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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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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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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 IFNγ by reverse transcription (RT) and fluorogenic PCR

To examine antigen-specific IFNγ 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 IFNγ 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 IFNγ 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 IFNγ mRNA were used for detection and quantification [30]. The RNA standard template used for quantitative determination of guinea pig-specific IFNγ 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 IFNγ 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 IFNγ 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

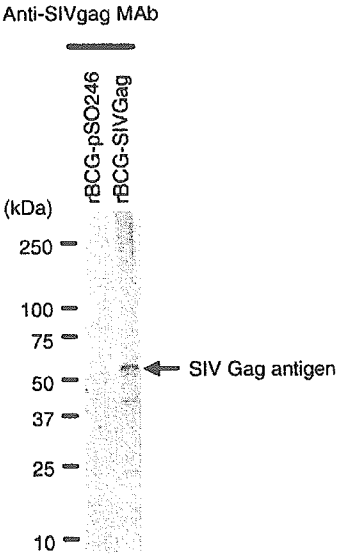

Figure 1 Western blot detection of SIV Gag in rBCG cell lysates. A 55-kDa molecule was identified, corresponding in size to the expected molecular weight of SIV Gag. Insertion of the empty plasmid 246 into rBCG (rBCG-pSO246) was used as a negative control.

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).

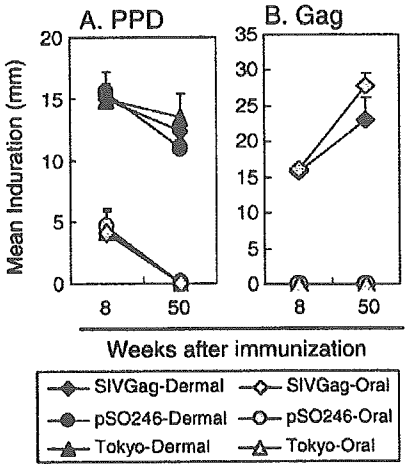

Figure 2 Induction of DTH skin reactions against (A) PPD and (B) SIV Gag p27 in guinea pigs intradermally or orally immunized with rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. DTH induction was performed at 8 and 50 weeks post-immunization. PPD (0.5 μg) and SIV Gag p27 (10 μg) were intradermally injected into 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.

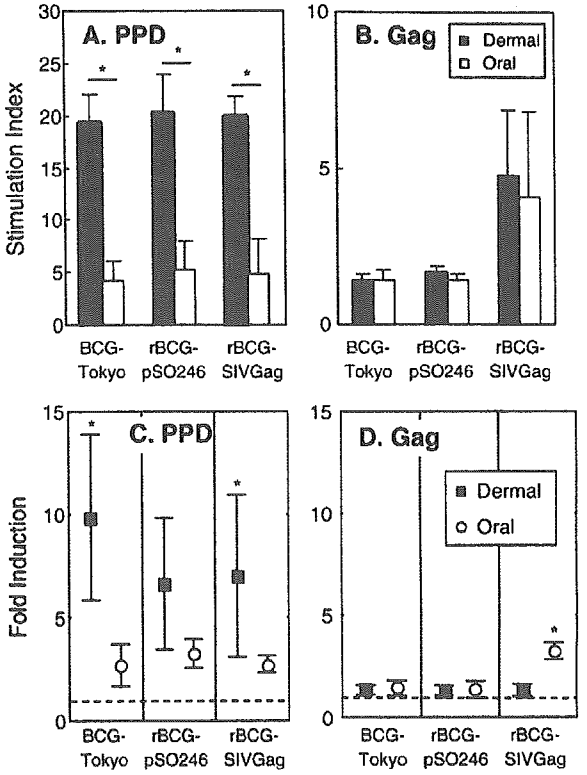

Figure 3 Induction of PPD- or SIV Gag-specific T cell proliferative responses (A, B) and IFNγ mRNA expression (C, D) in PBMC from guinea pigs intradermally or orally immunized with either rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. PBMC were isolated at week 20 after immunization and were cultured with or 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 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).

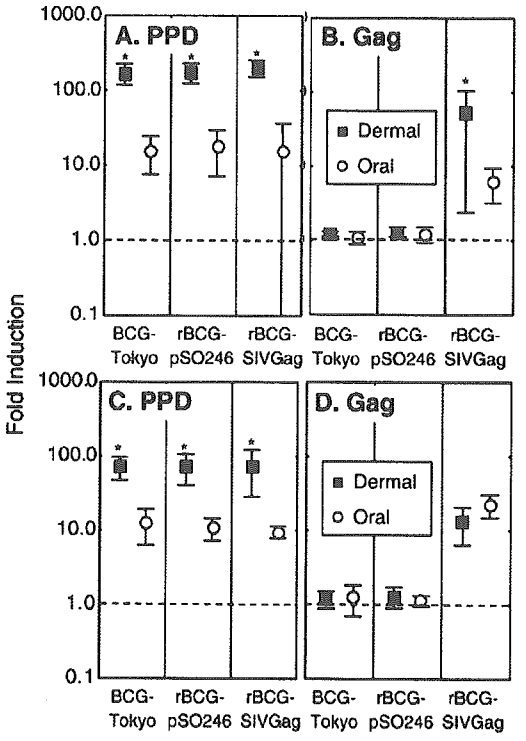

Figure 4 Profile of IFNγ responses at 50 weeks against PPD and SIV Gag p27 antigens in PBMC (A, B) and spleen cells (C, D) from guinea pigs intradermally or orally immunized with rBCG-SIVGag, BCG-Tokyo or rBCG-pSO246. Cells harvested 50 weeks after immunization were stimulated in vitro with antigen (20 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).

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).

