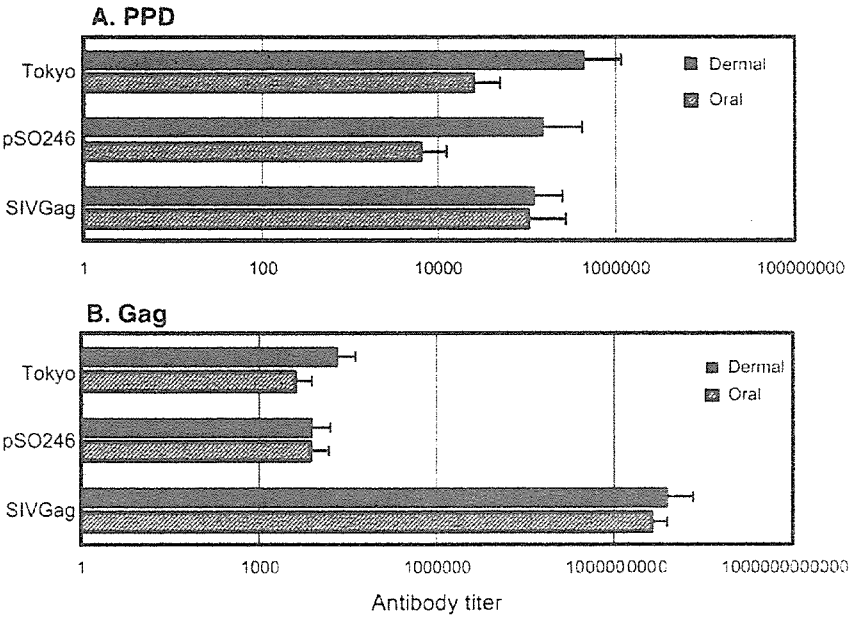

Figure 6 Induction of serum IgG responses against (A) PPD and (B) SIV Gag p27 antigens in guinea pigs intradermally (solid bars) or orally (hatched bars) immunized with either BCG-Tokyo, rBCG-pSO246 or rBCG-SIVGag 50 weeks after immunization. Shown are the mean values ± standard deviations.

significant levels of serum IgG specific for PPD and SIV Gag p27 were detected in all animals from both immunization groups. In comparison to animals inoculated with control preparations of BCG-Tokyo or rBCG-pSO246, the levels of Gag-specific serum IgG in the animals receiving rBCG-SIVGag were 10^6-fold higher, although low but detectable levels of non-specific IgG against Gag p27 antigen could be found in the animals receiving control inoculations compared to non-immunized healthy animals (Fig. 6B). As expected, animals inoculated with BCG-Tokyo and rBCG-pSO246 had serum IgG titers against PPD similar to those seen in the rBCG-SIVGag-immunized group (Fig. 6A).

Discussion

We report here that i.d. or oral inoculation with rBCG-SIVGag induces SIV- and tuberculosis-specific immune responses in guinea pigs, and these responses persist for at least 1 year (the duration of the study period). These findings suggest that rBCG-Gag might be used as an immunogen to elicit responses specific for HIV as well as tuberculosis at human doses.

In this study, we used BCG-Tokyo as a parental strain to develop a rBCG vector and confirmed that both the parental BCG and the rBCG had the same advantage of providing long-lasting, cell-mediated, PPD-specific immunity after a single i.d. inoculation. Furthermore, two oral inoculations of rBCG also produced levels of immunity against PPD identical to those seen with the parental BCG. We have evidence that both i.d. and oral inoculation of rBCG can elicit positive immunity against challenge by inhaled *M. tuberculosis* with similar efficacy to that conferred by vaccination with BCG-Tokyo [S. Haga et al., personal communication]. Thus, the ability to induce antigen-specific immunity and provide protective efficacy against *M. tuberculosis* may apply to rBCG as well as the BCG-Tokyo vaccine strain.

In studying the rBCG, we sought to determine how effectively BCG might express an inserted gene from a foreign immune deficiency virus. We found that rBCG produced intracellular SIV Gag protein at a level of 0.5 ng/mg. Even though i.d. inoculation dosages were small, 0.1 mg of rBCG-SIVGag and 0.05 ng of SIV Gag protein per animal, we achieved the induction of a strong immune response specific for SIV Gag that was maintained over a 50-week period of observation. Several possible attributes may contribute to the ability of rBCG to elicit potent immune responses: (i) the BCG bacillus is known to increase immune responses by acting as an adjuvant; (ii) the persistence of live rBCG in host cells may account for its ability to induce long-lasting, specific immunity; and (iii) secretory proteins derived from the mycobacteria, such as α-antigen (also known as MPB59 or antigen 85B), can elicit potent Th1 immune responses [33–35], which have been shown to be beneficial for controlling pathogenic infectious agents [36–39].

