ENHANCEMENT OF CELL-MEDIATED IMMUNE RESPONSE IN MICE BY WHOLE HIV-1 GAG IN *MYCOBACTERIUM BOVIS* BCG AS A LIVE VACCINE CANDIDATE

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Abstract. In this study, we employed a recombinant *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) harboring whole HIV-1 CRF01_AE gag DNA as a candidate vaccine to investigate specific cell-mediated immunity in BALB/c mice. Construction of the stable expression recombinant BCG was achieved by demonstrating by Western blot detection of protein of approximately 55 kDa. By a single injection of 0.1 mg of the recombinant HIV-1 gag protein expressing BCG subcutaneously into mice, after 2 weeks various specific cytotoxic T-lymphocyte (CTL) responses were exhibited against a single gag epitope of amino acid positions 294-304, and also against various peptide regions along the entire gag protein with moderate CTL activities (10-35% specific cell lysis), which increased to relatively high levels (50-68%) after one month. However, after two months activities were 3-3.7 fold lower. On the other hand, gag-specific lymphocyte proliferation was detected 9.3 fold higher than that of non-immunized mouse spleen cells. Our results indicate that in mice, BCG can be used as a recombinant live vector to induce cellular immune responses to HIV-1 gag antigen.

INTRODUCTION

Worldwide efforts are now being focused on the development of a vaccine to protect against infection by HIV. Protective vaccines have usually been designed to induce humoral antibodies to neutralize viruses. However, as the spread of HIV in human population is likely to occur frequently through exposure to intracellular viruses, it may be necessary to use vaccines that induce cell-mediated immunity.

Since live vaccines are among good vehicles to induce cellular immunity, *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) is an appropriate vector for developing into HIV vaccine because it has several advantages. BCG is the most widely used vaccine in the world especially in neonates to protect against tuberculosis. The cell wall of BCG has proven to be good adjuvant by itself for induction of cell-mediated immunity. Moreover, it has a large capacity to accept insertion of foreign DNA and is heat stable (Fortier *et al*, 1987; Pearce *et al*, 1988). At

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present, several plasmid vectors utilizing the replication origin from mycobaterial plasmid (Snapper *et al*, 1988, 1990; Ranes *et al*, 1990) or chromosomal integration of BCG have been developed and used to express foreign antigens (Husson *et al*, 1990; Lee *et al*, 1991). Some recombinant BCGs have been reported to induce protective humoral antibody (Langranderie *et al*, 1993; Langermann *et al*, 1994) or even immunoglobulinA (IgA) antibodies (Langranderie *et al*, 1993).

Regarding the investigation of HIV vaccine, recombinant BCG have already been employed to explore cellular immunities, such as cytotoxic T-lymphocytes (CTL) against HIV-1 V3 in mice (Kameoka *et al*, 1994) or even protective neutralizing immunoglobulin (IgG) in guinea-pigs and CTL in mice (Honda *et al*, 1995). Furthermore, there have been studies of gag-specific CTL in Simian immunodeficiency virus (SIV)infected - rhesus monkeys (Yasutomi *et al*, 1993), and different four recombinant BCG of SIV-Gag, Pol, Env and Nef have been introduced into monkeys simultaneously, which have been able to stimulate specific IgA, IgG antibodies, CTL and lymphocyte proliferation (Leung *et al*, 2000). HIV-1 gag is one of the most conserved viral proteins. Cross-clade CTL responses recognizing conserved epitopes in HIV-1 gag have been detected in HIV-1 infected people (Betts *et al*, 1997; Bertoletti *et al*, 1998; Durali *et al*, 1998; Lynch *et al*, 1998). Additionally, a strong gag-specific CTL response appears to correlate inversely with the viral load of HIV-1 infected patients (Clerici and Shearer, 1996).

In this study, the cellular immune responses as CTL and lymphocyte proliferation towards HIV-1 CRF01_AE gag were evaluated following introduction of the whole gag sequence into a recombinant DNA vector to determine whether it was appropriate to use BCG expressing HIV gag protein to develop as an HIV vaccine candidate.

