

Efficiency of Recombinant Bacille Calmette-Guérin in Inducing Humoral and Cell Mediated Immunities against Human Immunodeficiency Virus Type 1 Third Variable Domain in Immunized Mice

Young Jae Kim

Department of Laboratory Medicine, Sungkyunkwan University School of Medicine, Masan Samsung Hospital, Masan, Korea.

Received: January 8, 2010

Revised: March 26, 2010

Accepted: April 21, 2010

Corresponding author: Dr. Young Jae Kim,
Department of Laboratory Medicine,
Sungkyunkwan University School of Medicine,
Masan Samsung Hospital,
50 Hapseong 2-dong, Masan 630-723, Korea.
Tel: 82-55-290-6098, Fax: 82-55-290-6499
E-mail: kyj7514@unitel.co.kr

The author has no financial conflicts of interest.

Purpose: The third variable (V3) loop of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein has been intensively studied for AIDS vaccine development. Bacille Calmette-Guérin (BCG) is widely used to immunize against tuberculosis and has many advantages as a vaccine vehicle, such as low toxicity, adjuvant potential, low cost, and long-lasting immune-inducing capacity. This work was initiated to investigate the immunogenicity of recombinant BCG (rBCG-mV3) designed to express trimeric HIV-1 V3 loop (mV3) in rBCG-mV3-immunized animals. **Materials and Methods:** HIV-1 V3-concatamer was cloned into pMV261, a BCG-expression vector, and then rBCG-mV3 was constructed by introducing the recombinant plasmid (pMV-V3). The recombinant BCG was examined with regard to its expression of V3-concatamer and the genetic stability *in vivo* and *in vitro*. The immune responses induced by recombinant BCG were tested in immunized mice and guinea pigs. **Results:** The rBCG-mV3 expressed detectable amounts of V3-concatamer when induced by single heat-shock. The recombinant BCG was genetically stable and maintained the introduced mV3 gene for several weeks. V3-specific antibodies were clearly detected 6 weeks after inoculation. The antibody titer rapidly increased after immunization up to 10 weeks, and then maintained for over 4 weeks. IgG2a was prevalent in the V3-specific antiserum. The recombinant BCG was also effective in inducing delayed-type hypersensitivity responses in the immunized guinea pigs. rBCG-immunized mice retained substantial amounts of V3-specific T cells in the spleen, even 5 months after the first immunization. **Conclusion:** Recombinant BCG-mV3 is very efficient in inducing humoral and long-lasting cell-mediated immunity against HIV-1 V3 in the immunized animals.

Key Words: HIV-1 vaccine, V3 domain, recombinant BCG, immunized animal

© Copyright:

Yonsei University College of Medicine 2011

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

The third variable (V3) region of human immunodeficiency virus type 1 (HIV-1)

gp120 has been termed the principal neutralizing determinant of the virus¹ and is involved in HIV-1 viral entry.^{2,3} For efficient entry into target cells, HIV-1 requires specific chemokine receptors (CCR5, CXCR4) as well as the primary receptor, CD4.^{4,5} The specific amino acids located in the V3 loop of HIV-1 gp120 play an important role in determining cellular tropism and viral preference for the specific chemokine co-receptor for viral entry.⁶ HIV-1 strains that enter T cell lines prefer CXCR4 as a co-receptor, resulting in the syncytium-inducing phenotype.⁷ CCR5 and, less commonly, CCR3 or CCR2b, are preferred by HIV-1 strains that enter macrophages, resulting in the macrophage-tropic phenotype.⁸ However, V3-specific antibody is rarely detected in the serum of patients with AIDS for V3 itself is not highly immunogenic.⁹ Therefore, the V3 loop can be a useful target for vaccine development against HIV-1. Several approaches have been attempted to intensify the immunogenicity of V3, such as substitution of amino acids,^{10,11} fusion with HBV surface Ag¹² or toxoid,¹³ and construction of multimers.¹⁴

Bacille Calmette-Guérin (BCG) is a live, attenuated bovine tubercle bacillus widely used to immunize against tuberculosis. BCG has many advantages as a vaccine vehicle, such as low toxicity, adjuvant potential, low cost, and long-lasting immune-inducing capacity. Since Stover, et al.^{15,16} developed a shuttle vector (pMV261) that could replicate in BCG and *Escherichia coli*, many researchers have con-

structed recombinant BCG vaccines expressing foreign antigens with sufficient immunogenicity to induce humoral and cellular immunity that could protect the host from viral, bacterial, or parasitic infections.^{17,18} Whereas, current development of HIV-1 vaccine is strongly recommended to induce humoral and cellular immunity against HIV-1 after vaccination.¹⁹⁻²¹ In this aspect, recombinant BCG can be a fascinating vaccine vector for the development of HIV-1 vaccine.¹⁹⁻²¹

In the present study, recombinant BCG (rBCG-mV3) was constructed which expressed the trimeric HIV-1 V3 loop (mV3), and the immunogenicity of rBCG-mV3 has been determined in experimental animals.

MATERIALS AND METHODS

All of the experiments were performed based on the process shown in Fig. 1.