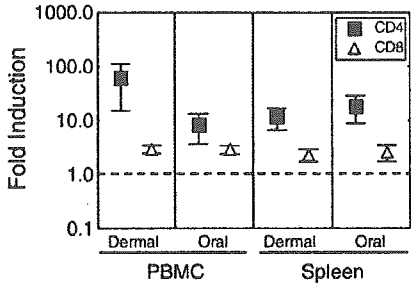

Figure 5 Profile of IFNγ responses at 50 weeks against SIV Gag p27 antigen in PBMC and spleen cells from guinea pigs intradermally or orally immunized with rBCG-SIVGag. Cells harvested 50 weeks after immunization were stimulated in vitro with antigen (20 μg/ml of SIV Gag p27) for 4 days. After separation of $CD4^+$ and $CD8^+$ T cell subsets, total RNA was extracted, and 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; ■, $CD4^+$ T cell subsets; △, $CD8^+$ T cell subsets) ± standard deviations.

To further investigate Gag-specific IFNγ 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 IFNγ 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 IFNγ 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 IFNγ 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 IFNγ 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 IFNγ responses were also determined for i-IEL from the large intestines of guinea pigs receiving rBCG-SIVGag. The mean values of PPD-specific IFNγ responses were 1.1 ± 0.4 and 0.7 ± 0.1 for the i.d. and oral groups respectively, while those for Gag-specific IFNγ 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 IFNγ responses of 1.0 ± 0.2 and 1.1 ± 1.0, respectively, while the means for Gag-specific IFNγ responses were 1.4 ± 0.2 and 1.3 ± 0.7, respectively. LPL from the large intestines exhibited no significant increase in antigen-specific IFNγ 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 IFNγ 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,

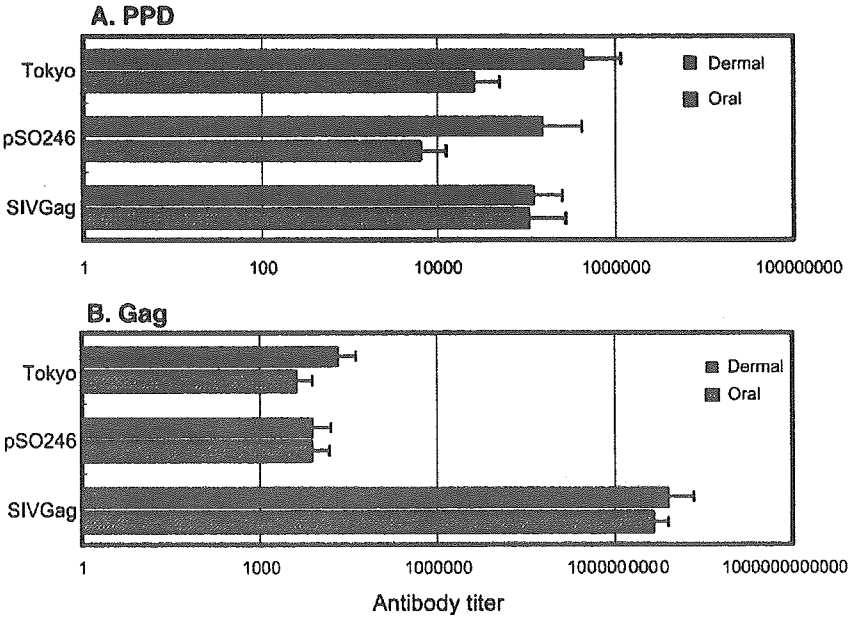

Figure 6 Induction of serum IgG responses against (A) PPD and (B) SIV Gag p27 antigens in guinea pigs intradermally (solid bars) or orally (hatched bars) immunized with either BCG-Tokyo, rBCG-pSO246 or rBCG-SIVGag 50 weeks after immunization. Shown are the mean values ± standard deviations.

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.

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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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[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.

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Plasmid

A highly efficient mammalian expression vector, pJW4303, was used for efficient expression of HIV env gp120 of the NL432 strain (pJWNL432) (13). The Ag85B expression vector pcDNA-Ag85B has been constructed by cloning a PCR product that possesses an Ag85B of *M. kansasii* open reading frame lacking a signal sequence into *Kpn*I-*Apa*I sites of pcDNA 3.1 (11).

Peptide synthesis

The peptides used in this study were an HIV-1 env helper epitope (315–329; RIQRGPGRAFVTIGK; p18) and CTL epitope (318–327; RGPGRAFVTI; p18-I10) in association with the class II MHC molecule I-A^d and the class I MHC molecule H-$2D^d$, respectively (14).

Priming to BCG

Six- to 8-wk-old female mice were primed to BCG by i.p. inoculation of 0.01 mg (dry weight) of BCG (Japan BCG Laboratory).

Immunization

Four weeks after BCG priming, groups of mice were i.m. injected four times with 100 μg of pJWNL432 mixed with or without 100 μg of pcDNA-Ag85B, and then the site of inoculation was immediately given an electric pulse by an Electric Square Porator (T820; BTX) to express both Ags of Ag85B and HIV gp120 in the same tissue, as previously described (15). Pulses were delivered to the muscle using a pair of electrode needles. Eight electric pulses of 50 V were delivered at a rate of one pulse per second. Each electric pulse was 99 ms in duration. Resistance was monitored with a graphic pulse analyzer (Optimizer 500; BTX). To test the dose dependency of Ag85B as an adjuvant, mice primed with BCG were coadministered various doses of pcDNA-Ag85B. Insufficiency of the amount of DNA by reduction of pcDNA-Ag85B was compensated for by mock DNA pcDNA3.1, the original expression vector of pcDNA-Ag85B, to equalize the total volume of administered DNA.