The rBCG-SIVGag used here was able to effectively elicit long-term, SIV Gag-specific DTH, proliferative and IFNγ responses in PBMC and splenocytes from either i.d. or orally immunized guinea pigs. Previous reports have shown that the degree of HIV-specific DTH responsiveness, which generally depends on the intensity of helper T cell function, correlates with clinical stability in infected individuals [40,41]. Furthermore, several lines of evidence support the importance of maintaining helper T cell function in controlling viral infection and replication [42,43]. A related study has suggested that the maintenance of HIV-1 Gag-specific proliferative responses helps preserve Gag-specific CTL activity [44]. In addition, IFNγ has been shown to play an important role in controlling HIV-1 and SIV replication [45–48] and *M. tuberculosis* infection [49–51]. In a recent study of HIV-1-infected subjects, the production of IFNγ in response to Gag was associated with a lower viral load set point [52]. Based on these observations, it is conceivable that immunization with rBCG-SIVGag might help control viral load and curb disease progression, although this has yet to be tested in the appropriate animal models.

In the present study, the levels of IFNγ mRNA induced in response to both PPD and Gag were elevated at week 50 compared to week 20. The cells were re-stimulated in vitro with the respective antigens; therefore, our results reflect the IFNγ reaction of effector cells that were differentiated from memory T cells and activated by in vitro restimulation. This finding might be attributed to the number of memory T cells or the ability of the memory cells to differentiate into effector cells. The amount of rBCG-produced Gag antigen is very low, perhaps resulting in low levels of Gag-specific IFNγ responses at week 20. However, once the Gag-specific IFNγ response was evoked by rBCG-Gag, the response was maintained for more than 1 year after immunization. As shown in Fig. 6, serum IgG against PPD and Gag was detected at high levels even at week 50 in rBCG-Gag-immunized guinea pigs, suggesting persistent antigenic stimulation by chronic rBCG infection. Such chronic infection might gradually lead to an increase in memory T cell counts and/or enhancement of differentiation into effector cells.

In guinea pigs, IFNγ-specific tetramers, ELISPOT assays and flow cytometric analyses remain to be developed. Hence, we relied on a method for quantitative determination of antigen-specific IFNγ mRNA expression in $CD4^+$ and $CD8^+$ T cell subpopulations using real-time RT-PCR. Our results indicate that long-lasting IFNγ responses against SIV Gag p27 induced by rBCG-SIVGag inoculation occur mainly in the $CD4^+$ T cell population and not the $CD8^+$ T cell population at the 50-week time point. However, it is unclear whether a $CD8^+$ T cell IFNγ response to Gag p27 may have occurred because the level of Gag-specific IFNγ expression was normalized by using the amount of rRNA in the sample. Namely, in case of a low frequency of Gag-specific memory $CD8^+$ T cells in the sample, it may be difficult to accurately detect enhancement of the Gag-specific IFNγ expression even if such memory $CD8^+$ T cells are activated by restimulation with Gag antigen.

The current study demonstrates that i.d. immunization with rBCG-SIVGag or BCG-Tokyo induces significantly higher DTH responses to PPD than does oral immunization with the same vaccines. Interestingly, guinea pigs lacking a PPD-specific DTH response 8 weeks after oral inoculation with rBCG-SIVGag still exhibited significant proliferative and IFNγ responses to the PPD antigen. Moreover, a significant IFNγ response to PPD was generated in guinea pigs in which a PPD-specific DTH reaction was no longer detected at week 50. These results clearly indicate that a DTH reaction to PPD does not necessarily reflect proliferative and IFNγ responses [30].

