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Specific Immune Response and Pathological Findings in BALB/c Mice Inoculated with Recombinant BCG Expressing HIV-1 Antigen

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SUMMARY Recombinant BCGs (rBCGs) containing extrachromosomal plasmids with different HIV-1 insert sequences: *nef*, *env* (V3J1 and E9Q), *gag* p17 or whole *gag* p55 were evaluated for their immunogenicity, safety and persistent infection in BALB/c mice. Animal injected with, rBCG-pIJKV3J1, rBCG-pSO *gag* p17 or rBCG-pSO *gag* p55 could elicit lymphocyte proliferation as tested by specific HIV-1 peptides or protein antigen. Inoculation with various concentration of rBCG-pSO *gag* p55 generated satisfactory specific lymphocyte proliferation in dose escalation trials. The rBCG-pSO *gag* p55 recovered from spleen tissues at different time interval post-inoculation could express the HIV protein as determined by ELISA p24 antigen detection kit. This result indicated that the extrachromosomal plasmid was stable and capable to express Gag protein. It was also demonstrated that rBCGs did not cause serious pathological change in the inoculated animals. The present study suggested the role of BCG as a potential vehicle for using in HIV vaccine development.

BCG (bacillus Calmette-Guérin), a live attenuated *Mycobacterium bovis*, has been used to vaccinate against tuberculosis worldwide especially in developing countries. The vaccine is incorporated as a part of the Expanded Program on Immunization (EPI) based upon its low cost and low adverse effect.[1,2] It can be given at birth or any time thereafter; and a single dose can produce long-lasting immunity for several to 10 years.[3,4] Moreover, feasibility of BCG as a gene vector for delivering foreign antigens has generated novel candidate vaccines which induced immune response against several pathogens including bacteria,[5-7] protozoa,[8] and viruses.[9-15]

Recombinant BCGs containing HIV-1 sequences (rBCG-HIV-1) have been shown to induce efficient humoral and cellular immune responses in animals.[9-14] Thus, its role as a current HIV vaccine

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candidate is proposed. rBCG-HIV-1 was constructed by transforming the BCG parental strain with the extrachromosomal recombinant plasmid containing HIV DNA insert.[16,17] However, those studies have not determined the most important characteristics of rBCGs: the stability of the recombinant plasmid to persist in rBCGs, and the capability of these rBCGs to express the HIV antigen persistently.

BCG has a long history and is well known about its attenuation. BCG dissemination after immunization is rare, except that occurs in immune compromised hosts.[1] An ideal BCG recombinant vaccine is not only to express the foreign antigen constantly, but also to generate good and protective immune response, and most of all, is to be safe for the vaccinees.[18] Persistence of rBCG-HIV-1 in secondary lymphoid organ without causing pathological changes is the evidence to prove that the genetic recombination does not interfere with the attenuation of BCG parental strain.

The present study investigated the rBCGs which contained different HIV-1 DNA sequences on their immunogenicity, persistence and safety upon inoculating into BALB/c mice. rBCG-pSO *gag* p55 dose escalation response (0.01, 0.1 and 1 mg/mouse) was also measured. The immunogenicity was assayed by lymphocyte proliferation (LP); the safety of the inoculated mice was examined by histopathological study; and the stability of rBCG-pSO *gag* p55 recovered from the inoculated mice to express Gag protein was determined by p24 antigen ELISA.

MATERIALS AND METHODS

Animals

Female BALB/c mice, aged 8-11 weeks, were provided by the National Institute of Health (NIH), Nonthaburi, Thailand. All animals were maintained under a specific pathogen free condition in biosafety level 2 animal facilities at the NIH. Animal care system was followed after the guideline of the Institute Ethical Committee on Experimental Animal Welfare.