Plasmid construction and expression

V3 was amplified by polymerase chain reaction (PCR) from cDNA of HXB2 with PCR primers covering the V3 region. Sense 5' ACC CGG GAT CCA TGT ACA AGA CCC AAC AAC-3' (BamHI), antisense 5' CTA CA CAA TTC AAG GTT ACA ATG TGC TT-3' (EcoRI), sense 5' CTA CA GAA

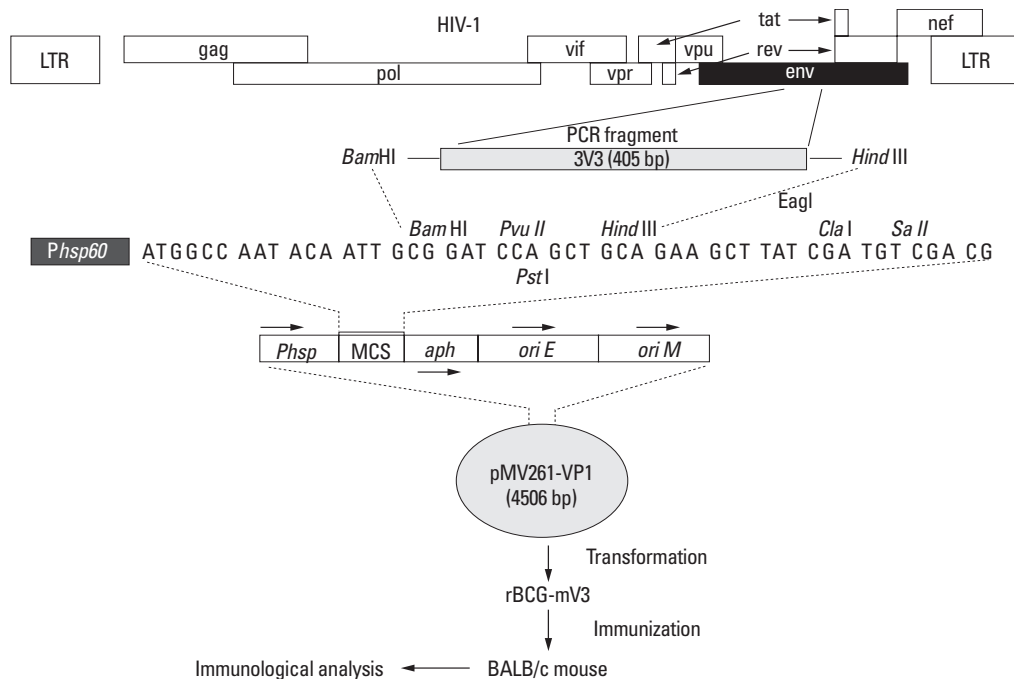


Fig. 1. Schematic illustration of experimental procedure. *Phsp60*, promoter for heat shock protein; MCS, multiple cloning site; *aph*, gene conferring kanamycin resistance as a selective marker; *oriE*, *E. coli* origin of replication; *oriM*, mycobacterial origin of replication; HIV, human immunodeficiency virus; V3, third variable.

TTC ATT AAT TGT ACA AGA CC-3' (EcoRI), antisense 5' CAA GT CTG CAG AAT GTT ACA ATG TGC TT-3' (PstI), sense 5' CAA GT CTGCAG ATT AAT TGT ACA AGA CC-3' (PstI) and antisense 5' GCA TTA AGC TTA AAT GTT ACA ATG TGC TTG TC-3' (HindIII) primers were designed from the sequence of HXB2, as reported previously.²² PCR-amplified cDNA were eluted from agarose gel and digested with BamHI, EcoRI, PstI, and HindIII. The PCR fragments of the 135bp of V3 DNA was ligated, and re-amplified with the BamHI sense & HindIII antisense primer set. The 405bp of V3 trimer (mV3) fragments were ligated into pRSET B (Invitrogen, Carlsbad, CA, USA) and pMV261, and henceforth referred to as pRSET-mV3 and pMV-mV3, respectively. Recombinant pRSET-mV3 plasmid was introduced into an *E. coli* strain containing an episome of T7 RNA polymerase, BL21 (DE3). The pRSET-mV3/BL21 (DE3) clone was cultured in modified M9 broth (1% Bacto-tryptone, 0.4% glucose, 0.5% NaCl, 1 mM MgSO₄, 0.3% KH₂PO₄, 0.6% NaH₂PO₄, and 0.1 mM NH₄C). Recombinant mV3 synthesis was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. pMV-mV3 recombinant plasmid was transformed into the *Mycobacterium bovis* BCG 1173-P2 Pasteur strain, as described previously.¹⁷ Cells were plated on Middlebrook 7H10 agar (Difco Laboratory, Detroit, MI, USA) containing albumin, dextrose, catalase (ADC) enrichment, and kanamycin (25 µg/mL). Kanamycin-resistant colonies were sub-cultured in Middlebrook 7H10 liquid medium containing ADC enrichment and kanamycin for 1 week. mV3 expression was induced by treating the rBCG-mV3 positive clones at 45°C for 2 hours, as described previously.¹⁷ To extract plasmid DNA from the positive BCG clones, the cell pellets were re-suspended and incubated with 400 µL of glucose-Tris-EDTA/20 mg lysozyme at 37°C for 2-3 hours. Cells were disrupted by adding 300 µL of lysis buffer (2.5% SDS and 0.3% NaOH), and 250 µL of Sol III [3M potassium acetate (pH 5.2)] to the reaction mixture. Supernatant was precipitated by adding the same volume of isopropanol.