It is possible that a T cell subset mediating a DTH response to PPD [53] was transiently localized at a cutaneous area by oral inoculation with rBCG-SIVGag. However, oral immunization with rBCG-SIVGag effectively induced Gag-specific DTH responses over a 50-week period. These findings indicate that oral inoculation of guinea pigs with rBCG-SIVGag engenders distinct DTH kinetics between PPD and Gag antigens, suggesting that different T cell subsets may be responsible. It has been shown that T cells activated by oral vaccination of humans with common BCG preferentially express a mucosal homing $\alpha 4\beta 7$ molecule associated with T cell trafficking to mucosa, resulting in a failure of the immunization regimen to induce PPD-specific DTH responses [54]. However, in this study of guinea pigs, it is unclear why oral rBCG-SIVGag inoculation persistently elicited vigorous Gag-specific DTH responses. It is possible that intracellular expression of SIV Gag within BCG inoculated orally may not be efficiently recognized by antigen-presenting cells in gut-associated lymphoid tissue (GALT). Namely, SIV Gag antigens might be processed and presented after migration of rBCG-SIVGag-infected macrophages to systemic compartments (e.g., the spleen). In the oral immunization group, a Gag-specific T cell subset may then home to systemic compartments including a cutaneous area, rather than to the mucosa. It will be important in future studies to investigate whether mucosal and cutaneous homing molecules are expressed on PPD- and Gag-specific T cell subsets that are induced by oral inoculation with rBCG-SIVGag.

Intradermal or oral vaccination of guinea pigs with rBCG-SIVGag resulted in the production of serum IgG directed to SIV Gag p27 and PPD. Although it is unclear how Gag-specific IgG affects HIV-1 infection or replication in vivo, a reduction in anti-Gag antibody levels has been shown to correlate with the onset of disease progression [55,56]. More recently, it has been reported that IgG2 directed against Gag is associated with a low viral load and high levels of antigen-specific IFNγ production [57]. We were unable to determine the IgG subclass of the serum antibodies in the immunized guinea pigs because of the lack of species-specific reagents. However, it is possible that the Gag-specific IgG observed here might be classified into a subclass corresponding to IgG2 of humans, since a significant Gag-specific IFNγ response was detected. In addition, we previously found that nasal immunization of mice with rBCG Env V3 induced not only antigen-specific IFNγ but higher levels of V3 antigen-specific serum IgG2 than IgG1 [58].

Thus, we have shown that a rBCG vaccine can induce antigen-specific immunity to viral as well as bacterial antigens. It is especially interesting to note that significant levels of Gag-specific immunity were induced by inoculation with rBCG-SIVGag at the dose and route commonly used for BCG vaccination in humans (0.1 mg by i.d. inoculation). These findings suggest that a rBCG-based vaccine targeting the HIV-1 Gag region might be an effective immunogen. Currently, many candidate HIV-1 vaccines are multivalent, utilizing several viral proteins for the induction of broadly reactive virus-specific immune responses. However, recent studies have shown the effectiveness of SIV vaccines expressing a single viral Gag protein, including Mamu-A*01 macaques immunized with either SIV Gag DNA [59] or adenovirus type 5 vectors expressing SIV Gag proteins [60]. Results using these vaccines indicate that expression of Gag alone is sufficient to induce significant efficacy in the macaque model.

However, there are certain drawbacks to using rBCG as a live, vector-based vaccine. One of the most serious concerns is that it might interfere with immunity induced by other tuberculosis vaccines that are based on the same BCG vector. Pre-existing immunity in BCG-vaccinated individuals may lead to rapid neutralization of a rBCG vaccine. However, the anamnestic effect of BCG vaccination would be irrelevant if our ultimate goal is reached—that is, the replacement of common BCG vaccination by administration of a rBCG vaccine to newborns who have no pre-existing immunity to BCG. While it is clear that the safety of a rBCG vaccine must be established for use in humans, BCG-Tokyo may be one of the most suitable BCG substrains to use as an HIV vaccine vector because it is less virulent than other substrains and its inoculation does not cause severe systemic infection in immune deficient animals [61–64]. Based on these findings, rBCG based on BCG-Tokyo may have promise as a suitable vector for an HIV/AIDS vaccine.

Although the current study did not directly address the efficacy of the rBCG-SIVGag vaccine against viral challenge due to the failure of guinea pigs to support infection with HIV or SIV, our results open up the possibility of i.d. immunization with a single, human dose of rBCG-HIVGag against both HIV and tuberculosis; an immunization regimen that might one day replace the common BCG vaccine without requiring any variation in the current dose or protocol.

Acknowledgments

We would like to thank Dr. Michio Ohba of Kyushu University for his insightful comments during our discussion of this study. This work was supported by funding from the Japan Health Sciences Foundation and the Ministry of Health, Labor and Welfare, Japan.