MATERIALS AND METHODS

Construction of recombinant BCG/HIV-1 gag

The whole DNA sequence of HIV-1 gag isolate subtype CRF01_AE (GenBank accession no. AY863146) was amplified with a pair of primers, 5'-ATATATCAATTGATCTAGCG GAGGCTAGAAGGAGAGAG-3', and 5'-ATATATGGATCCCTAATACTGTATC ATCTG CTCCTGTAT-3', whose 5'-ends were modified to comply with the *Mun*I and *Bam*HI restriction site, respectively. The amplified fragment was then digested with *Mun*I and *Bam*HI and subsequently inserted into the respective enzymes-digested pUC19 vector, with hsp60 promoter downstream of the *Sac*I site introduced by the appearance of the *Mun*I site upstream of the multi-cloning site of the vector and the ampicillin resistance gene. This construct was designated as pUChsp-gagE. Following addition of *Kpn*I linker at the *Eco*RI site of pUChsp-gagE, the recombinant plasmid was able to be digested with *Kpn*I and ligated with pSO246 plasmid (Matsumoto *et al*, 1996) harboring the mycobacterial origin of replication and kanamycin resistance gene to generate the recombinant HIV-1gagE plasmid as shown in Fig 1. This recombinant plasmid was then transformed into BCG strain Tokyo72. The transformed BCG (rBCG/HIV-1gagE) was cultured by plating on 7H10 agar containing 1.9% Middlebrook 7H10 (Difco, BD Diagnostic Systems, Sparks, MD), 0.5% glycerol, 10.0% Middlebrook OADC serum (Difco, BD Diagnostic Systems, Sparks, MD), and 10 μg/ml kanamycin for 3-4 weeks until colonies appeared. The culture was then enriched by transferring a colony into the 7H9 broth containing 0.47% Middlebrook 7H9(Difco, BD Diagnostic Systems, Sparks, MD), 0.05% glycerol, 10.0% Middlebrook ADC serum (Difco, BD Diagnostic Systems, Sparks, MD) with kanamycin and culturing for 2-3 weeks.

Screening for the gene expression of rBCG/HIV-1gagE

The rBCG/HIV-1gagE cell lysate which was prepared by sonication and was electrophoresed in 8-20% gradient polyacrylamide gel (Laemmli, 1970) and subjected to Western-blot analysis by reacting with anti-HIV-1 gag p24 epitope monoclonal antibody kindly provided by Professor K Ikuta, the Research Institute for Microbial Diseases, Osaka University, Japan (Matsuo *et al*, 1992).

Determinations of the HIV-1 gag protein expression yield and stability

To subculture the bacteria, 1.0 ml of rBCG/HIV-1gagE culture was inoculated into 50.0 ml of fresh 7H9 medium containing 10 μg/ml kanamycin and maintained until the presence of the gag protein could be detected by Western blot analysis and/or by a commercial HIV-1p24 antigen ELISA (Murex Biotec, Kent, UK). After that, cells were subcultured serially as described above. To determine the amount of gag protein in each culture, 5.0 ml of the culture were centrifuged to collect cells, which were sonicated to obtain cell lysate. Appropriately diluted lysate was assessed with HIV-1p24 antigen ELISA and compared against a standard curve of HIV-1 p24 protein (US Biologicals, Swampscott, MA).

Mouse immunization

rBCG/HIV-1gagE (0.1 mg or $2x10^6$ cfu) was injected subcutaneously once into each 6-8 week old BALB/c mouse (H-2d). After specific periods of time, mice were sacrificed and the spleens were taken to test for cell-mediated immunity. Another group of mice was injected with recombinant BCG containing only the DNA vector pSO246 as immunization control.