Immunohistochemical analysis

Immunized leg muscles were examined immunohistochemically for in vivo expression of HIV gp120 and Ag85B. Three days after injection, the tibialis anterior muscle was removed, fixed with 4% paraformaldehyde in PBS, and embedded in paraffin wax. Serial sections were prepared and deparaffinized and then incubated with proteinase K for 5 min at room temperature (gp120) or heated by microwave oven three times for 5 min each time (Ag85B) to reactivate the Ag. After incubation with 3% H_2O_2/methanol for 30 min to quench endogenous peroxidase activity, the sections were blocked with normal serum and incubated with anti-HIV gp120 Ab (OEM Concepts) diluted 1/100 or rabbit anti-Ag85B antiserum (16) diluted 1/250 for 30 min at room temperature. Subsequently, the sections were additionally incubated with a biotinylated secondary Ab and HRP-labeled avidin-biotin complex (ABC-peroxidase staining kit Elite; Vector Laboratories). They were then reacted with 0.5% 3.3′-diaminobenzidine tetrachloride and 0.01% H_2O_2 to visualize the bound Abs. Sections incubated with an irrelevant Ab instead of the primary Ab were used as negative controls. Sections were slightly counterstained with hematoxylin.

Delayed-type hypersensitivity (DTH) responses

DTH responses to HIV were elicited by injecting 5 μg of p18 peptide into the footpad of each immunized mouse. The degree of footpad swelling 24 h after the injection was measured using a micrometer and was expressed as the mean increment ± SE of three mice per group (11).

Determination of cytokine production

Spleen cells from immunized mice (5×10^6) were cultured with 2.5×10^6 mitomycin C (MMC)-treated autologous spleen cells labeled with p18 peptide in 24-well culture plates at a volume of 2 ml. After incubation at 37°C in a humidified incubator (5% CO_2) for 48 h, culture supernatants were collected and analyzed for IFN-γ (BioSource International) or IL-4 (Quantikine; R&D Systems) production by an ELISA according to the manufacturer's protocol.

RT-PCR

Total RNA was isolated from leg muscles of the site of immunization using TRIzol (Invitrogen Life Technologies), then reverse transcribed to cDNAs using a SuperScript system (Invitrogen Life Technologies). The resulting cDNA was amplified using TLR sequence-specific primers for 30 cycles of PCR (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min). The following primers corresponding to each TLR were used: 5′-ATGGCAGAAGAT GTGTCCG-3′ and 5′-GTCACCATGGCCAATGTAGG-3′ for TLR2, 5′-TGGATTCTTCTGGTGTCTTCC-3′ and 5′-AGTTCTTCACTTCGCAA CGC-3′ for TLR3, 5′-CTGGCATCATCTTCATTGTCC-3′ and 5′-GCTTAGCAGCCATGTGTTCC-3′ for TLR4, 5′-CAGAACCTTCCTG GCTATTGC-3′ and 5′-AGAGGTTGACCAGACCTTGG-3′ for TLR9, and 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ and 5′-CTTCTG CATCCTGTCAGCAATGCC-3′ for β-actin.

Generation of CTL effector cells

Effector cells were derived from spleen cells as precursor CTLs. $CD8^+$ T cells were purified with anti-CD8 magnetic beads (Miltenyi Biotec) by positive selection according to the recommended protocol (MACS system). Aliquots of 1×10^6 $CD8^+$ T cells were cocultured with 5×10^6 MMC-treated autologous spleen cells labeled with p18-I10 peptide at 37°C in a 5% CO_2 atmosphere. Two days after stimulation, human rIL-2 (Shionogi) was added to all wells at a final concentration of 5 ng/ml. The effector cells generated were harvested after 5 days of culture.

Cytotoxicity assay

MHC-matched (A20.2j) and unmatched (FBL-3) target cells (2×10^6) were incubated at 37°C in a 5% CO_2 atmosphere with or without 10 μg/ml p18-I10 peptide for 16 h. Then the target cells were washed and labeled with ^{51}Cr. The ^{51}Cr-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 10%. Percent lysis was calculated as [(experimental release − spontaneous release)/(100% release − spontaneous release)] × 100. All experiments were performed more than three times, and each group consisted of three mice.

Blocking of cytolysis

Blocking of cytolysis was performed by a method previously described (13). ^{51}Cr-labeled target cells were preincubated at 37°C for 20 min with anti-H-2 K^d, D^d, or L^d mAb (Meiji Institute of Health Science), and effector cells were then added. In a separate experiment, effector cells were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) at a 1/50 dilution with complement (Sigma-Aldrich) for 20 min at 37°C, and then labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h ^{51}Cr release assay.

Evaluation of HIV gp120-specific $CD8^+$ T cells by ELISPOT assay

The number of gp120-specific, IFN-γ-secreting cells was determined by ELISPOT assay. Briefly, 96-well nitrocellulose plates (Millipore) were each coated with 8 μg/ml anti-mouse IFN-γ mAb R4-6A2 (BD Pharmingen) in 100 μl of PBS. After overnight incubation at 4°C, the wells were washed three times with PBS. Then 100 μl of complete medium supplemented with 10% FCS was added to each well, and the plates were incubated at 37°C for 1 h. Triplicate samples of $CD8^+$ T cells separated from the spleen were plated in 2-fold dilutions from 5×10^5 to 6.25×10^4 cells/well. The p18-I10-labeled MMC-treated P815 cells were used as APCs. APCs (1×10^5) were added to each well, and the plates were incubated for 24 h in a 37°C incubator with a 5% CO^2 atmosphere. After stimulation, plates were washed intensively with PBS containing 0.05% Tween 20 and incubated overnight at 4°C with a solution of 2 μg/ml biotinylated anti-mouse IFN-γ mAb XMG1.2 (BD Pharmingen). Afterward, plates were washed with PBS containing 0.05% Tween 20 and 100 μl of streptavidin-alkaline phosphatase (Mabtech) at a 1/1000 dilution was added to each well. Spots were visualized using alkaline phosphatase color development buffer (Bio-Rad) and counted using KS ELISPOT (Zeiss).