Anti-mV3 antiserum preparation

The pRSET-mV3-transformed *E. coli* BL21 (DE3) culture was harvested, resuspended in lysis buffer [50 mM Tris-Cl (pH 8.0), 1 mM EDTA and 100 mM NaCl], and then lysed by an ultrasonic dismembrator. The lysate was dissolved in buffer A [6 M guanidine HCl, 0.1 M Na-phosphate and 0.01 M Tris-Cl (pH 8.0)], centrifuged, and the supernatant was applied to the 50% slurry of a Ni²⁺-NTA agarose col-

umn (Quiagen, Chatsworth, CA, USA) at a flow rate of 10-15 mL/hour. The column was sequentially washed with 10 column-volumes of buffer A, 5 column-volumes of buffer B [8 M urea, 0.1 M Na-phosphate, and 0.01 M Tris-Cl (pH 8.0)], and 3 column-volumes of buffer C [buffer B (pH 6.8)]. Purified proteins were eluted with buffer D [buffer B (pH 4.5), supplemented with 200 mM EDTA], dialyzed against phosphate buffered saline (PBS), and then re-dissolved in 0.2 M sodium bicarbonate buffer containing 0.02% SDS (pH 7.4) overnight. Small amounts of the eluted samples were tested for purity on 12% SDS-PAGE. Ten 6-week-old female BALB/c mice were used to raise antiserum. Recombinant mV3 protein was mixed with the same volume of Freund complete adjuvant by sonication. A protein-adjuvant emulsion containing 25 µg of immunogen was injected subcutaneously into each mouse. The first immunization was followed by two booster injections every 2 weeks with 20 µg of the protein mixed with incomplete adjuvant. The final immunization was given 2 weeks after the second booster by injecting 10 µg of recombinant protein via the tail vein. Blood was collected from the ophthalmic vein of immunized mice, from which antiserum was prepared and used for Western blot analysis.

Analysis of mV3 expression in rBCG

As described previously,¹⁷ recombinant BCG-mV3 transformants were cultured in Middlebrook 7H9 broth media containing 25 µg/mL of kanamycin. When the cells were grown to 1×10⁶ cells/mL, the culture was heat-induced, and then harvested at the requested time points. The cells were washed in PBS plus 0.05% Tween 80 and resuspended in 1/20-volume of radioimmunoprecipitation assay buffer. Culture lysates were analyzed by Western blot hybridization with anti-mV3-antiserum prepared from the previous step and goat-antimouse IgG (Sigma Chemical Co., St. Louis, MO, USA).

Immunization of BALB/c mice with rBCG-mV3

BALB/c female mice, 5-6 weeks old, were inoculated intraperitoneally with heat-induced rBCG-mV3 and control BCG at a concentration of 1×10⁷ cells/mouse.

Genetic stability of the rBCG-mV3 *in vivo*

Immunized BALB/c mice were killed 4, 8, and 16 weeks after inoculation. Spleens were homogenized in PBS with 0.05% Tween 80 (pH 7.5) and were then cultured on the Middlebrook 7H11 agar plate supplemented with oleic

acid, albumin, dextrose, catalase (OADC) enrichment (Difco Laboratory), and 25 µg/mL of kanamycin. Kanamycin-resistant colonies were subcultured in Middlebrook 7H9 liquid medium containing ADC enrichment and kanamycin. Lysates from these cultures were tested for mV3 expression by Western blot, and plasmid DNA extracted from the recovered BCG clone was tested for its integrity by PCR with mV3-covering PCR primer set.

Antibody titration and IgG isotyping

Blood was obtained from the ophthalmic vein from BALB/c mice every other week beginning 5-6 weeks after immunization, and antiserum was assessed by ELISA to measure

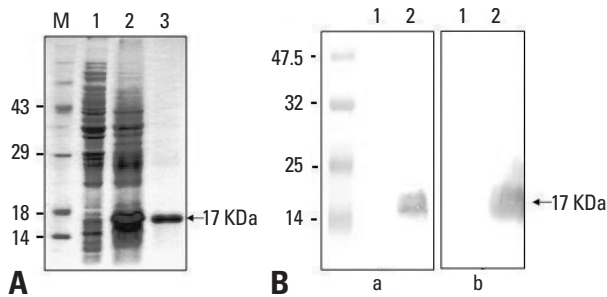


Fig. 2. (A) V3-trimer was cloned into pRSET-B vector, and expressed in *E. coli* BL21 (DE3). Whole cell lysates or purified protein was separated on a SDS-PAGE. Lane 1, pRSET/B control vector-transformed cell lysate; lane 2, pRSET-mV3-transformed cell lysate; lane 3, Ni²⁺-NTA resin-purified recombinant mV3 protein. (B) Western blot analysis of V3-concatamer (three V3) expressed in bacteria with 2 different antisera. pRSET/B control vector-transformed cell lysate (1) and pRSET-mV3-transformed cell lysate (2) were separated on a SDS-PAGE, and then assessed by Western blot analysis with mouse anti-3V3-antibody (a) and rabbit anti-gp120 antibody (b). SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; V3, third variable.

