PRIME-BOOST VACCINATION USING RECOMBINANT MYCOBACTERIUM BOVIS BCG AND RECOMBINANT VACCINIA VIRUS DIs HARBORING HIV-1 CRF01_AE GAG IN MICE: INFLUENCE OF IMMUNIZATION ROUTES

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Abstract. We have previously reported that live vector-based HIV-1 gag vaccine candidate using BCG as a vector was achievable in BALB/c mice. Although the gag-specific CTL induced by this live candidate vaccine is significantly high, persistence of CTL remains unclear. Thus, efforts were made to explore the potential of recombinant Vaccinia virus DIs strain harboring the same HIV-1 CRF01_AE gag gene (rVaccinia/HIV-1gagE) present in the BCG construct, using different immunization routes. After one month following a single subcutaneous (s.c.) injection of rBCG/HIV-1gagE, higher CTL responses were recognized against various peptide epitopes along the whole gag protein compared to that by intradermal (i.d.) route. A prime-boost regimen having only rDIs/HIV-1gagE injected i.d. induced very low CTL levels. However, within two months, by priming with rBCG/HIV-1gagE s.c. and boosting with rVaccinia/HIV-1gagE intravenously (i.v.), CTL levels were greater (20-68% specific cell lysis) than those obtained by priming and boosting both i.d. (18-35%). After seven months, both prime-boost immunization with rBCG/HIV-1gagE s.c. and with rVaccinia/HIV-1gagE either i.v. or i.d. sustained similar CTL levels. Our studies exhibit that the prime-boost vaccination of rBCG/HIV-1gagE following by rVaccinia/HIV-1gagE i.d. could be used to elicit prolonged CTL responses as well as memory T-cells in mice, which might be more practical than using i.v. route.

INTRODUCTION

Recombinant Vaccinia virus is an attractive anti-HIV-1 vaccine delivery system due to its ability to elicit cell-mediated immunity as well as humoral antibodies (Collado *et al*, 2000; Ishii *et al*, 2002; Shen *et al*, 2002). Furthermore, highly attenuated Vaccinia virus mutants that represent safe live vectors appropriate as vaccine constructs have been generated (Moss 1996; Paoletti, 1996). A number of different Vaccinia recombinant viruses expressing HIV antigens have been developed as potential HIV vaccines (Rodriguez *et al*, 1989; Collado *et al*, 2000). This virus can accommodate large pieces of foreign DNA for expression and is retained

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as virion-like particle by some strains (Moss, 1996; Paoletti, 1996), and has been claimed to be safe and immunogenic (World Health Organization 1972).

We have recently shown that recombinant BCG containing the whole HIV-1 gag gene was able to elicit cell-mediated immunity in mice (Promkhatkaew *et al*, 2009). However, use of recombinant BCG as a single vaccine may stimulate only high specific CTL response for a limited period of time. Thus, in this study attempts were made to apply Vaccinia virus as a live HIV vaccine candidate in order to exhibit better and more prolong immune response. Vaccinia virus DIs strain was chosen as it is a highly attenuated mutant strain that is not capable of replicating in mammalian cells in vitro (Ishii *et al*, 2002). Moreover, DIs has been shown to induce high cytotoxic T-lymphocyte (CTL) levels in mice (Ishii *et al*, 2002), and to protect monkeys from simian-human immunodeficiency virus (SHIV) (Izumi *et al*, 2003).

Currently, a prime-boost approach based on the use of different vectors as priming agents and a recombinant Vaccinia virus as a booster agent shows promise as an effective vaccination strategy against pathogens, due to a marked enhancement of specific CD8+ T cell immune response (Li *et al*, 1993; Hanke *et al*, 1998; Miyahara *et al*, 1998; Gonzalo *et al*, 1999; Izumi *et al*, 2003). It is expected that by priming with one vaccine facilitating cellular immunity, a population of memory T-cells will exist, and after boosting with other vaccine vehicles, routes or means of antigen presentation to the immune system, this may stimulate the immune response due to the primed memory T-cells. Immunization routes should be taken into consideration when designing and evaluating vaccines, therefore in the present study, we evaluated in mice two live vector-based vaccine models, namely, recombinant BCG and Vaccinia virus presenting HIV-1 gag protein, individually as well as prime-boost vaccination strategies.