Recombinant BCG-HIV-1

Seven kinds of rBCG-HIV-1 used in the present study were derived from BCG-Tokyo parental strain transformed with recombinant plasmid containing the HIV DNA insert sequence of different lengths as follows:1) rBCG-pSO *gag* p55 (pSO246 plasmid [19] inserted with HIV-1 whole *gag* gene); 2) rBCG-pSO *gag* p17 (pSO246 plasmid inserted with 12 mer *gag* sequence TLWCVHQRIEVK); 3) rBCG-pSO *nef* (pSO246 plasmid inserted with 10 mer *nef* sequence QVPLRPMTYK); 4) rBCG-pIJKE9Q (pIJK-1 plasmid[16] inserted with 9 mer gp120 V3 sequence TIGPGQVFY); and 5) rBCG-pIJKV3J1 (pIJK-1 plasmid inserted with 19 mer gp120 V3 sequence NTRK-SIHIGPGRAFYATGS). HIV-1 sequences in recombinants Nos. 1 to 4 were derived from a CRF01_AE isolate, while the sequence in recombinant No. 5 was derived from subtype B isolate. Recombinants Nos.2 and 3 used MPB64 gene from BCG [20] and recombinants Nos. 4 and 5 used alpha antigen gene from *Mycobacterium kansasii* (alpha-K) [16] as the carrier protein gene for fusion with the DNA fragment encoding the corresponding HIV-1 sequence. Diagrams of DNA insertion into the plasmid are shown in Fig. 1. The sixth and seventh kinds of rBCGs were derived from BCG-Tokyo strain transformed with either pSO246 or pIJK-1 plasmid backbone. These two kinds of rBCG were included as the controls in the related experiments. Both pSO246 and pIJK-1 contained kanamycin resistant gene as the selective marker.

Bacterial cultivation

BCG Tokyo 172 parental strain and rBCG seeds were grown in Middlebrook 7H9 broth (BBL, Becton Dickinson, MD, USA) supplemented with 10% albumin-dextrose complex (BBL) and 0.05% Tween-80 (Sigma, MO, USA) with an exception that the culture media for rBCGs was added with 30 μg/ml of kanamycin (Sigma).

Preparation of BCG cell lysates

BCG Tokyo 172 was cultured until the growth density of about 1-1.5 optical density at 470 nm was obtained. The bacterial culture was harvested and centrifuged at 1,000 × *g* for 10 minutes at 4ºC. The bacterial pellet was suspended in phosphate buffered saline (PBS) to the concentration of 1 mg/ml before sonication and centrifugation at 10,080 × *g* for 5 minutes at 4ºC. The supernatant was collected and diluted with PBS for using as specific positive control antigen in lymphocyte proliferation assay. Lysate of rBCG-pSO *gag* p55 was prepared in

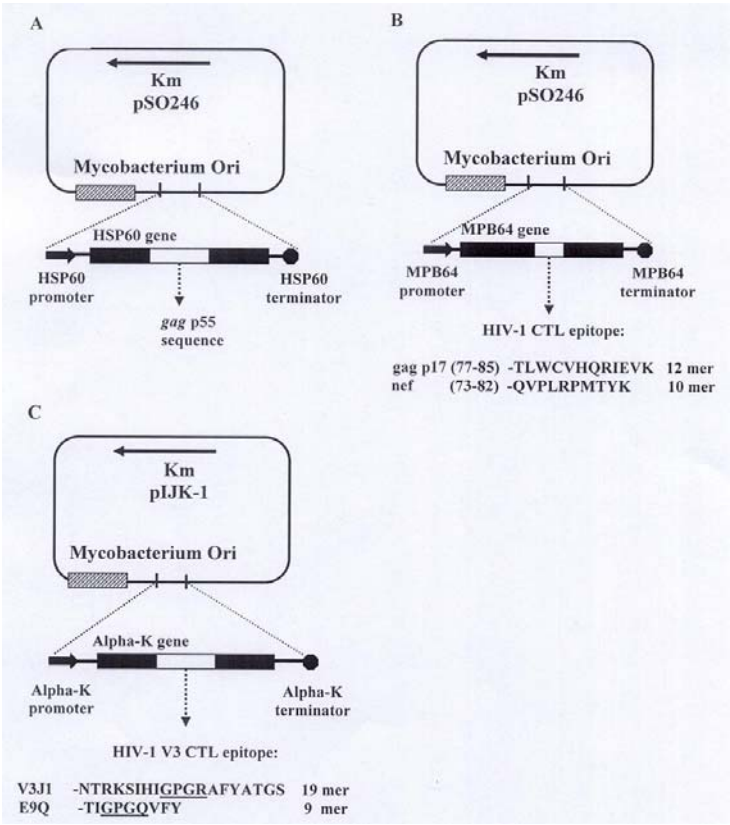

Fig. 1 Diagrams of DNA insertion into the expression plasmid for construction of rBCG-HIV-1. (A) Recombinant pSO246 plasmid inserted with HSP60 flanking HIV-1 whole *gag* gene**.** (B) Recombinant pSO246 plasmid inserted with MPB64 fusion protein flanking CTL epitope of HIV-1 *gag* p17 or *nef*. (C) Recombinant pIJK-1 plasmid inserted with Alpha-K fusion protein flanking CTL epitope of HIV-1 V3J1 or E9Q.