Cytotoxic T-lymphocyte (CTL) assay employing ^{51}Chromium Release Assay

In every assay, at least ten mice were immunized with the same immunogen in each test group. Immunized and control mouse spleens were harvested and prepared as single spleen cells. Before performing the assay, all spleen cells from the same immunization group were pooled and 10^7 cells (used as effector cells) were incubated for 5 days with various HIV-1 gag peptides: HIV-1 gag p24 (amino acids 287-301, QGPKEPFRDYVDRFY), or ten different mixtures of five overlapping 20 mer-peptides which spanned a range of 50-60 amino acids serially one after another, starting from the first to the last amino acid to cover the whole sequence of HIV-1 gag protein (498 amino acids). For the preparation of target cells, p815 mouse (H-2d) cells were either incubated for 3 hours with gag peptide (amino acids 287-301) or infected overnight with recombinant Vaccinia virus harboring the whole HIV-1 gag gene (Ishii *et al*, 2002), and then labeled with $Na^{51}CrO_4$. Effector cells were mixed with target cells in various ratios for 5 hours before removing supernatants to determine release of radioactivity due to lysis of target cells. Experiments were conducted in duplicate.

Lymphocyte proliferation assay

Immunized spleen cells were incubated in complete medium for 3 days with or without mouse p815 cells infected with recombinant Vaccinia virus expressing the whole HIV-1 gag protein, designated as "Stimulator" as described elsewhere (Nishino *et al*, 1994). Then 1 μCi of [^{3}H]-thymidine was added to the cells, which were incubated overnight, filtered through a 96-well plate membrane filter and washed with water. The filter was counted for the amount of [^{3}H]-thymidine incorporated into cells.

RESULTS

Construction of recombinant BCG expressing HIV-1 gag protein

A recombinant plasmid that contained

HIV-1 gag DNA was constructed. The procedure required the construction of a modified plasmid that facilitates the insertion of foreign DNA without disrupting BCG chromosome necessary for the growth of the organism. This involved the use of two plasmids, pUChsp and pSO246 that contained *Mycobacterium* hsp60 promoter and the origin of replication, respectively. pUC-hsp is an *E. coli-Mycobacterium* shuttle vector in which the gag gene was initially inserted so as to place it in-frame with the translation initiation codon and under the regulatory control of the hsp60 promoter. This recombinant construct contained the whole 1,494 bp gag gene.

Cell lysate from transformed BCG (rBCG/HIV-1gagE) was screened for the gag protein expression by Western blotting using with anti-gag p24 epitope monoclonal antibodies. Fig 2 shows that the lysate of rBCG/HIV-1gagE BCG contained a major protein band of approximately 55 kDa, the estimated size of the whole gag protein. Some smaller protein bands were also observed. Lysate from BCG transformed with only the pSO246 vector without the gag gene insert and the culture medium of the rBCG/HIV-1gagE culture showed no immuno-reactive proteins.

Using a commercial HIV-1 p24 antigen ELISA with a standard curve of HIV-1 p24 protein, the yield of gag protein expressed in rBCG/HIV-1gagE was estimated as ranging from 0.26 to 0.45 mg/liter of culture. On the other hand, rBCG/HIV-1gagE was found to stably express HIV-1 gag protein even after thirty subculturing.

CTL activity against a single HIV-1 p24 peptide

Spleen cells were isolated from BALB/c mice 2 weeks after they had been immunized once with a subcutaneous injection of either 0.1 mg ($2x10^6$ cfu) of rBCG/HIV-1gagE or BCG transformed with only the vector DNA (pSO246) as an immunization control. De-