Study of protection from vaccinia virus expressing HIV env gp120

The protective ability in immunized mice against systemic infection of recombinant vaccinia virus (rVV) was analyzed by real-time detection PCR as described previously (16). Twelve weeks after the first immunization, mice were challenged i.p. with 5×10^7 PFU of rVV carrying the HIV IIIB gp120 gene (rVV-HIV gp120). Five days after the challenge, the ovaries were harvested and homogenized, and DNA was isolated using a Genomic DNA Isolation kit (Promega). Primers (forward, 5′-GTTCCT TCGCCAACAGGTTAA-3′; reverse, 5′-ACTCGCGATCCTCAAAATG C-3′) and a TaqMan probe (5′-FAM-TTGGAAGCGCCACGGTTACAT TCACT-3′) were selected from the core 4b gene of vaccinia virus. Amplification and detection were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). After incubation at 50°C for 2 min, amplification was begun at 95°C for 10 min, followed by 45 two-step cycles of 95°C for 15 s and 60°C for 60 s.

Statistical analysis

Statistical analysis was performed using Mann-Whitney's U test and the Kruskal-Wallis test. Values are expressed as the mean ± SD. A 95% confidence limit was taken as significant ($p < 0.05$).

Results

In vivo expression of HIV gp120 and Ag85B

It has been reported that $CD4^+$ T cells play a critical role in the generation of CTLs at the site of Ag presentation (17). To induce the effect of Ag85B as an adjuvant for augmentation of cellular immune responses, two DNA vaccines, encoding HIV gp120 and Ag85B, were mixed and administered simultaneously using in vivo electroporation. Three days after immunization, transgene expression was assessed by immunohistochemical analysis in serial sections. Except for slight transient inflammation, no pathological changes were detected in muscles after DNA injection and in vivo electroporation (data not shown) (15). Both HIV gp120 (Fig. 1*A*) and Ag85B (Fig. 1*B*) were observed inside bundles of muscle cells and connective tissue among the muscle fascicles in the same area. Transgene expressions were only seen in the area between the electrode needles.

Coadministration of Ag85B DNA induces strong HIV gp120-specific Th1-type immune responses

As in cases of tuberculosis, one of the important markers of Th1-mediated acquired immunity (not synonymous with protection) is the DTH response. To confirm the ability of Ag85B to induce Th1 responses against coadministered Ag, immunized mice were injected with HIV env helper epitope p18 into footpads, and HIV gp120-specific DTH responses were assessed. As shown in Fig. 2, mice coadministered pcDNA-Ag85B showed greater footpad swelling than mice not administered pcDNA-Ag85B. The effectiveness of Ag85B for inducing Th1-type immune responses to vaccine Ag was augmented by BCG sensitization. In contrast, no significant responses were observed in nonimmunized mice and immunized mice injected with a control peptide (data not shown).

Next, to determine the effect of Ag85B on the patterns of Th1/Th2 cytokine production, we quantified the production of IFN-γ and IL-4 by ELISA in supernatants obtained from 48-h cocultures of peptide-pulsed syngeneic APCs with spleen cells of immunized mice. The production of IL-4 from spleen cells stimulated by p18 in mice immunized with both pcDNA-Ag85B and pJWNL432 was much less than that in mice immunized with pJWNL432 alone. Relatively high levels of HIV gp120-specific IFN-γ production were observed in mice coadministered pcDNA-Ag85B. Furthermore, these Th1-type immune responses were clearly observed when mice were sensitized by BCG inoculation before DNA immunization (Fig. 3). These results are in accordance with the results for DTH responses against HIV gp120 in in vivo experiments and indicate that predominant HIV gp120-specific Th1 responses were induced by coadministration of pcDNA-Ag85B.

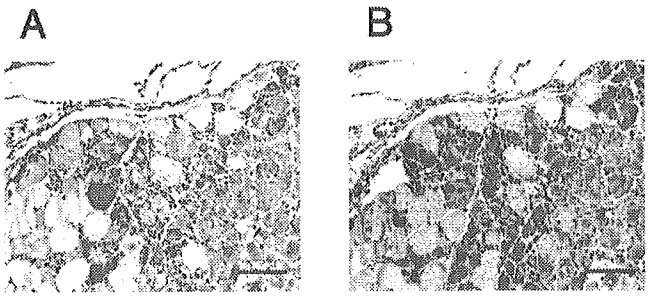

FIGURE 1. Immunostaining of serial sections of muscle tissue from a mouse 3 days after electric administration of pJWNL432 mixed with pcDNA-Ag85B. HIV gp120 (*A*) and Ag85B (*B*) were observed in muscle cells and connective tissue among the muscle fascicles in the same area. Bars represent 100 μm.

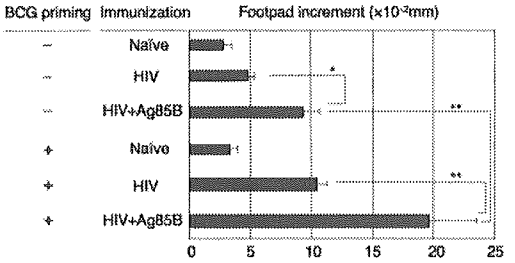

FIGURE 2. Anti-HIV gp120 DTH responses in immunized mice. BCG-primed or unprimed mice were immunized with pJWNL432 with or without pcDNA-Ag85B. The helper epitope peptide of HIV gp120 (p18) was injected into the footpads of immunized mice. The degree of footpad swelling was measured 24 h after the challenge. The results are expressed as the mean footpad increment ± SE of five mice per group. *, $p < 0.01$; **, $p < 0.001$.