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Ag85B of Mycobacteria Elicits Effective CTL Responses through Activation of Robust Th1 Immunity as a Novel Adjuvant in DNA Vaccine[1]

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CD4$^+$ T cells play a crucial role in CTL generation in a DNA vaccination strategy. Several studies have demonstrated the requirement of CD4$^+$ T cells for the induction of a sufficient immune response by coadministrating DNAs. In the present study we investigated the effectiveness of Ag85B of mycobacteria, which is known to be one of the immunogenic proteins for Th1 development, as an adjuvant of a DNA vaccine. HIV gp120 DNA vaccine mixed with Ag85B DNA as an adjuvant induced HIV gp120-specific Th1 responses, as shown by delayed-type hypersensitivity, cytokine secretion, and increasing HIV-specific CTL responses. Moreover, these responses were enhanced in mice primed with *Mycobacterium bovis* bacillus Calmette-Guérin before immunization of HIV DNA vaccine mixed with Ag85B DNA. Furthermore, these immunized mice showed substantial reduction of HIV gp120-expressing recombinant vaccinia virus titers compared with the titers in other experimental mice after recombinant vaccinia virus challenge. Because most humans have been sensitized by spontaneous infection or by vaccination with mycobacteria, these findings indicate that Ag85B is a promising adjuvant for enhancing CTL responses in a DNA vaccination strategy. *The Journal of Immunology,* 2005, 175: 2541–2547.

The use of adjuvant in vaccination is thought to be useful for enhancing the immune responses to various pathogens and tumors. One of the major advantages of plasmid DNA vaccination is the induction of MHC class I-restricted CTL responses through endogenous production of an Ag similar to viral infection (1). However, plasmid DNA immunization does not fully elicit cellular immune responses against infectious pathogens in some cases. Unlike viral infection, generation of CTLs after DNA vaccination appears to be critically dependent on functions of CD4$^+$ T cells, such as secretion of Th1 cytokines, which facilitate CTL expansion and activity (2), and activation of professional APCs through CD40-CD40L interaction to increase the expression of costimulatory molecules (3–5). Accordingly, simultaneous activation of CD4$^+$ T cells, especially Th1 cells, during priming is a promising strategy for the generation of substantial CTL responses when using a noninflammatory Ag expression system. In many cases, some helper epitopes are already present in a DNA vaccine, and epitope-specific CD4$^+$ Th cell responses are induced after vaccination. However, because CD4$^+$ T cell help for CTL generation does not require a pathogen including a CTL epitope, pathogen-specific CD4$^+$ T cell responses are not necessary for eliciting pathogen-specific CTL immunity (6, 7). This prompted us to use an appropriate molecule as an adjuvant for the induction of an effective CTL response by the activation of CD4$^+$ T cells.

Mycobacterium bovis bacillus Calmette-Guérin (BCG),[3] a currently available vaccine to prevent tuberculosis, is thought to have powerful immunogenic adjuvant activity that augments cell-mediated immune responses by induction of several Th1 cytokines (8). It is also well known that CFA, which contains heat-killed mycobacteria, augments immune responses by activating Th cells. However, the specific proteins that elicit Th1 immunity of BCG are not clear. One immunogenic protein that can induce a strong Th1-type immune response in hosts sensitized by BCG is Ag85B (also known as α Ag or MPT59) (9). Ag85B belongs to the Ag85 family, which participates in cell wall mycolic acid synthesis (10). Moreover, Ag85B is one of most dominant protein Ags secreted from all mycobacterial species, shows extensive cross-reactivity between different species, and has been shown to induce substantial Th cell proliferation and vigorous Th1 cytokine production in humans and mice infected with mycobacterial species, including individuals vaccinated with BCG (9). We previously showed that inoculation of Ag85B-transfected tumor cells enhances the immunogenicity of tumor-associated Ags and elicits a strong tumor-specific CTL response (11). In the present study we evaluated the effectiveness of Ag85B from *Mycobacterium kansassi* as an adjuvant for enhancing cellular immune responses induced by DNA vaccine.

Materials and Methods

Mice

In mice, unlike humans, studies using inbred and congenic strains have demonstrated different fastness against BCG infection among each strain (12). To give resistance to BCG infection, BALB/c (BCG-susceptible strain) × C3H/HeN (BCG-resistant strain; CC3HF1, H-2$^{d/k}$) female mice were used in this study. The mice were housed at the Laboratory Animal Center of Mie University School of Medicine.

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Received for publication February 25, 2005. Accepted for publication May 31, 2005.

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[1] This work was supported by Health Science Research Grants from the Ministry of Health, Labor, and Welfare of Japan and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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[3] Abbreviations used in this paper: BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; DC, dendritic cell; DTH, delayed-type hypersensitivity; FN, fibronectin; MMC, mitomycin C; rVV, recombinant vaccinia virus.

0022-1767/05/$02.00