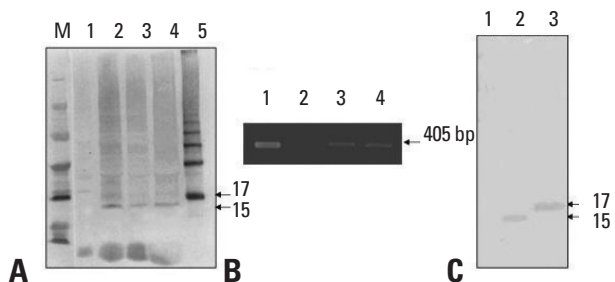


Fig. 3. (A) Western blot analysis of rBCG-mV3 lysates with anti-V3 antiserum. Cultures of rBCG-mV3 were heat-induced at 45 °C for 2 hours in the log phase, and the same amounts of cells were harvested from the culture in 2 week intervals. M, marker; lane 1, BCG-pMV control; lane 2, BCG-mV3 (2 weeks); lane 3, BCG-mV3 (4 weeks); lane 4, BCG-mV3 (6 weeks) after a single heat-shock; lane 5, mV3-expressing *E. coli* lysate as a positive control. (B) PCR amplification of the plasmid extracted from the kanamycin-resistant rBCG that was isolated from the spleen of immunized mice was performed with V3-specific primer set. Each lane shows a pMV-mV3 positive control (1), pMV261 negative control (2), and plasmid recovered from the rBCG-mV3-immunized mice 4 weeks (3) and 8 weeks after immunization (4). (C) Western blot analysis of rBCG-mV3 recovered from immunized mice with anti-mV3 sera. Each lane shows BCG-pMV (1), rBCG-mV3 (2), and pRSET-mV3 / *E. coli* BL21 (DE3) lysate (3). BCG, Bacille Calmette-Guérin; PCR, polymerase chain reaction.

mV3-specific antibodies, as described previously.¹⁷ EIA plates were coated with 0.5 µg/mL of the purified recombinant mV3 overnight. After the plates were washed 3 times with 0.2% Tween 20 in PBS (PBS-T) and blocked with 2% blotto/0.5% bovine serum albumin (BSA), 100 µL of the serum samples (1 µL of serum in 100 µL binding buffer) were added to each well and incubated at 37°C for 1 hour. Plates were washed 3 times with PBS-T buffer, and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Sigma Chemical Co.). P-nitrophenyl phosphate solution was added to each well, and then read by an ELISA reader (Molecular Devices Inc., St. Louis, MO, USA) at 405 nm. Analysis of mV3-specific antibodies was performed with a mouse Ig isotyping kit (Pharmingen, San Diego, CA, USA).

DTH response in immunized guinea pigs

As described previously,¹⁷ guinea pigs were administered 1×10^8 cells of live rBCG-mV3 and control BCG intraperitoneally. Four weeks later, the mice were boosted with the same amount of cells via the same route. Two weeks after boosting, the animals were skin tested by intradermal injection of purified recombinant mV3 protein and purified protein derivative of tuberculin (PPD). Delayed type hypersensitivity (DTH) responses were evaluated by measuring erythema 48 hours after skin injection.

T cell proliferation assay

Spleens were removed from mice 10 weeks and 5 months after the primary injection. The splenocytes of each immunization group ($n = 4$) were pooled, and 2×10^5 cells/well in RPMI-1640 containing 2% mouse serum were cultured in 96-well plates in the presence or absence of antigen for 120 hours, labeled with [³H]-thymidine (1 mCi/well) for 8 hours prior to harvesting, and then counted on a scintillation counter.

RESULTS

Recombinant mV3 protein and polyclonal anti-mV3 antibody

Recombinant mV3 proteins were purified from pRSET B-mV3/*E. coli* BL21 (DE3) using a Ni²⁺-NTA resin column (Fig. 2A). Polyclonal antibody specific to mV3 protein was obtained from BALB/c mice immunized with the recombinant protein (Fig. 2B). In order to determine whether or not the mV3 protein shared similar properties with the other V3

proteins, recombinant protein was assessed by Western blot analysis with anti-gp120 polyclonal antibodies. The mV3 protein was detected by mouse anti-V3 and rabbit anti-gp120 polyclonal antibodies (Fig. 2B). These results suggest that V3-specific antibodies induced by mV3-immunization are likely to interact with wild type V3 motif.

Genetic stability of rBCG-mV3 *in vitro* and *in vivo*

pMV-mV3 recombinant plasmid was transformed into BCG by electroporation, as described in the Materials and Methods. The rBCG-mV3 stably expressed mV3 over 6 weeks when examined every 2 weeks (Fig. 3A). BALB/c mice were immunized with rBCG-mV3. The rBCG-mV3 isolated from the mice 8 weeks after immunization still harbored the inserted mV3 (Fig. 3B) and expressed mV3 protein (Fig. 3C). These data indicate that the rBCG-mV3 is genetically stable, not only *in vitro*, but also *in vivo*.