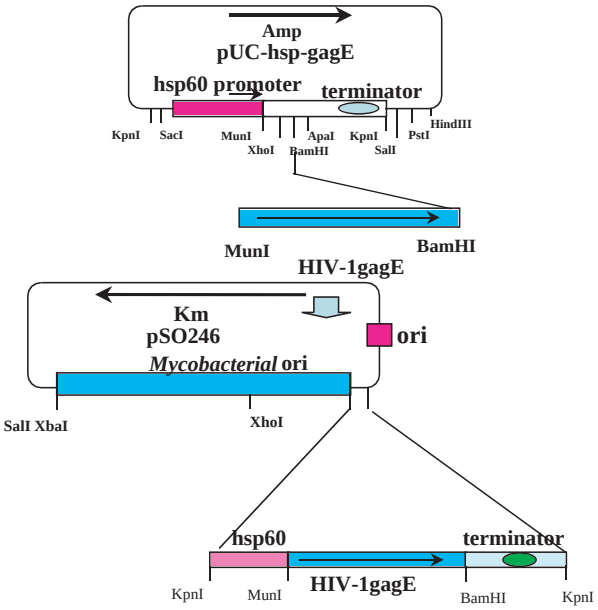

Fig 1–Scheme of construction of HIV-1 CRF01_AE gag DNA into plasmid vectors containing *Mycobacterium* promoter and origin of replication to generate rBCG/HIV-1gagE.

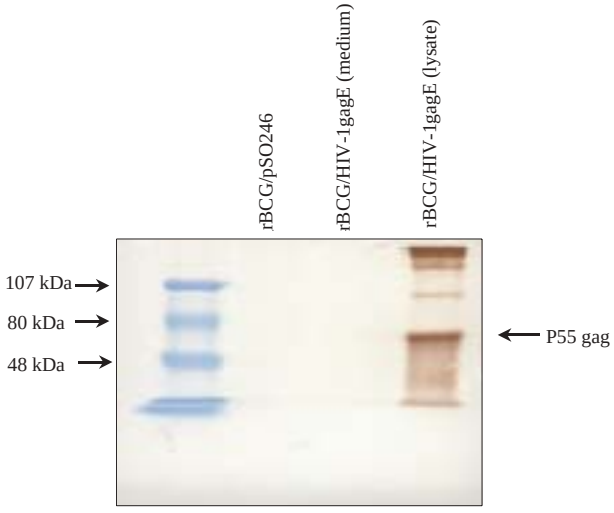

Fig 2–Western blot analysis of the cell lysates obtained from rBCG/HIV-1gagE, recombinant BCG transformed only with the DNA vector (pSO246), and the culture medium of rBCG/HIV-1gagE. Gels were reacted with anti-HIV-1 p24 (amino acids 287-301) monoclonal antibody.

tection of CTL response induced by the gag construct was performed against stimulation with a specific p24 peptide (gag amino acids 287-301). In order to obtain most specific target cells, P815 mouse cells were also pulsed with the same p24 peptide. After labeling with ^{51}Cr, the ability to lyse target cells containing p24 peptide was assumed to represent specific CTL activity. As shown in Fig 3, specific cell lyses were observed in duplicate dose-response curves against increasing ratios of effector to target cells. These results indicated that rBCG/HIV-1gagE could induce effector cells to lyse HIV-1 p24 peptide expressed target cells 5 to 15 fold higher than spleen cells injected with recombinant BCG without the gag gene insert (immunization control).

CTL activity against various peptides of HIV-1 gag protein

In this study, three different consecutive mouse immunizations were performed and spleen cells were tested separately. After a single injection with rBCG/HIV-1gagE, CTL activity studies were conducted by stimulating 10^7 spleen cells separately with each pool of different gag peptides. In total, ten cell cultures with ten different pools of five peptides were examined for CTL response to each peptide region, involving 50 peptides needed to cover the entire HIV-1 CRF01_AE gag protein sequence (498 amino acids). Target cells expressing all HIV-1 gag peptides were prepared by infecting P815 mouse cells with Vaccinia virus harboring the whole HIV-1 gag before ^{51}Cr-labeling. For the control group, non-immunized mouse spleen cells were cultured with the pool of all 50 HIV-1 gag peptides (20 μg each peptide).