the same manner except for the replacement of PBS by Tris-EDTA buffer. rBCG-pSO *gag* p55 cell lysates were used in the experiment on the determination of Gag protein expression by the detection of p24 antigen.

ELISA for p24 antigen

rBCG-pSO *gag* p55 lysate was assayed for the presence of HIV-1 p24 antigen by ELISA (Vironostika, BioMerieux, Boxtel, Netherlands) using the protocol as described in the kit instruction. Briefly, 100 µl of the test solution was added into each well of the anti-p24 coated microELISA plate and incubated at 37 ºC for one hour before washing. The anti-HIV-1 labeled with horseradish peroxidase was added into each well and the plate was further incubated at 37ºC for one hour. After washing, the TMB chromogenic substrate was added into each well and incubated for 30 minutes at room temperature until the color was developed. The reaction was stopped by adding with 1 M sulfuric acid, and the plate was measured under a spectrophotometer using dual wavelength at 450-630 nm. The cut-off value was calculated according to the kit instruction. The amount of p24 antigen present in the test sample was determined from the standard curve derived from the assay on known concentrations of the reference p24 antigen.

p24 antigen blocking assay

The specificity of the result on HIV-1 p24 antigen detection was confirmed by blocking assay using anti-p24 polyclonal antibody (Catalog No. ARP406) kindly provided by Centralised Facility for AIDS Reagent, National Institute for Biological Standards and Control, UK. The supernatant of rBCG-pSO *gag*

p55 cell lysate at the dilution of 1:50 was mixed with an equal volume of anti-p24 polyclonal antibody or mixed with PBS as the mock control. The mixtures were incubated for one hour at room temperature to allow the binding of anti-p24 polyclonal antibody with its specific epitopes on Gag p55 protein present in the bacterial cell lysate. After incubation, the mixtures were tested by ELISA HIV-1 p24 antigen detection kit. Based on the cut-off O.D., the result of the test supernatant blocked with anti-p24 polyclonal antibody should be negative, while the supernatant mixed with PBS should be positive.

Animal inoculation

Cultures of rBCGs prior tested for protein expression were centrifuged at 1,000 × *g* for 10 minutes at 4ºC. The bacterial pellets were washed once with normal saline, and then adjusted to the concentration of 1 mg/ml in normal saline. Based on previous titration, this suspension contained about 2×10^7 bacterial cells/ml.[11] The fresh bacterial suspension were diluted to the desired dose in a final volume of 0.1 ml, and then injected subcutaneously once into BALB/c mice.

Isolation of rBCGs from splenic cells

Recovery of rBCGs from splenic cells was conducted in mice inoculated with 0.1 mg/0.1 ml/mouse. Two inoculated mice were sacrificed for spleens and skin tissues at injection site at the intervals of 3, 7, 14, 28 and 60 days post inoculation. Each spleen was cross sectionally cut into three pieces; the middle piece together with skin tissue at injection site were put into 10% formalin solution for pathological study, while the remaining pieces from the two mice were pooled together and gently ground on a 0.7 µm cell strainer (Falcon, Becton Dickinson, NJ, USA) to obtain single cell suspension before inoculating into bottles of MB/BacT process medium (Organon Teknika, NC, USA or BacT/ALERT MP, BioMérieux, NC, USA) in duplicate. The bottles were then incubated in MB/BacT 3D Mycobacteria Detection System automated machine (Organon Teknika) for recovery of rBCGs for 60 days. Once the signal of bacterial growth was shown, one ml of the culture medium was harvested and inoculated into 50 ml of Middlebrook 7H9 broth supplemented with 10% albumin-dextrose complex, 0.05% Tween 80 and 30 µg/ml of kanamycin for growth expansion. Only rBCGs could grow in the medium containing kanamycin; and growth turbidity was confirmed for their presence by acid fast bacilli (AFB) staining.