Recombinant BCG-mV3 was efficient in inducing V3-specific humoral immunity which persisted for at least 14 weeks

After a single heat shock, the rBCG-mV3 was injected into

BALB/c mice. Six weeks later, V3-specific antibody was assessed in the antiserum collected from the immunized mice every 2 weeks. As shown in Fig. 4A, mV3-specific polyclonal antibodies were induced and maintained for at least 14 weeks. The amount of V3-specific polyclonal antibodies rapidly increased in the immunized mice, reached a maximum at week 10, and then lasted for over 4 weeks (Fig. 4A). The specific antibodies completely disappeared when examined 10 months after primary inoculation (data not shown). The specificity of polyclonal antibodies to mV3 was further evidenced by Western blot analysis with purified mV3 and control BCG lysates (Fig. 4B). However, HIV-1 replication was not efficiently blocked by the antiserum (Fig. 4C).

Recombinant BCG-mV3 elicited mV3-specific DTH responses in guinea pigs

In general, recombinant BCG is well-known to induce strong DTH responses against BCG and introduced foreign antigens in immunized animals.^{23,24} The mV3-specific DTH was assessed in the guinea pigs immunized with rBCG-mV3. Two weeks after the second inoculation, animals were tested for the reciprocal DTH response to mV3 protein and PPD.

Table 1. DTH Response Against Recombinant mV3 in Guinea Pigs

Immunization	Skin reaction (diameter; mm)		
	Purified mV3 protein		PPD
	5 µg / 0.1 mL	10 µg / 0.1 mL	5 TU / 0.1 mL
None	0	0	0
BCG-pMV	0	0	12.5 ± 0.82
BCG-mV3	3.5 ± 0.41	4.0 ± 0.71	14.5 ± 0.95

DTH, delayed type hypersensitivity; PPD, purified protein derivatives; TU, tuberculin unit.

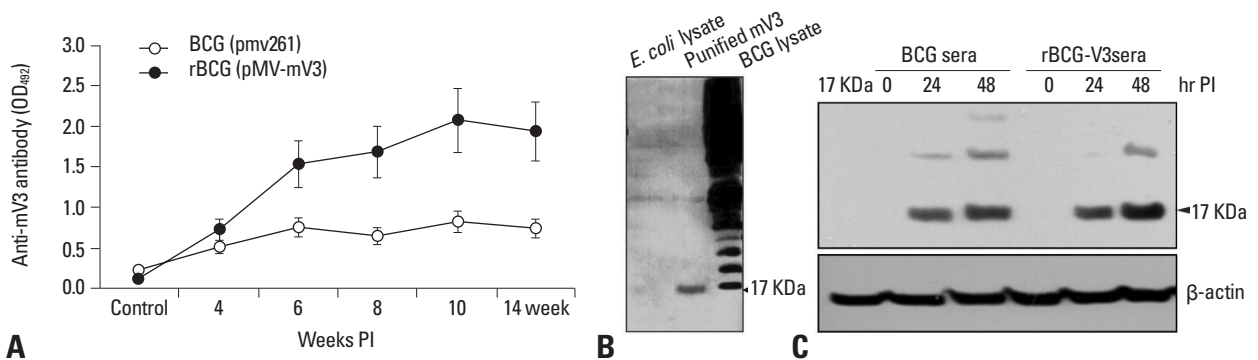


Fig. 4. rBCG-mV3 induces V3-specific antibody in BALB/c mice. (A) Six-week old BALB/c mice were inoculated with control (transformed with pmv261 empty vector) and recombinant BCG (rBCG-mV3) at a concentration of 1×10^7 cells/mouse intraperitoneally. The titer of anti-V3 antibody in the serum obtained from the immunized mice every 2 weeks were assessed by ELISA as described in the Materials and Methods. Anti-serum was collected from the rBCG-mV3-immunized mice 6 weeks after primary inoculation. **(B)** Cell lysates or purified mV3 were examined by Western blot analysis with the antiserum obtained from the mice immunized with rBCG-mV3. The arrow indicates the band of purified mV3 protein. **(C)** C8166 human T cell line was infected with 1 MOI of HIV-1 (HXB2), which was pre-incubated with mouse anti-rBCG-mV3 sera or control anti-BCG sera at 37 °C for 1 hr. Cells were harvested and lysed at the indicated time point and then p24 antigen was assessed by Western blot analysis with anti-p24 monoclonal antibody. OD, optical density; PI, postinoculation; MOI, multiplicities of infection; BCG, Bacille Calmette-Guérin.

The DTH responses to mV3 in the immunized animals were positive 6 weeks after primary inoculation (Table 1). Recombinant BCG-mV3 induced relatively weak DTH response against mV3 protein compared with that against PPD in immunized guinea pigs, which was attributable to the amount of mV3 weakly expressed in rBCG-mV3.