MATERIALS AND METHODS

Constructions of recombinant BCG and recombinant Vaccinia virus having HIV-1gag gene

Construction of recombinant BCG having HIV-1 gag gene (rBCG/HIV-1gagE) has been described previously (Promkhatkaew *et al*, 2009). To construct the recombinant Vaccinia-DIs virus expressing the same HIV-1 gag gene (rDIs/HIV-1gagE), the whole DNA sequence of HIV-1 gag isolate subtype CRF01_AE (GenBank Acc. No. AY863146) was prepared by PCR as described previously (Promkhatkaew *et al*, 2009). The amplified DNA was then made blunt-ended and inserted into *Sma*I site of a plasmid containing Vaccinia virus early/late promoter p7.5 that has already been modified to contain a *Hin*dIII site at the position of *Eco*RI site (Ishii *et al*, 2002). In order to obtain an appropriate plasmid for transfection into the Vaccinia virus DIs, plasmid pUC/DIs was generated by inserting a 1.9 kbp of gene-deleted Vaccinia virus DIs fragment into pUC vector (Ishii *et al*, 2002). Finally, the DNA fragment containing HIV-1 gag gene adjacent to p7.5 promoter was inserted into pUC/DIs at the *Hin*dIII site (Fig 1). This recombinant plasmid (pUC-DIs-gagE) was then transfected into the Vaccinia-DIs virus which was designated as rDIs/HIV-1gagE. For control virus, only pUC/DIs was transfected into Vaccinia-DIs (designated as DIs/LacZ). Recombinant virus was cultured in primary chicken embryonic (CEF) cells and the titer was determined as described (Morita *et al*, 1977; Ishii *et al*, 2002).

Screening for gene expressions from rBCG/HIV-1gagE and rDIs/HIV-1gagE

Western blot analysis of gene expression in cell lysate of rBCG/HIV-1gagE

was performed as described previously (Promkhatkaew *et al*, 2009). Western blot analysis of lysate of CEF cells infected with rDIs/HIV-1gagE was conducted as described (Ishii *et al*, 2002), and gels were immunostained with anti-HIV-1gag p24 monoclonal antibody, kindly provided by Professor K Ikuta, the Research Institute for Microbial Diseases, Osaka University, Japan (Matsuo *et al*, 1992).

Mouse immunization

rBCG/HIV-1gagE cells were washed and resuspended in normal saline solution and 0.1 mg (approximately $2x10^6$ cells) were injected with different routes once into 6-8 weeks old BALB/c (H-2d strain) mice. To immunize mice with rDIs/HIV-1gagE, virus grown in CEF cells was purified by centrifugation through 36% sucrose at 18,000*g* for 90 minutes and resuspended in phosphate-buffered saline. Virus titer was then determined. rDIs/HIV-1gagE ($1x10^6$ pfu) was injected once or twice into mice through different routes. For trials of prime-boost immunizations, mice were inoculated with a single dose of rBCG/HIV-1gagE for various periods of time prior to a single injection of rDIs/HIV-1gagE. In control group, mice were injected with the same amounts of recombinant BCG harboring only pSO246 or rDIs/LacZ.

Cytotoxic T-lymphocyte (CTL) assay

At least ten BALB/c mice were tested in each group. After immunizing with an individual immunogen or prime-boost immunization, immunized and control mouse spleens were harvested and prepared as isolated spleen cells. To perform as effector cells, spleen cells from the same immunization protocol were pooled and 10^7 cells were incubated for 5 days with different HIV-1 CRF01_AE gag peptide mixtures. Each mixture comprised 5 overlapping serial 20-mer peptides of 50-60 amino acids in length (amino acid positions 1-60, 51-110, 101-160, 151-210, 201-260, 251-310, 301-360, 350-408, 399-456, and 449-498), resulting in ten mixtures that covered the whole sequence of HIV-1 gag protein (498 amino acids). For the target cells, 10^7 P815 mouse (H-2d strain) cells were infected with rDIs/HIV-1gagE for 18 hours and labeled with 100 µCi $Na^{51}CrO4$ for 90 minutes at 37ºC under an atmosphere of 5% CO_2. Cells were then washed with RPMI-1640 medium and resuspended (10^5cells/ml) in RPMI-1640 medium supplemented with 10% FBS. Then $5x10^3$ target cells together with two-fold serial dilutions of $5x10^5$ effector cells were placed into U-bottom microtiter plate (to produce effector: target cell ratios of 100:1, 50:1, 25:1, and 12.5:1) and incubated for 5 hours. Spontaneous- and total- ^{51}Cr release was determined by radioactivity counting of target cells with the presence of medium and following treatment with 5% Triton X-100, respectively, and radioactivity counting of the target cells in the presence of effector cells was designated as Sample release. Percent specific cell lysis was calculated as follows: (Sample release - Spontaneous release) / (Total release - Spontaneous release) x100. Experiments were conducted in duplicate.