The capability to express Gag protein of the recovered rBCG-pSO *gag* p55 was determined by p24 antigen ELISA kit as described above.

Immunogenicity of rBCGs in the injected mice

A) Fixed dose response to rBCGs containing short insert sequence

Mice injected with rBCGs containing short HIV-1 inserted sequences: rBCG-pSO *gag* p17, rBCG-pSO *nef*, rBCG-pIJK E9Q, rBCG-pIJK V3J1 or the recombinant control (rBCG-pSO246 or rBCG-pIJK-1) at the inoculum size of 0.1 mg/0.1 ml/mouse were sacrificed for spleens at four weeks after inoculation for LP assay.

B) Dose escalation response to rBCG containing complete* gag *sequence

Mice injected with rBCG-pSO *gag* p55 or rBCG-pSO 246 control at dose of 0.01, 0.1, and 1 mg/0.1 ml/animal were sacrificed for spleens at 2 and 4 weeks after injection for LP assay.

Lymphocyte proliferation assay

Spleens were gently ground on a 0.7 µm cell strainer (Falcon) in RPMI-1640 (Gibco, NY, USA) supplemented with 10% fetal calf serum (Hyclone, Belgium) plus penicillin and streptomycin in order to obtain a single cell suspension. The mononuclear cells were separated from splenic cell suspension by using Lymphocepal II (IBL, Japan), then, adjusted to the concentration of 1×10^5 cells /200 µl/well and cultured in triplicate in a 96 well U-plate in presence of the corresponding specific synthetic peptides (Chiron Mimotopes, Australia): GagP17: TLWCVHQRIEVK, Nef: QVPLRPMTYK, E9Q: TIGPGQVFY; V3J1: NTRKSIHIGPGRAFYATGS at concentration of 5, 2.5 and 1.25 µg/well. Upon dose escalation response, the mononuclear cells from mice injected with rBCG-pSO *gag* p55 at different inoculum sizes were stimulated with recombinant GagP55 protein (10, 5 and 2.5 µg/well) or with Gag P24 pooled overlapping peptides (5, 2.5 and 1.25 µg/well). Recombinant GagP55

protein was kindly provided by National HIV Repository and Bioinformatic Center, Thailand; and the GagP24 pooled overlapping peptides comprising 22 peptides of 20 mers with 10 amino acid overlapping was contributed by Centralised Facility for AIDS Reagent, National Institute for Biological Standards and Control, UK (Catalog No. 788.1-788.22). Mononuclear cells from mice inoculated with rBCG-pIJK-1 and rBCG-pSO246 were included as the mock controls in every test run.

BCG cell lysate at dilutions of 1:10 and 1:20 and PPD (2 and 1 μg/well) were used as the specific positive control antigen and culture media was used as the negative control antigen.

After 6 days of stimulation, each well was added with 1 μCi of ^{3}H labeled thymidine (Amersham, NJ, USA) and further incubated for 16 hours before counting for ^{3}H thymidine incorporation in a Beta-counter machine (Packard Bioscience Company, Model Top Count, USA). The mean of counts per minute (CPM) was calculated from the triplicate wells. Stimulation index (SI) was determined by dividing the mean CPM from the wells stimulated with the test antigen with the mean CPM from the wells without antigen (medium control). SI of ≥ 2 was considered to be positive for LP.

Pathological study

Formalin-fixed tissues from the spleen and skin at injection site were processed, embedded, sectioned, and stained conventionally at the Department of Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. In each tissue sample, slides stained by hematoxylin & eosin were used for pathological study and slides stained by Ziehl-Neelsen method were used for detection of AFB.

RESULTS

HIV short insert sequences induced lymphocyte proliferation

Immunogenicity of rBCGs containing short HIV insert sequence was determined by LP assay at 4 weeks after injection. Among four types of rBCG (rBCG-pSO *gag* p17, rBCG-pSO *nef*, rBCG-pIJK E9Q and rBCG-pIJKV3J1), only rBCG-pIJKV3J1 and rBCG-pSO *gag* p17 could elicit the specific response against HIV-1 peptides. Nevertheless the response was at low level. Meanwhile, mice inoculated with either of these rBCGs yield positive LP response when stimulated with PPD. The result was reproducible in one additional set of experiments in different lot of mice. The average CPMs from those experiments were calculated and presented in term of SI as shown in Fig. 2.