Cellular immune responses in mice immunized with rBCG-mV3

Sometimes there is a discrepancy between the DTH response and T cell proliferation. The T cell responses in immunized mice were examined. The splenocytes of mice immunized with rBCG-mV3 or control BCG were isolated 10 weeks and 5 months after immunization, and subjected to a T cell proliferation assay. The stimulation index (SI) was determined, as described in the Materials and Methods. The SI in response to mV3 in mice immunized with rBCG-mV3 was about three times as strong as that in mice immunized with control BCG (BCG-PMV), and the SI to mV3 assessed 5 months after immunization was markedly enhanced compared with that assessed 10 weeks after immunization (Fig. 5A). These results imply that rBCG-mV3 is able to produce and maintain the memory T cells against mV3 in immunized animals during induction of cellular immunity against BCG.

mV3-specific IgG2a was prevalent in anti-sera

It is well established that IgG1 and IgG3 represent the Th2

response,^{25,26} and IgG2a results from the Th1 response.²⁶ Based on IgG isotyping analysis, V3-specific IgG2a was prevalent in the sera of mice immunized with recombinant BCG-mV3, whereas IgG1 and IgG3 isotypes were relatively lower (Fig. 5B). This means that rBCG-mV3 was very effective in inducing Th1-oriented cell-mediated immunity in immunized mice.

DISCUSSION

A rBCG-mV3, expressing the trimer of HIV-1 V3, was constructed by introducing pMV-mV3 plasmid into BCG as a vaccine vector. Based on the information that Gly-Pro-Gly-Arg-Ala-Phe residues (GxGRxF) are highly conserved in HIV-1 variants^{27,28} and play a crucial role in the interaction with chemokine receptor,²⁹ these sequences have been included into the V3-trimer.

Not only humoral immunity but also Th1-oriented cellular immunity are essential for protection against HIV-1 infection.³⁰ BCG is well-known for its safety and efficacy in the induction of strong cell-mediated immunity.³¹ However, even a BCG as a live attenuated vaccine caused tuberculosis-like disease in simian immunodeficiency virus (SIV)-infected monkeys is probably due to a loss of T-cell immunity.³² Therefore, it is advisable to develop a recombinant BCG as a preventative vaccine rather than therapeutic vaccine against HIV-1.³³

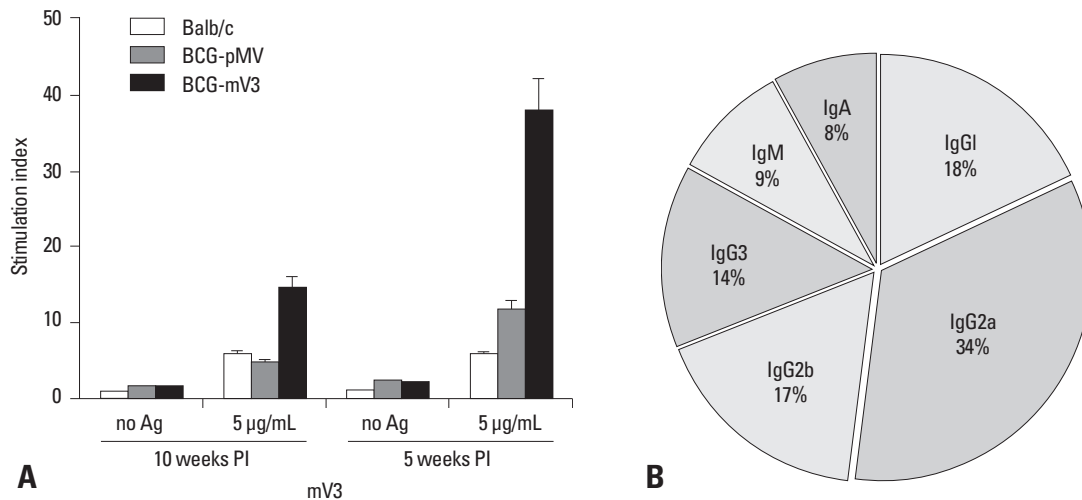


Fig. 5. (A) Lymphocyte proliferation assay in response to recombinant-mV3 antigen stimulation in T cell-enriched splenocytes of immunized mice. Spleens were removed from 3 mice per group 10 weeks and 5 months after immunization with rBCG-mV3 and control BCG (BCG-PMV), respectively. Lymphocyte proliferation assay was performed by measuring the [³H]-thymidine incorporation, and is expressed as the mean \pm SD stimulation indices in triplicate and three independent experiments. (B) Anti-mV3 antibody isotyping was performed using a monoclonal antibody-based mouse Ig isotyping kit and expressed by percentage for each isotype and subclass. Anti-serum was obtained from mice 10 weeks after immunization with rBCG-mV3. Anti-serum was pre-cleared with BCG lysates and then used for ELISA. PI, postinoculation; BCG, Bacille Calmette-Guérin.

In the present study, we have examined the humoral and Th1-oriented cell-mediated immunities in mice and guinea pigs immunized with recombinant BCG expressing multiple HIV-1 V3 domains. The mV3 protein expressed in BCG was detected by Western blot analysis with mouse anti-mV3 serum and anti-gp120 polyclonal antibodies (Fig. 2B). Even though V3 has high sequence variations among HIV-1 variants, it still has some conserved region which can be targeted for vaccine development.³⁴

The mV3 proteins expressed in *E. coli* were easily detected by polyclonal antibody against V3 protein, but hardly detected when expressed in rBCG-mV3 due to the limited amount of expression (Fig. 3). Nonetheless, the immune response to mV3 was not as weak as shown in mice immunized the rBCG-mV3 (Figs. 4 and 5). The rBCG-mV3 injected into mice survived over 8 weeks and produced the mV3 protein for the same period *in vitro* and *in vivo* (Fig. 3). Thus, a single immunization of rBCG-mV3 would be enough to induce specific immunity without an additional boosting injection. mV3-specific antibodies lasted longer than 14 weeks after immunization (Fig. 4).