RESULTS

Construction of rDIs/HIV-1gagE expressing HIV-1 gag protein

rDIs/HIV-1gagE was constructed to contain same HIV-1 gag gene that present in rBCG/HIV-1gagE. HIV-1 gag gene was inserted plasmid pUC-vvp7.5 to be in-frame with the initiation codon of the p7.5 promoter. Lysates of CEF cells infected with rDIs/HIV-1gagE and rDIs/LacZ were electrophoresed and subjected to Western blot analysis. A protein band of approximately 55 kDa protein band was observed in lysate from rDIs/HIVgagE-infected CEF cells,

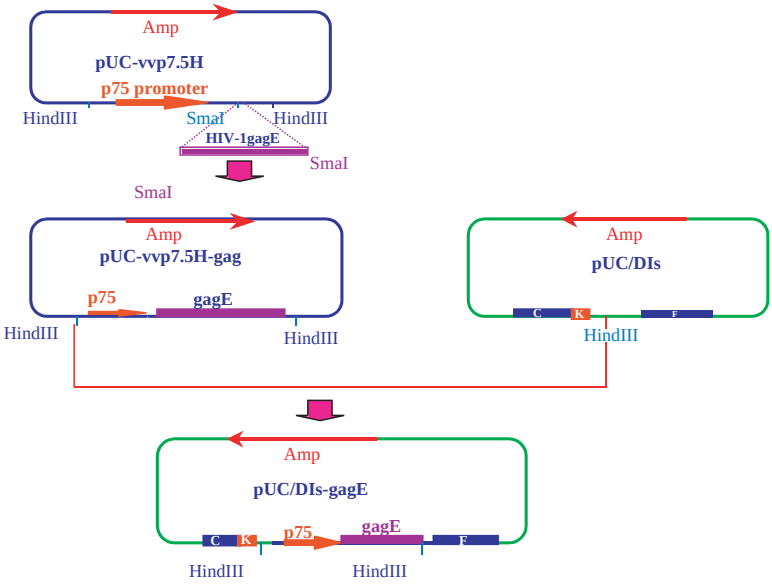

Fig 1–Scheme of construction of HIV-1 CRF01_AE gag DNA into plasmid having Vaccinia virus p7.5 promoter and insertion of 1.9 kbp of gene-deleted Vaccinia virus DIs fragment into pUC vector for transfection into Vaccinia virus to generate rDIs/HIV-1gagE.

whereas this band was absent in lysates from rDIs/LacZ-infected cells (Fig 2).

Immunization with rBCG/HIV-1gagE alone

To observe HIV-1 gag-specific CTL response resulting from immunization with rBCG/HIV-1gagE alone, mice were injected subcutaneously (s.c.) once with the immunogen. As shown in Fig 3, after one month following immunization by this route, specific cell lysis was induced using almost all peptides along the gag protein sequence (ten different gag peptide mixtures), with some peptide mixtures showing small induction but some high percentage of cell lysis. However, when a single dose of rBCG/HIV-1gagE was introduced into mice by an intradermal (i.d.) route, after the same duration of time lower percent cell lysis was observed: 6-12% versus 10-53% by s.c. route at 100:1 effector:target cell ratio. These numbers had already been adjusted to take into account specific cell lysis from control cells.

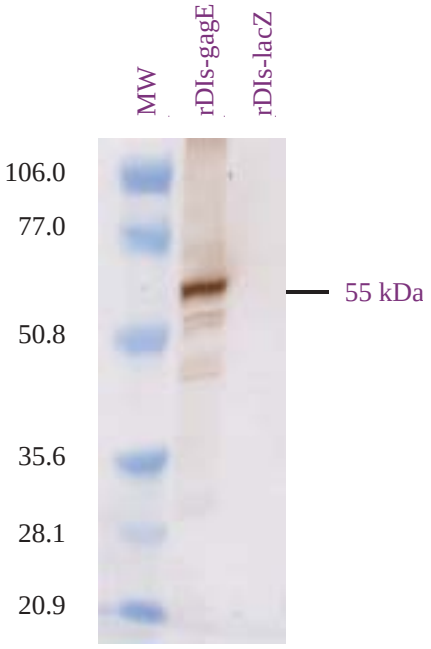

Fig 2–Western blot analysis of cell lysates obtained from rDIs/HIV-1gagE- and rDIs/LacZ-infected CEF cells. Gel was reacted with anti-HIV-1 p24 (amino acids 287-301) monoclonal antibody.