rBCG-pSO *gag* p55 dose escalation in LP assay

Regarding dose escalation experiments in mice inoculated with different inoculum sizes of rBCG-

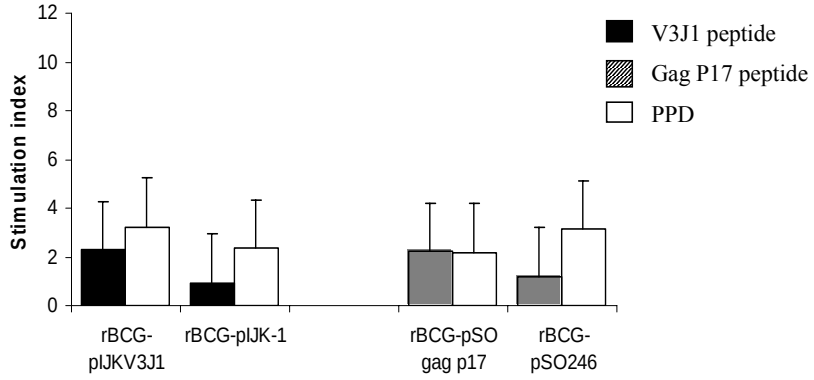

Fig. 2 Specific lymphocyte proliferation induced by rBCG-HIV-1 short insert sequences: rBCG-pIJK V3J1, rBCG-pSO *gag* p17 and backbone controls.

pSO *gag* p55 or rBCG-pSO246, LP assays against HIV epitopes have demonstrated positive results as tested at 2 and 4 weeks after injection, and in the dose response manner i.e., the dose of 1 mg/mouse gave stronger response than that of 0.1 mg/mouse; and the dose of 0.01 mg/mouse did not induce LP response. It was shown that recombinant Gag P55 protein elicited stronger LP response than Gag P24 pooled overlapping peptides. And as specific positive control antigens, BCG cell lysate gave stronger response than PPD. The results were reproducible in the second set of experiments using the other lot of mice. The SI values calculated from those two sets of experiments are shown in Table 1.

Isolation of rBCG-HIV-1 from the inoculated mice

No AFB could be recovered from splenic cells of mice inoculated with either kind of rBCGs at three days after inoculation. Dissemination of rBCGs from the inoculation site into spleen was shown at 7 days after inoculation. The inoculated rBCGs could persist in splenic cells for at least 28 days or may last as long as 60 days post-inoculation in mice inoculated with rBCG-pSO *gag* p55 (Table 2).

Expression of *gag* gene of the recovered rBCGs

Prior to animal inoculation, one ml of rBCG-pSO *gag* p55 cell suspension at O.D. of 1-1.5 was lysed and kept at -70ºC. At each interval post-inoculation, the rBCGs isolated were subpassaged once for expanding propagation before lysing. The original rBCGs together with the recovered rBCGs at the same concentration were tested simultaneously for expression of HIV Gag protein by the detection of p24 antigen using commercial ELISA. The result showed that the recovered rBCG-pSO *gag* p55 was still able to express p24 antigen. Although, the amount of protein expressed on day 7 was slightly increased, it markedly declined later on as seen in the rBCGs recovered at days 28 and 60 (Table 3). The specificity of p24 antigen detection system was confirmed by blocking assay using polyclonal antibody against p24 antigen. The

Table 1 Lymphocyte proliferation response to rBCG-pSO *gag* p55 by dose escalation

Inoculum/dose	Stimulation index in response to various antigens*			
	rGag P55 protein	Gag P24 pooled peptides	PPD	BCG-cell lysates
2 weeks after injection				
rBCG-pSO *gag* p55				
0.01 mg	1.71	1.27	1.27	2.238
0.1 mg	4.02	1.47	3.5	6.57
1 mg	7.67	2.68	6.58	9.89
rBCG-pSO246				
0.01 mg	0.78	1.12	1.62	1.1
0.1 mg	1.09	0.84	1.89	2.35
1 mg	1.64	0.72	2.77	9.14
4 weeks after injection				
rBCG-pSO *gag* p55				
0.01 mg	2.64	1.64	1.93	2
0.1 mg	3.47	1.84	2.18	2.05
1 mg	9.17	2.65	6.72	5.91
rBCG-pSO246				
0.01mg	0.87	1.02	1.14	1.28
0.1 mg	0.71	1.03	1.98	2.21
1 mg	1.03	1.43	3.21	5.01

*Average CPMs from two independent experiments were used in the calculation for SI.