The mV3-containing the conserved sequence (GxGRxF-GPGRAF) was expected to produce a neutralizing antibody against various HIV-1 subtypes. rBCG-mV3 induced substantial amounts of anti-mV3 antibody in the serum of immunized mice. Unfortunately, the V3-specific antiserum, obtained from mice immunized with rBCG-mV3, was not able to inhibit the HIV-1 replication *in vitro*, indicating that the antiserum does not contain any neutralizing antibody. This was due to the lack of appropriate V3-conformational epitope after expression in BCG. However, rBCG-mV3 was very effective in inducing V3-specific antibodies which might be involved in opsonization of the virus *in vivo*, followed by facilitating virus clearance in vaccinated animals. rBCG-mV3 also induced a strong humoral immunity against control BCG itself (Fig. 4B). However, we are unable to rule out the possibility that the construction of recombinant BCG to express HIV-1 V3 multimer might cause some changes of the characteristics of original BCG.

Cell-mediated immunity plays a key role in the inhibition of AIDS progression.^{19,20,34,35} In the present study, antigen-specific DTH response in guinea pigs (Table 1) and high titer of V3-specific IgG2a in recombinant BCG-immunized mice (Fig. 5B) suggest that recombinant BCG also induced strong cell-mediated immunity and immune memory against the introduced foreign gene. While it is well-known that BALB/c mice have a bias tendency towards Th2 im-

munity, recombinant BCG is such a good Th1 immune stimulator that it pushes for Th1 development even in BALB/c mice, as shown previously in IL-12-mediated Th1 development in BALB/c mice.³⁶ However, to make it clearer, DTH response in immunized BALB/c mice remains to be traced by measuring footpad swelling, as reported previously.³⁷

In summary, recombinant BCG expressing mV3 efficiently induced humoral and cellular immunities against the V3 domain. Particularly noteworthy is that rBCG-mV3 induced long-lasting mV3-specific cellular immunity in immunized animals.

REFERENCES

1. Javaherian K, Langlois AJ, LaRosa GJ, Profy AT, Bolognesi DP, Herlihy WC, et al. Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. *Science* 1990; 250:1590-3.
2. Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD, et al. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 1996;85:1135-48.
3. Sullivan N, Sun Y, Sattentau Q, Thali M, Wu D, Denisova G, et al. CD4-Induced conformational changes in the human immunodeficiency virus type 1 gp120 glycoprotein: consequences for virus entry and neutralization. *J Virol* 1998;72:4694-703.
4. Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996;381:661-6.
5. Wu L, Gerard NP, Wyatt R, Choe H, Parolin C, Ruffing N, et al. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* 1996;384:179-83.
6. Speck RF, Wehrly K, Platt EJ, Atchison RE, Charo IF, Kabat D, et al. Selective employment of chemokine receptors as human immunodeficiency virus type 1 coreceptors determined by individual amino acids within the envelope V3 loop. *J Virol* 1997;71:7136-9.
7. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 1996;272:872-7.
8. Zhu T, Mo H, Wang N, Nam DS, Cao Y, Koup RA, et al. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* 1993;261:1179-81.
9. Emini EA, Putney SD. Human immunodeficiency virus. *Biotechnology* 1992;20:309-26.
10. Shioda T, Oka S, Ida S, Nokihara K, Toriyoshi H, Mori S, et al. A naturally occurring single basic amino acid substitution in the V3 region of the human immunodeficiency virus type 1 env protein alters the cellular host range and antigenic structure of the virus. *J Virol* 1994;68:7689-96.
11. Wolfs TF, Zwart G, Bakker M, Valk M, Kuiken CL, Goudsmit J. Naturally occurring mutations within HIV-1 V3 genomic RNA lead to antigenic variation dependent on a single amino acid substitution. *Virology* 1991;185:195-205.
12. Fomsgaard A, Nielsen HV, Bryder K, Nielsen C, Machuca R, Bruun L, et al. Improved humoral and cellular immune responses against the gp120 V3 loop of HIV-1 following genetic immunization with a chimeric DNA vaccine encoding the V3 inserted into