One month after immunization, the specific lysis achieved from rBCG/HIV-1gagE-immunized spleen cells against peptide groups 1 to 10 is shown in Fig 4. All data are means of three consecutive immunization

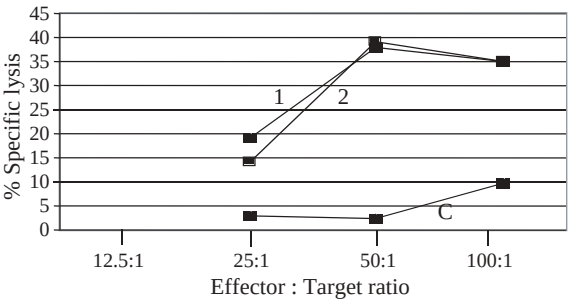

Fig 3–CTL response (percent specific lysis) after immunization of ten mice once with rBCG/HIV-1gagE for 2 weeks and restimulation with only a single peptide (amino acids 249-304). CTL activity was obtained in duplicate experiments (1 and 2). Immunization with rBCG/pSO246 was performed as immunization control (C).

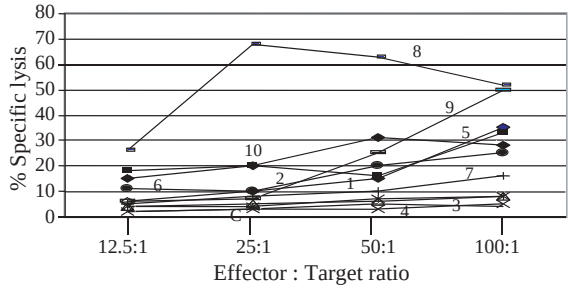

Fig 4–CTL response (percent specific lysis) after one month following immunization of twelve mice once with rBCG/HIV-1gagE and restimulation with ten groups of different gag peptides. CTL activity against each peptide group is designated nos. 1-10. Immunization with rBCG/pSO246 was performed as immunization control (X). Mean specific lysis from three consecutive experiments were used to plot the curves.

groups. Some showed high CTL recognition, as seen in No. 8 with the highest specific lysis of 68% at effector:target cell of 25:1. The rBCG/HIV-1gagE construct could induce CTL that recognized many gag peptides at different activity levels as seen in curves 1, 2, 6, 7, 8, 9 and 10, some of which were obvi-

ously high while some were moderate (numbers 6 and 7). Nevertheless, these results also showed that some gag regions seemed not to be recognized by the immunized cells (numbers 3, 4, and 5, when the cut-off value was 10% cell lysis). In the case of the immunization control, which was obtained from spleen cells immunized with vector DNA-transformed BCG and stimulated with all gag peptides, no CTL activity was detected (less than 5% cell lysis). From the attempts to detect specific lysis against 10 different mixtures of gag peptides at various effector:target cell ratios, comparative results were obtained from CTL induction at a fixed effector:target cell ratio of 100:1 were also demonstrated (Fig 5).

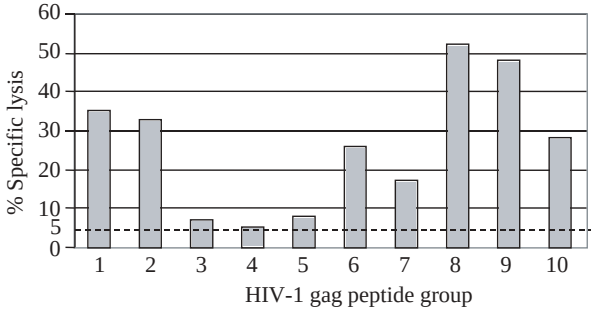

Fig 5–CTL response (percent specific lysis) after one month following immunization of twelve mice once with rBCG/HIV-1gagE and restimulation with ten groups of different gag peptides. CTL activity against each peptide group is designated nos. 1-10. The effector:target cell ratio was 100:1.

After two months of immunization, some specific lysis was found but most of them had decreased 3 - 3.7 folds (comparing the lowest and highest percent cell lysis) (Fig 6). The highest specific lysis after two months was only 23%, while that after one-month duration was as high as 68%. In this study, the gag peptides used to stimulate the immunized spleen cells were contained in only five different mixtures of ten peptides each, so that the fifty peptides were reduced to five groups, designated as numbers 1, 2, 3, 4 and 5. Similarly, mouse cells of immunization control still showed percent specific lysis of less than 5%.