Table 2 Recovery of rBCGs from splenic cells of the inoculated mice at different time intervals after inoculation

rBCGs	Bacterial recovery at different days after inoculation (days)				
	3	7	14	28	60
rBCG-pSO *gag* p55	neg	pos	pos	pos	pos
rBCG-pSO *gag* p17	neg	pos	pos	pos	ND*
rBCG-pSO *nef*	neg	pos	pos	pos	ND
rBCG-pIJK E9Q	neg	neg	pos	pos	ND
rBCG-pIJK V3J1	neg	pos	pos	pos	ND
rBCG-pSO246	neg	pos	pos	pos	ND
rBCG-pIJK-1	neg	pos	pos	pos	ND

*ND, Not done

Table 3 Expression of p24 antigen from rBCG-pSO *gag* p55 recovered from the inoculated mice

Days after inoculation	Amount of p24 antigen by	
	O.D.*	μg of p24 antigen per mg of rBCGs
0	0.461	2.415
7	0.616	3.341
28	0.441	2.296
60	0.211	0.924

*Cut-off O.D. = 0.093

blocking and unblocking assays of the same test sample were run simultaneously; and complete blocking was found in all test samples originated from days 0, 7, 28, and 60 after inoculation (data not shown).

Pathological study of spleen and skin tissues

Microscopic examinations of skin tissues at injection sites from mice inoculated with any kind of rBCGs revealed similar results. Abscess formation was found on day 3 post-inoculation. On days 7 and 14, the lesion progressed to abscess with central necrosis. Numerous AFB were found in necrotic debris, frequently in macrophages. On day 28, the abscess resolved, and necrosis disappeared together with a decrease in numbers of neutrophils and AFB. However, increasing numbers of foamy macrophages were observed within the lesion (Fig. 3). There was no granuloma formation in any animals observed throughout the study period.

Examination of the spleens from all mice inoculated with any kind of rBCGs at any time post-inoculation revealed no granuloma; and the change in number and size of lymphoid follicles was not found. At any time after inoculation, no AFB were visualized microscopically (Fig. 4). In addition, the significant pathologic change was not observed as compared the spleens of the inoculated mice to those of the non-inoculated ones.

Conclusively, there was no difference in the histological findings of spleens or skins of mice inoculated with either rBCGs or BCG vaccine strain at any interval after the inoculation.

DISCUSSION

Among various gene vehicles used in the development of HIV vaccine, BCG has such several advantages that it has been administered safely to