Alteration of TLR mRNA expression after Ag85B DNA administration

Various proteins derived from pathogens promote Th1 responses through stimulation of TLRs and subsequently through secretion of cytokines (18). We therefore compared TLR mRNA expression profiles at the site of DNA injection with electroporation in pcDNA-Ag85B-immunized mice and mice immunized with pcDNA alone using RT-PCR. Ag85B stimulated the expression of TLR2, TLR3, and TLR4 mRNA, whereas mock immunization with electroporation resulted in only modest increases in the levels of these TLR mRNAs (Fig. 4). TLR9 mRNA was not detected in either group of mice (Fig. 4). Plasmid pcDNA3.1 has immunostimulatory sequence (six 5′-GACGTC-3′), whereas TLR9 mRNA was not detected in either group of mice under these conditions (Fig. 4). Positive reactions, however, were observed in both Ag85B-injected tissues and control tissues using high cycles (>60) of PCR (data not shown). This positive reaction was not thought to be the effect of Ag85B. These results suggested that Ag85B immunization plays a role in enhancement of the expression of these TLRs, although the possibility of indirect responses by cytokine production cannot be ruled out (18).

Ag85B enhances anti-HIV gp120-specific CTL responses

$CD8^+$ cells from BCG-primed mice and unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were stimulated in vitro with p18-I10-pulsed syngenic splenocytes, and the

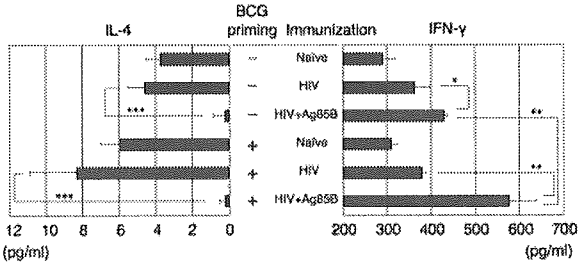

FIGURE 3. Induction of HIV gp120-specific Th1 immune responses by spleen cells obtained from immunized mice. Spleen cells obtained from BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were stimulated with p18-labeled spleen cells, and supernatants were assessed for cytokine concentrations. The results are expressed as the mean concentration ± SE of five mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

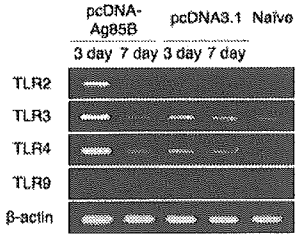

FIGURE 4. TLR mRNA expression profiles of the DNA injection site with electroporation in mice immunized with pcDNA-Ag85B or pcDNA3.1 alone. Total RNA was isolated 3 and 7 days after injection and was analyzed by RT-PCR for TLR2, TLR3, TLR4, and TLR9 mRNA expression. Equality of the RT reaction of isolated RNA between samples was confirmed by amplification of β-actin. Data are representative of three independent experiments.

lytic activities of the cells against p18-I10-pulsed target cells were assessed. Effector cells from mice immunized with pJWNL432 and pcDNA-Ag85B showed higher levels of p18-I10-specific lytic activity than effector cells from BCG-unprimed mice. Moreover, these cytolytic activities were clearly enhanced by BCG sensitization in mice immunized with pJWNL432 mixed with pcDNA-Ag85B (Fig. 5*A*). These effector cells from immunized mice were cultured in a medium containing anti-CD4 or anti-CD8 mAb during the ^{51}Cr release assay. Anti-CD8 mAb inhibited cytolysis against target cells pulsed with the peptide, whereas anti-CD4 mAb did not affect this effector cell function (Fig. 5*B*). Therefore, these results indicate that effector cells expressed CD8 and used this molecule to recognize the target cells. Furthermore, lytic activities of peptide-specific effector cells from immunized mice against MHC-matched or mismatched target cells labeled with the peptide were assessed. These p18-I10-specific effector cells lysed MHC-matched, H-2^d target cells, but not mismatched, H-2^b target cells pulsed with the peptide (Fig. 5*C*). Moreover, the functions of these p18-I10-specific effector cells were inhibited by anti-H-2D^d mAb, but not by anti-H-2K^d mAb or anti-H-2L^d mAb (Fig. 5*D*).

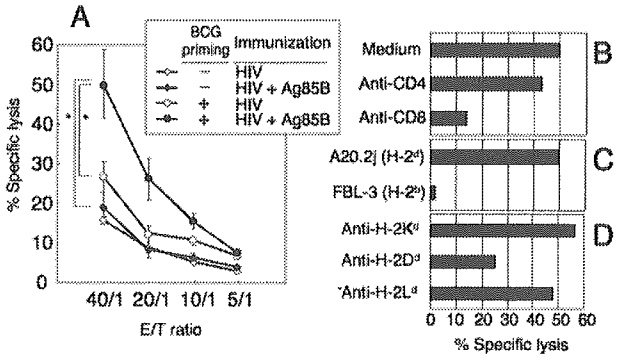

FIGURE 5. Spleen cells from BCG-primed mice coadministered pJWNL432 and pcDNA-Ag85B showed high levels of HIV gp120-specific MHC class I-restricted lytic activity. *A*, CD8$^+$ T cells were purified from spleens of BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B, and the cells were then stimulated with p18-I10-labeled spleen cells and assessed for lytic activities against p18-I10-pulsed target cells. *B*, Lytic activities of effector cells were assessed in the presence of anti-CD4 mAb, anti-CD8 mAb with complement, or medium. The E:T cell ratio was 40:1. *C*, Lytic activities of effector cells against p18-I10-pulsed H-2^d or H-2^b target cells were assessed. The E:T cell ratio was 40:1. *D*, Effector cells were examined for p18-I10-specific lytic activities in the presence of anti-H-2K^d, anti-H-2D^d, or anti-H-2L^d mAb. The E:T cell ratio was 40:1. Each value is the mean percentage of the specific lysis values obtained from five mice. *, $p < 0.01$.