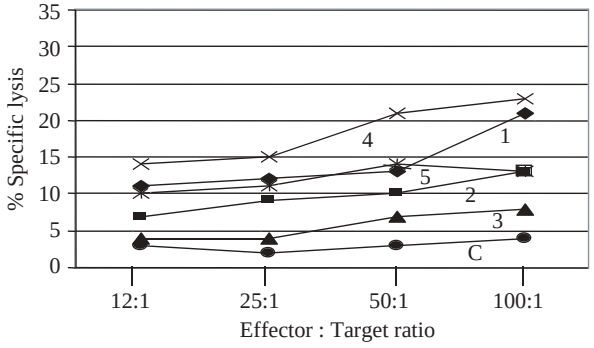

Fig 6–CTL response (percent specific lysis) after two months following immunization of ten mice once with rBCG/HIV-1gagE and restimulation with five groups of different gag peptides. CTL activity against each peptide group is designated nos. 1-5.

Specific lymphocyte proliferation response

After a single immunization of rBCG/HIV-1gagE in BALB/c mice, spleens were harvested at week 6 and spleen cells isolated. The spleen cells were stimulated by incubation for 3 days with P815 mouse cells to present the gag antigen as a 'stimulator', while the same immunized cells were also separately incubated without the stimulator as control. At the same time, spleen cells from non-immunized mice (by injection with BCG transformed with only vector DNA) were processed similarly. A marked enhanced anti-gag antigen lymphoproliferative response was demonstrated with a stimulation index as high as 11.2, while in non-stimulated mouse spleen cells the stimulation index was only 1.2, approximately 9 fold lower than the gag-specific stimulated cells (Fig 7).

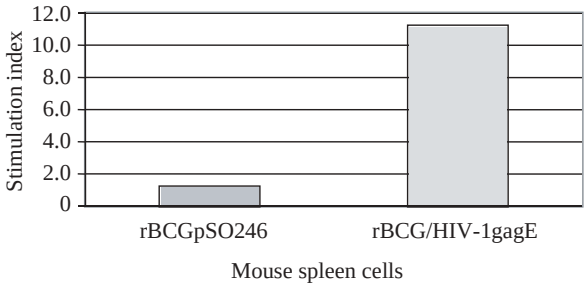

Fig 7–Specific lymphocyte proliferation resulting from mouse spleen cells two months after immunized with rBCG/HIV-1gagE. Stimulation index is ratio of incorporation of [^{3}H]-thymidine in stimulated to non stimulated spleen cells. As control, mice were immunized with BCG transformed with vector pSO246 (rBCGpSO246).

DISCUSSION

In the present study, an attempt was made to design an HIV vaccine candidate by investigating the immune response in BALB/c mice. The vaccine construct was based on a model of recombinant live vaccine by using BCG as a vector to harbor recombinant HIV-1 DNA, as BCG is one of the live microorganisms efficient in replication in human as well as in mice to express foreign proteins (Takeya *et al*, 1977; Ueda *et al*, 1978; Dannenberg, 1994; Fine *et al*, 1994; Edelman *et al*, 1998). To control the spread of HIV or progression of AIDS disease, which is characterized by cell-associated HIV that might attribute to cell-mediated immune responses, and since natural infection of microorganisms or live vaccines frequently induce cellular immunity and perhaps also humoral antibodies, experiments were carried out using a vaccine construct to determine some cell-mediated immune responses such as CTL and lymphoproliferative activities in immunized mice.

To construct a recombinant vaccine candidate, the whole gag DNA of isolated HIV-1 CRF01_AE was inserted into two appropriately modified plasmids containing mycobacterial promoter and an origin of replication. The target protein was detected only from lysate of the recombinant BCG, having the predicted size of approximately 55kD, as shown by Western blot assay using anti-gag epitope monoclonal antibody. The expression seemed to be in-frame with the iniation codon of the hsp60 promoter used in this study. However, there were some protein bands of lower and higher molecular weight reacting with the monoclonal antibody, which might be cleaved and aggregated gag products. Moreover, as the expressed protein could be detected even after the 30 continuous subculturing of the recombinant BCG, the construct seems to present a stable protein expression system appropriately for future utilization as a vaccine model.