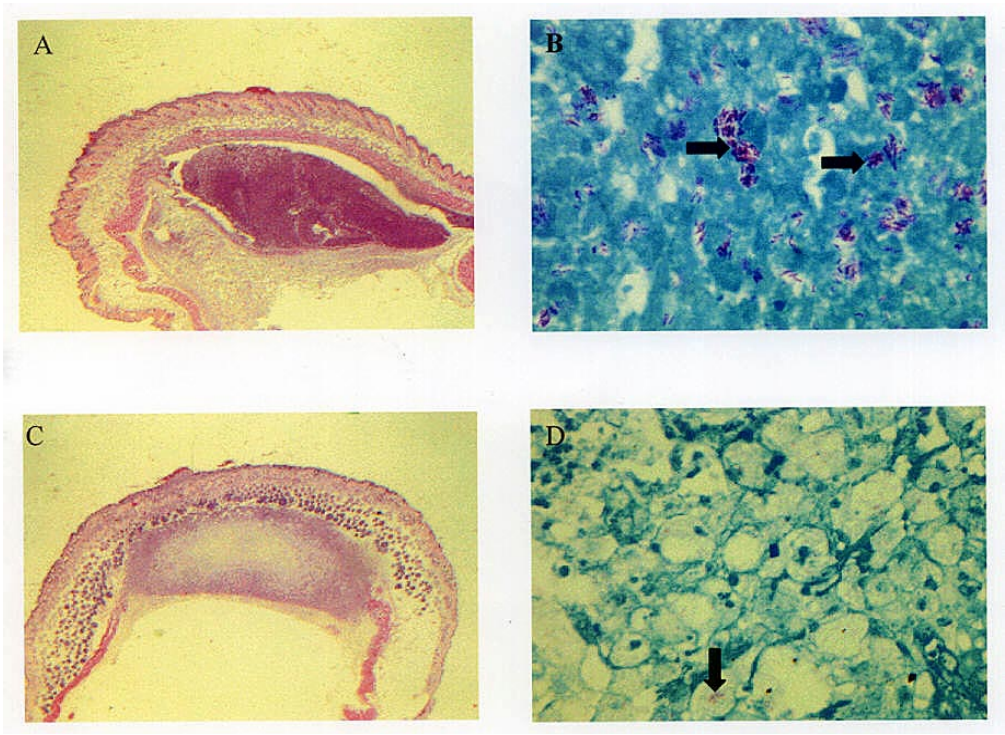

Fig. 3 Pathology of skin at injection site of mice inoculated with rBCGs. (A) Abscess formation at day 7 post-inoculation; dense content of pus is shown. (B) Numerous AFB (indicated by arrows) were observed in necrotic debris at day 7 post-inoculation. (C) Healing lesion appeared at day 28 post-inoculation. (D) Decreased numbers of neutrophils and AFB (indicated by an arrow) with an increased numbers of foamy macrophages were observed within the lesion on day 28 post-inoculation.

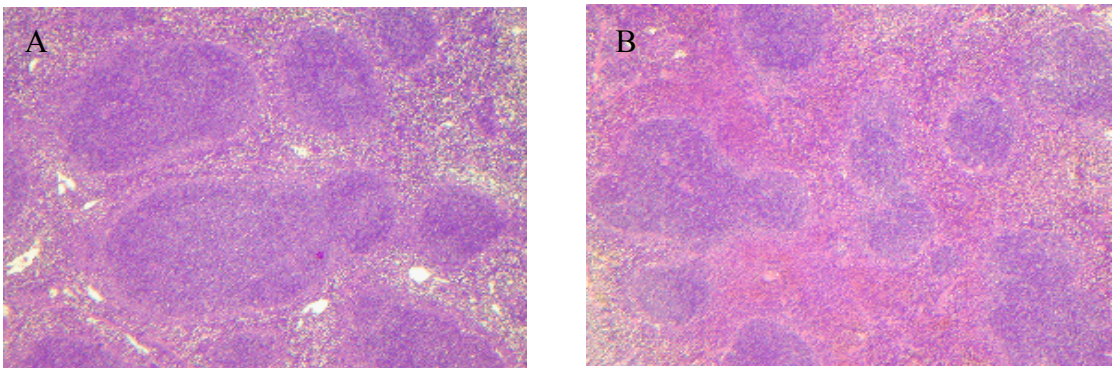

Fig. 4 Histological findings in spleen of mice inoculated with rBCGs. (A) Non-inoculated mouse. (B) rBCG inoculated mouse. The difference between the non-inoculated and the rBCG inoculated mice were not observed. No granulomatous reaction, no significant changes in numbers and sizes of lymphoid follicles, and no AFB were found.

billions of humans since 1960s;[2] it is affordable for developing countries, and BCG itself has an adjuvant effect which may enhance vaccine potency.[21,22] Regarding adverse events resulted from BCG vaccination, the localized effects are common, but the serious effects are rare.[1,2]

Recombinant BCGs with different foreign gene inserts of different microbial origins have been studied. The insert sequences of various sizes could express specific antigens which were immunogenic.[5-15] In 1990, Matsuo *et al*.[16] first reported the construction of rBCG expressing HIV-1 Gag p17 B-cell epitope. Subsequent reports by different groups of investigators have shown that rBCGs could express different kinds of HIV antigens.[9-14] In 1995, Honda *et al*.[11] reported that rBCG containing DNA fragment encoding 19 amino acids derived from V3 domain of Japanese consensus HIV-1 subtype B designated rBCG-pIJKV3J1 could induce both neutralizing antibody and cytotoxic T lymphocyte (CTL) activities. In addition, rBCG-pSOV3J1 was also shown to induce delayed type hypersensitivity and lymphocyte proliferation response in guinea pigs.[13]