- the hepatitis B surface antigen. *Scand J Immunol* 1998;47:289-95.
13. Mrsny RJ, Daugherty AL, Fryling CM, FitzGerald DJ. Mucosal administration of a chimera composed of *Pseudomonas* exotoxin and the gp120 V3 loop sequence of HIV-1 induces both salivary and serum antibody responses. *Vaccine* 1999;17:1425-33.
 14. Lu Y, Xiao Y, Ding J, Dierich M, Chen YH. Immunogenicity of neutralizing epitopes on multiple-epitope vaccines against HIV-1. *Int Arch Allergy Immunol* 2000;121:80-4.
 15. Stover CK, de la Cruz VF, Bansal GP, Hanson MS, Fuerst TR, Jacobs WR Jr, et al. Use of recombinant BCG as a vaccine delivery vehicle. *Adv Exp Med Biol* 1992;327:175-82.
 16. Stover CK, de la Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, et al. New use of BCG for recombinant vaccines. *Nature* 1991;351:456-60.
 17. Choi BK, Cho SH, Bai GH, Kim SJ, Hyun BH, Choe YK, et al. Prevention of encephalomyocarditis virus-induced diabetes by live recombinant *Mycobacterium bovis* bacillus Calmette-Guérin in susceptible mice. *Diabetes* 2000;49:1459-67.
 18. Connell ND, Medina-Acosta E, McMaster WR, Bloom BR, Russell DG. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guérin expressing the *Leishmania* surface proteinase gp63. *Proc Natl Acad Sci U S A* 1993;90:11473-7.
 19. Li F, Horton H, Gilbert PB, McElrath JM, Corey L, Self SG. HIV-1 CTL-based vaccine immunogen selection: antigen diversity and cellular response features. *Curr HIV Res* 2007;5:97-107.
 20. Mölder T, Adojaan M, Kaldma K, Ustav M, Sikut R. Elicitation of broad CTL response against HIV-1 by the DNA vaccine encoding artificial multi-component fusion protein MultiHIV--study in domestic pigs. *Vaccine* 2009;28:293-8.
 21. Yang OO. Retracing our STEP towards a successful CTL-based HIV-1 vaccine. *Vaccine* 2008;26:3138-41.
 22. Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, et al. Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* 1985;313:277-84.
 23. Honda M, Matsuo K, Nakasone T, Okamoto Y, Yoshizaki H, Kitamura K, et al. Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin vector candidate vaccine for human immunodeficiency virus type 1 in small animals. *Proc Natl Acad Sci U S A* 1995;92:10693-7.
 24. Sell S, Hsu PL. Delayed hypersensitivity, immune deviation, antigen processing and T-cell subset selection in syphilis pathogenesis and vaccine design. *Immunol Today* 1993;14:576-82.
 25. Kohm AP, Sanders VM. Suppression of antigen-specific Th2 cell-dependent IgM and IgG1 production following norepinephrine depletion *in vivo*. *J Immunol* 1999;162:5299-308.
 26. Martin RM, Lew AM. Is IgG2a a good Th1 marker in mice? *Immunol Today* 1998;19:49.
 27. Yu XF, Wang Z, Beyrer C, Celentano DD, Khamboonruang C, Allen E, et al. Phenotypic and genotypic characteristics of human immunodeficiency virus type 1 from patients with AIDS in northern Thailand. *J Virol* 1995;69:4649-55.
 28. Zvi A, Kustanovich I, Hayek Y, Matsushita S, Anglister J. The principal neutralizing determinant of HIV-1 located in V3 of gp120 forms a 12-residue loop by internal hydrophobic interactions. *FEBS Lett* 1995;368:267-70.
 29. Isaka Y, Sato A, Miki S, Kawachi S, Sakaida H, Hori T, et al. Small amino acid changes in the V3 loop of human immunodeficiency virus type 2 determines the coreceptor usage for CXCR4 and CCR5. *Virology* 1999;264:237-43.
 30. Brander C, Walker BD. T lymphocyte responses in HIV-1 infection: implications for vaccine development. *Curr Opin Immunol* 1999;11:451-9.
 31. Flynn JL. Recombinant BCG as an antigen delivery system. *Cell Mol Biol (Noisy-le-grand)* 1994;40 Suppl 1:31-6.
 32. Shen Y, Shen L, Sehgal P, Huang D, Qiu L, Du G, et al. Clinical latency and reactivation of AIDS-related mycobacterial infections. *J Virol* 2004;78:14023-32.
 33. Falk LA, Goldenthal KL, Esparza J, Aguado MT, Osmanov S, Ballou WR, et al. Recombinant bacillus Calmette-Guérin as a potential vector for preventive HIV type 1 vaccines. *AIDS Res Hum Retroviruses* 2000;16:91-8.
 34. McKinney DM, Skvoretz R, Livingston BD, Wilson CC, Anders M, Chesnut RW, et al. Recognition of variant HIV-1 epitopes from diverse viral subtypes by vaccine-induced CTL. *J Immunol* 2004;173:1941-50.
 35. Berzofsky JA, Ahlers JD, Derby MA, Pendleton CD, Arichi T, Belyakov IM. Approaches to improve engineered vaccines for human immunodeficiency virus and other viruses that cause chronic infections. *Immunol Rev* 1999;170:151-72.
 36. Shibuya K, Robinson D, Zonin F, Hartley SB, Macatonia SE, Somoza C, et al. IL-1 alpha and TNF-alpha are required for IL-12-induced development of Th1 cells producing high levels of IFN-gamma in BALB/c but not C57BL/6 mice. *J Immunol* 1998;160:1708-16.
 37. Aguilar D, Infante E, Martin C, Gormley E, Gicquel B, Hernandez Pando R. Immunological responses and protective immunity against tuberculosis conferred by vaccination of Balb/C mice with the attenuated *Mycobacterium tuberculosis* (phoP) SO2 strain. *Clin Exp Immunol* 2007;147:330-8.