These results indicated that effector cells elicited in immunized mice were CD8$^+$ and MHC class I-restricted CTLs and suggested that Ag85B has potent adjuvant activities for enhancement of CTL responses by being mixed with DNA vaccine Ag.

Ag85B increase the number of HIV gp120-specific, IFN-γ-secreting, CD8$^+$ T cells

ELISPOT assays were performed to compare the numbers of HIV gp120-specific, IFN-γ-secreting, CD8$^+$ cells in immunized mice. CD8$^+$ T cells purified from spleens of immunized mice were stimulated with peptide-pulsed P815 cells in ELISPOT filter plates coated with an IFN-γ capture mAb for 24 h. The numbers of spots were counted automatically using a KS ELISPOT system. In BCG-unprimed mice, the number of p18-I10-specific IFN-γ-secreting CD8$^+$ T cells was slightly increased in mice coadministered pcDNA-Ag85B (20.3 ± 10.0/10^6 cells) compared with that in mice immunized with pJWNL432 alone (14.0 ± 3.6/10^6 cells). In BCG-primed mice, however, the number of p18-I10-specific IFN-γ-secreting CD8$^+$ T cells was ~3.7-fold greater in mice coadministered pcDNA-Ag85B (96.7 ± 13.3/10^6 cells) than in mice immunized pJWNL432 alone (26.3 ± 5.1/10^6 cells; Fig. 6*A*). To confirm whether the improved CTL responses strictly depend on the presence of Ag85B, BCG-primed mice were coadministered various doses of pcDNA-Ag85B, and the frequency of anti-p18-I10-specific IFN-γ-secreting CD8$^+$ T cells was determined by ELISPOT assay. The number of anti-p18-I10-specific, IFN-γ-secreting, CD8$^+$ T cells was gradually increased by coadministration of Ag85B in a dose-dependent manner (Fig. 6*B*). In addition, dose dependency in improving the anti-p18-I10-specific response was not found in mice coadministered a control plasmid, which expresses an unrelated protein constructed by the same expression vector (data not shown). These results clearly indicate that the

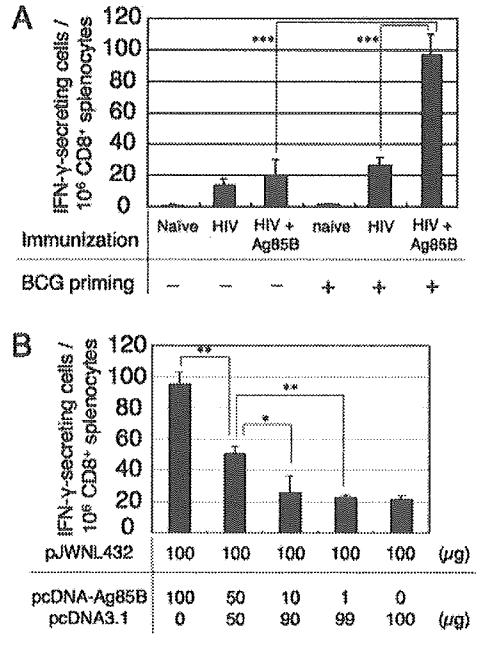

FIGURE 6. pcDNA-Ag85B coadministration in BCG-primed mice enhances HIV gp120-specific, IFN-γ-secreting cell frequency. CD8$^+$ T cells were purified from spleens of BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B (*A*) or with various doses of pcDNA-Ag85B (*B*) and were examined for IFN-γ production in an ELISPOT assay after stimulation with p18-I10-labeled P815 cells. Data are presented as the mean number of p18-I10-specific spots per 10^6 CD8$^+$ spleen cells ± SE of five mice per group. *, $p < 0.02$; **, $p < 0.01$; ***, $p < 0.001$.

anamnestic response to Ag85B could enhance the simultaneously induced CTL responses. These data also support the results for CTL responses and suggest that coadministration of pcDNA-Ag85B, especially in BCG-primed mice, induces high frequency, Ag-specific, responding $CD8^+$ T cells.

Ag85B enhances protective immunity against rVV-HIVenv infection

Finally, to determine the functional relevance of HIV gp120-specific CTLs generated by immunization with these DNA vaccines to eliminate the virus infection, immunized mice were challenged with 5×10^6 PFU of rVV-HIV gp120. Five days after the challenge, the mice were killed, and the ovaries were harvested and used for estimation of the vaccinia virus titer by RT-PCR, because the ovary is the organ in which the vaccinia virus preferentially replicates. The titers of rVV-HIV gp120 in mice coadministered pcDNA-Ag85B were much lower than those in mice immunized with pJWNL432 alone. Moreover, this inhibitory effect on replication of rVV gp120 was clearly demonstrated in mice primed with BCG before immunization (Fig. 7). These results indicated that immunization of mice with pJWNL432 mixed with pcDNA-Ag85B resulted in the generation of an effector T cell response capable of recognizing endogenously processed viral protein, and that DNA immunization inhibited the replication of rVV-expressing HIV gp120 in vivo.