The study on immunogenicity of rBCGs containing short HIV insert sequence demonstrated that only mononuclear cells from mice inoculated with rBCG-pIJKV3J1 and rBCG-pSO *gag* p17 could elicit specific lymphocyte proliferative response upon stimulating with the corresponding peptides (Fig. 2). However, the SI was relatively low (approximately 2 SI). The negative result together with the low LP response could be explained by length of the insert sequences such that *gag* p17 had 12 mers and V3J1 had 19 mers, while the others had 10 mers or less. Moreover, the HIV-1 T-cell epitopes of our insert sequences were designed to match with human MHC molecule (HLA-A2, A11), thus, they may not fit mouse MHC. Previous investigators had shown good LP response against peptide epitopes which were suitably matched with mouse MHC.[23,24]

Regarding escalation of the inoculum sizes, the higher injecting dose elicited stronger LP response both to Gag and bacterial proteins. In addition, the response at 4 weeks were higher than those at two weeks

Hanson *et al*.[18] recognized that BCG or rBCG expressing PspA of *Streptococcus pneumoniae* or OspA of *Borrelia burgdorferi* rapidly disseminated and established foci of infection in a variety of organs including spleen, liver and lung upon intravenous inoculation, and remained persistent for as long as 60 days. Nevertheless, it has not been shown that the recovered rBCGs could either harbour the insert sequence or express the foreign antigen. Hiroi *et al*.[10] found that single nasal immunization of rBCG-V3J1 in mice could induce and maintain high levels of long term HIV-specific serum IgG response for longer than 12 months without any booster dose. It was proposed that such long lasting antibody production reflected the stability of rBCG-V3J1 in an expression of HIV-1 antigen. However, the study did not explore this issue.

The present study demonstrated that as early as 7 days after subcutaneous inoculation, rBCG disseminated to spleen, and the persistence of the bacteria was evidenced throughout 28 days or even 60 days (with the study on rBCG-pSO *gag* p55 only) of observation (Table 2). It appeared that the size of HIV DNA inserts did not affect time of persistence of the bacteria in spleen. In addition, the present study has demonstrated that HIV-1 *gag* DNA insert sequence was persistent in the rBCGs with stable expression as determined by the ELISA p24 antigen assay of the bacterial lysates obtained from rBCG-pSO *gag* p55 recovered from splenic cells at different time intervals. The persistence of rBCGs may have such an advantage that it may prolong time of expression of the HIV DNA insert sequence, but the disadvantage is the chance of bacterial dissemination and complication.

The skin tissues at injection site together with spleens of the inoculated BALB/c were examined for a pathological change. Abscess formation in the skin tissue was observed on the third day post-inoculation, followed by progression of lesion and spontaneous healing on day 28 of observation. Even though AFB were detected, no granuloma was found in any skin lesion throughout the study period. Similarly, AFB and granuloma were not seen in any spleen sections, regardless of the negative or positive recovery of rBCGs from the splenic cells from mice sacrificed at days 3, 7, 14, 28 and 60 after inoculation. In addition, the results of pathological study were similar among mice inoculated with various kinds of rBCGs. In Thailand, BCG vaccine is administered intradermally to children within 24 hours after birth at dose of 0.05 mg in 0.1 ml.[25] Thus, the amount of rBCG used in this study was approximately equal to two newborn doses.

Therefore, the present study demonstrated that rBCGs containing extrachromosomal recombi-

nant plasmid with different HIV-1 DNA insert sequences at relatively high dose for small animals could be administered subcutaneously without serious pathological effect. The recombinant plasmids persisted in the recovered rBCGs, and their ability to express the foreign protein remained intact.

Accumulative data suggested that small HIV epitope in the vaccine design may limit vaccine efficiency only to certain HLA alleles. The present study also demonstrated weak immune response when rBCG containing short insert sequences were used. On the other hand, several pieces of evidence suggested that Gag epitopes should be included in the HIV vaccine design. Gag epitopes are conserved across different HIV subtypes. They also induce cytotoxic T lymphocyte (CTL) activity which control HIV disease progression, and these CTL activities also acted against cross subtype infection.[26-29] We also showed that rBCG-pSO *gag* p55 at appropriate dose could also induce significantly lymphocyte proliferation response in mice. However, anti-HIV Gag antibody in these mice has not been detected. Nevertheless, rBCG-HIV-1 whole *gag* should be appropriated for further investigation as the candidate rBCG based HIV vaccine.

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