All immunization in this study was done by single injection of rBCG/HIV-1gagE subcutaneously into BALB/c mice. After 2 weeks, CTL activity induced in the mice was determined. In this case, a gag peptide having amino acids 294-304 in the p24 region was used to stimulate immunized spleen cells *in vitro* and successful CTL induction in mice was observed. More obviously, after 1 month, when immunized spleen cells were stimulated with peptides spanning the entire gag protein by separate incubations with different mixtures of five overlapping 20-mer peptides in order to demonstrate specific CTL reflected from different regions, variable degrees of CTL activities were observed, some of which were moderate (10-35%) while some were relatively high (50-68%). Additionally, at a fixed effector:target cell ratio of 100:1, CTL responses recognizing different gag epitopes in many regions were more easily observed.

Based on amino acid positions, CTL recognition of HIV-1 gag in mice might be in the regions of HIV-1 p17 (peptide groups 1

and 2), with lower recognition were among the end region of p24 (groups 6 and 7) and in p6, while the highest was in the regions of p2, p7 and p1 (groups 8 and 9). However, the peptides in the first half of p24 were observed to show no specific CTL induction (groups 3, 4, and 5). In the case of CTL activity against one specific peptide, position 294-304, in the first experiment, which was among peptide group 6, good specific cell lysis was exhibited. The different results observed between the first and second experiment might be due to individual immunogenic susceptibility of the different groups of mice studied. Alternatively, pulsing with a single peptide to the target cells might induce a stronger antigen expressed target than by infection with recombinant Vaccinia virus, which might induce clusters of gag peptides expressed *in vitro*. However, the reason to use recombinant Vaccinia virus infection to generate specific target cells was that it was expected to express most of the specific antigens, as the recombinant DNA inserted into the virus was the same HIV-1 DNA used to construct the live vaccine candidate.

When the immunization was prolonged until two months, although CTL activities against different portions of the gag peptides existed, they were 3-3.7 fold lower than those of the one month immunization. This decline might be affected by insufficient CTL induction process owing to the immunogen itself, or it may have progressed to be another immune response such as memory T-cells. This should be proven by boosting with the same vaccine candidate or with other vaccine vehicle harboring the same immunogen.

In the present study, the effect of rBCG/HIV-1gagE immunization on lymphoproliferation was also investigated. Anti-gag antigen spleen cell proliferation was significant compared to the non-stimulation control. This verified that not only CD8+ T cells were induced to elicit CTL response, but CD4+T helper cell related immune induction was also revealed by the same immunization. In contrast, detection in plasma after 2 weeks, 1 and 2 months of the single injection, showed no anti-gag antibodies, using either p17, p24, or p55 proteins by Western blot (data not shown). It may be postulated that rBCG/HIV-1gagE immunization might not appropriate to stimulate B cells as opposed to T cells, or that amount of immunogen used might be too small that the stimulation was geared towards cell-mediated immunity rather than humoral antibody as shown in other reports (Bretschu *et al*, 1992; Hosken *et al*, 1995; Levy, 1998).

The findings from this study suggest that BCG can be used as a live vector to deliver HIV-1 gag as a vaccine candidate in mice by stimulating gag-specific cell-mediated immunity as determined *in vitro*. Although, specific T cell recognition epitopes were not identified, by this immunization regimen HIV-1 gag could be exhibited efficiently to contain T cell epitopes that were substantially recognized. Despite the large DNA insert, the large protein expressed intracellularly was processed *in vivo* into multiple peptides to trigger the cellular immunity in mice. This recombinant BCG-/HIV-1gagE vaccine might be more advantageous than a recombinant BCG expressing only single gag epitope, which failed to induce protective efficacy in monkeys (Yasutomi *et al*, 1995).

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