Discussion

DNA vaccination is a practical and effective way to induce cellular immune responses, especially a CTL response, and has shown great promise for initiating cellular immune responses that are regulated for the prevention of various disease such as tumors, HIV, tuberculosis, hepatitis C virus, and malaria. In humans and large animal models, however, immune responses induced by DNA vaccination are not sufficient for prevention or control of infection. Thus, there is a need to increase the potency of DNA vaccines for use in humans. In the present study we investigated the effectiveness of a novel approach for enhancing the ability of a DNA vaccine to induce cellular immune responses by using previously experienced immunogenic proteins that induce a strong Th1-dominant immune response.

$CD4^+$ T cells play a critical role in the generation and maintenance of CTL responses in a noninflammable vaccination strategy including conventional plasmid DNA vaccination. Convincing evidence that dendritic cells (DCs) are the principal cells for priming

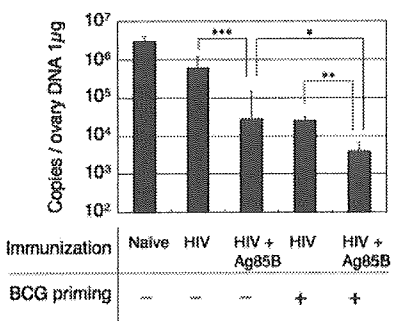

FIGURE 7. pcDNA-Ag85B coadministration in BCG-primed mice enhances HIV gp120-specific protective immunity. BCG-primed or unprimed mice immunized with pJWNL432 with or without pcDNA-Ag85B were challenged with 5×10^7 PFU of rVV-HIV-gp120. The bars show the virus as the log of the number of virus copies in ovaries of mice. The data represent the mean copies of virus obtained from five mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

$CD8^+$ T cells in DNA vaccination through direct transfection of DNA (19–21) or cross-presentation of a vaccine-derived Ag has been presented (22–24). Activation of $CD4^+$ T cells is required for maturation of DCs through CD40 signaling (3–5); however, this requirement is not sufficient in many cases of DNA vaccine immunization (25, 26). Various studies on compensation for the insufficiency of $CD4^+$ T cell help by coadministration of costimulatory molecules, such as B7-1 or B7-2 (27, 28), or by treatment with a CD40 agonist during immunization have therefore been conducted (26). Another $CD4^+$ T cell-mediated helper effect for induction of CTL by DNA vaccine is thought to be the production of cytokines for enhancement of activity and proliferation of CTLs (29). Cognate $CD4^+$ T cell help is important for inducing pathogen-specific CTLs (30), and cognate $CD4^+$ T cell help should be induced to elicit HIVgp120-specific CTLs by injection of DNA vaccine. The Ag85B in our system enhances this cognate manner and polarizes Th1-type immune responses as a vaccine adjuvant. Numerous studies have focused on the effectiveness of coadministration of Th1 cytokine-encoding plasmids, such as IL-2, IL-12, IL-15, IL-18, IL-23, and IFN-γ, for enhancing CTL responses (31, 32). However, adjuvant effects of cytokines on CTL generation are different (33, 34), suggesting that it is necessary to augment the immune responses by these strategies for administration not only of the combination of cytokines codelivered, but also for the timing of administration (35, 36). The expression of both Ag85B and HIV Ag in the same tissues provides this advantage, because Ag85B is thought to be a strong potentiator of Th1-type cytokines. In fact, our results showed production of IFN-γ from spleen cells after stimulation with HIV Ag (Fig. 3).

BCG is an important clinical tool because of its strong immunostimulatory properties. Humans as well as resistant mouse strains infected with BCG predominantly exhibit a Th1 cytokine secretion profile (37). Although specific Ags eliciting Th1 cell responses in mycobacteria are not yet known, a recent study suggested that one of the immunogenic proteins for Th1 development is Ag85B (9). Apparently, strong Th1 responses have been elicited in vitro from purified protein derivative-positive asymptomatic individuals using purified Ag85B (38–40). Furthermore, vaccination of mice with plasmid DNA encoding Ag85B induced strong Ag85B-specific CD4 T cell proliferation and vigorous IFN-γ secretion, resulting in the protection of further *Mycobacterium tuberculosis* infection (41). We have also shown that Ag85B-specific recall responses enhance tumor-specific cellular immune responses in Ag85B gene-transfected tumor cell immunization (11). One possible reason for Th1 domination by Ag85B is that the immunogenic Th1-inducing helper epitope, known as peptide-25, is included in Ag85B protein (42, 43). Peptide-25 was able to stimulate proliferation and a high amount of IFN-γ production in *M. tuberculosis*-primed cells (42). It remains unclear why peptide-25 can induce potent Th1 responses; however, several recent studies have suggested that the avidity of the peptide for its specific TCR may be strong enough to induce Th1 development (9, 44). It is now generally accepted that MHC class II-dependent activation of $CD4^+$ T cells, mainly Th1-polarized cells, potently enhances concomitantly existing unrelated CTL responses (7, 44). According to this line of reasoning, coadministration of Ag85B DNA is a promising tool for enhancement of CTL responses through Ag85B-specific Th cell proliferation and Th1 polarization in a DNA vaccination strategy.

The roles of some proteins and peptides in the polarized development of Th1 cells have been reported, and Ag85B is considered to be one such protein. In fact, we found therapeutic effects of Ag85B on Th2-type allergic disease, asthma, and atopic dermatitis (unpublished observations). The mechanisms, however, are still

not clear. Various products with adjuvant activities, such as LPS, CpG motif, or polyinosinic-polycytidylic acid, involve TLRs and show augmentation of Th1-type immune responses (18). Bacterial components, mycobacterial lipoprotein, bacterial peptidoglycan, and flagellin, also associate with TLRs (18). A correlation between the adjuvant activities of Ag85B and TLRs has not been found. Mycobacteria can bind some TLRs and show typical Th1-type immune responses (45). In a transfection model using Chinese hamster ovary cells (which are relatively deficient in TLRs), the expression of TLR2 or TLR4 conferred responsiveness to both virulent and attenuated *M. tuberculosis* (46). Lipoarabinomannan, a major mycobacterial cell wall component, appears to resemble the cell wall component of Gram-negative bacterial LPS. TLR2 was shown to be necessary for signaling of mycobacterial LPS lipoarabinomannan (47). An undefined, heat-labile, cell-associated, mycobacterial factor was found to be the ligand for TLR4 (47). Ag85B might be included in one of these factors, if it is involved in innate immunity through TLRs. In fact, our results showed enhancement of the expression of TLR2, TLR3, and TLR4 in Ag85B DNA-injected mice (Fig. 4). Because it has been reported that not only microbial components, but also several cytokines regulate the expression of TLRs, there is the possibility of secondary responses for the expression of TLRs by induction of cytokine (18).

Another important biological role of Ag85B is binding of fibronectins (FNs) (48–50). FNs are a family of high molecular weight glycoproteins found in plasma and tissues and are involved in cell motility and adhesion, regulation of cell morphology, phagocytic function, and wound healing (51). Many integrin-binding sites have been identified in amino acid sequences of FNs (52), and adhesion of FN-binding proteins to FNs helps the phagocytosis of proteins into integrin-expressing APCs, especially monocytes, macrophages, and DCs (53). Binding of FNs to human monocytes enhances the phagocytic function of monocytes for bacilli (51), and inhibition of FN-integrin receptor interaction can prevent *M. kansasii* phagocytosis (54). Moreover, Ag85B from *M. tuberculosis* and FNs synergistically stimulate TNF-α expression in human monocytes (55), suggesting that the binding ability of Ag85B with FNs influences not only the enhancement of incorporation of Ags into phagocytic cells, but also the construction of the Th1 milieu at the site of injection.

The results of the present study suggest that coadministration of Ag85B DNA has several potential advantages over other genetic adjuvants due to the existence of multiple mechanisms for elicitation of CTL responses by a DNA vaccine. The results also showed the effectiveness of mycobacterial sensitization for enhancing adjuvanticity of Ag85B. Because most humans have been sensitized by spontaneous infection or by vaccination with mycobacteria, this finding is valuable for the possible use of Ag85B as a genetic adjuvant of a DNA vaccine. The results of this study have provided evidence of the potential utility of Ag85B for the development of a DNA vaccination strategy for successful human use.

Disclosures

The authors have no financial conflict of interest.

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Rapid Communication

Ubiquitination of APOBEC3 proteins by the Vif–Cullin5–ElonginB–ElonginC complex

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Abstract

APOBEC3 proteins are antiviral host factors for a wide variety of retroviruses. HIV-1 Vif overcomes the antiviral activity of APOBEC3G by ubiquitinating the protein. In this study, we examined the ability of Vif to antagonize other family members of APOBEC3 proteins, together with its mechanism. Using HIV infectivity, virion incorporation, immunoprecipitation, and in vitro ubiquitin conjugation assays, we show that the ability of Vif to inhibit antiviral activity of APOBEC3 proteins positively correlates with its ability to bind and ubiquitinate these proteins by a Vif–Cullin5–ElonginB–ElonginC (Vif–BC–Cul5) complex. These results suggest that Vif exhibits its anti-APOBEC3 activity by the ubiquitin ligase activity of the Vif–BC–Cul5 complex.

Keywords: HIV-1; Cytidine deaminase; Virion incorporation; Ubiquitin; Cullin5–ElonginB–ElonginC complex

Introduction

APOBEC3G (A3G) is a potent antiretroviral host factor (Sheehy et al., 2002). It deaminates cytidine to uridine in nascent minus-strand viral DNA, inducing G-to-A hypermutation in the plus-strand viral DNA (Harris et al., 2003; Mangeat et al., 2003; Shindo et al., 2003; Zhang et al., 2003). HIV-1 Vif protein overcomes the antiviral activity of A3G by targeting it for ubiquitin-dependent degradation (Marin et al., 2003; Sheehy et al., 2003; Stopak et al., 2003). Vif interacts with cellular proteins, Cullin5 (Cul5), Elongin B (EloB), Elongin C (EloC), and Rbx1 through its novel SOCS-box motif to form a ubiquitin ligase (E3) complex (Vif–BC–Cul5) and functions as a substrate recognition subunit of the complex (Kobayashi et al., 2005; Mehle et al., 2004; Yu et al., 2003, 2004b). A3G belongs to the APOBEC superfamily of cytidine deaminases (Jarmuz et al., 2002), and several studies have reported that other members of this family such as APOBEC3B (A3B) and APOBEC3F (A3F) also have an antiviral activity on HIV-1, while the involvement of Vif in antagonizing these enzymes remains controversial (Bishop et al., 2004; Liddament et al., 2004; Wiegand et al., 2004; Zheng et al., 2004). We have recently reported that the Vif–BC–Cul5 complex could indeed work as an E3 ligase by an in vitro ubiquitin conjugation assay using the purified Vif–BC–Cul5 complex (Kobayashi et al., 2005). Using the assay, here, we show that the Vif–BC–Cul5 complex ubiquitinates A3F as well as A3G, but not A3B. We also demonstrate that the ability of Vif to inhibit antiviral activity of APOBEC3 proteins positively correlates with its ability to bind and ubiquitinate these proteins by the Vif–BC–Cul5 complex, indicating that Vif exhibits its anti-APOBEC3 activity by the ubiquitin ligase activity of the Vif–BC–Cul5 complex.

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