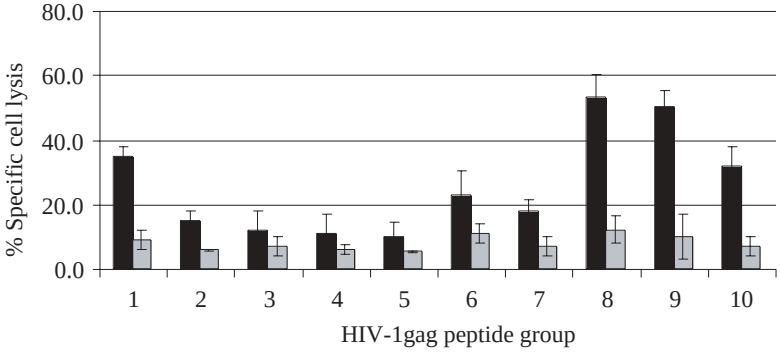

Fig 3–CTL response (percent specific lysis) after immunization of 10 mice once with rBCG/HIV-1gagE and and restimulation after one month with 10 different gag peptide groups. Dark bar, subcutaneous injection; shaded bar, intradermal injection. Error bars represent ± standard deviation.

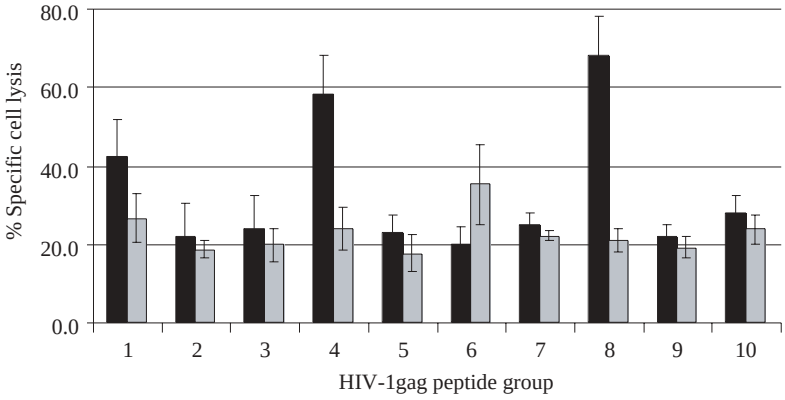

Fig 4–CTL response (percent specific lysis) after immunization of 12 mice with the prime-boost regimen of rBCG/HIV-1gagE and rDIs/HIV-1gagE and restimulation after 2 months against 10 different gag peptide groups. Dark bar, subcutaneous rBCG/HIV-1gagE and intravenous rDIs/HIV-1gagE; shaded bar, intradermal rBCG/HIV-1gagE and intradermal rDIs/HIV-1gagE. Error bars represent ± standard deviation.

Prime-boost immunization with rBCG/HIV-1gagE and rDIs/HIV-1gag

One month after immunization with rBCG/HIV-1gagE, mice were boosted with 10^6 pfu rDIs/HIV-1gagE through a number of different routes and sacrificed after one month. In mice primed through s.c. route and boosted by intravenous (i.v.) injection, CTL activities against all ten different gag peptide mixtures induced 20-68% cell lysis at 100:1 effector:target cell ratio (Fig 4). On the other hand, mice immunized by i.d. route and boosted by the same route, lower CTL activities were induced against almost all peptide mixtures (18-35% cell lysis), in particular against peptide mixtures 1, 4, and 8 (1.6-3.3 fold lower compared to s.c./i.v. route) (Fig 4).

Mice were also left for 6 months after priming with rBCG/HIV-1gagE s.c. and then boosted i.v., or for 6 month after priming i.d. and boosting by the same route. One month after boosting, CTL activities from both groups of mice were reduced (19-35% cell lysis for s.c./i.v. route and 13-26% cell lysis for i.d./i.d. route) (Fig 5).

Immunization with rDIs/HIV-1gagE alone

rDIs/HIV-1gagE was used as the only immunogen for both priming and boosting in order to investigate whether rDIs/HIV-1gagE by itself could induce CTL response. Recombinant virus was injected by i.v. route one month prior to boosting through the same route. One month after boosting, low CTL activities were observed (7-23% cell lysis) (Fig 6).

DISCUSSION

We had previously shown that BCG

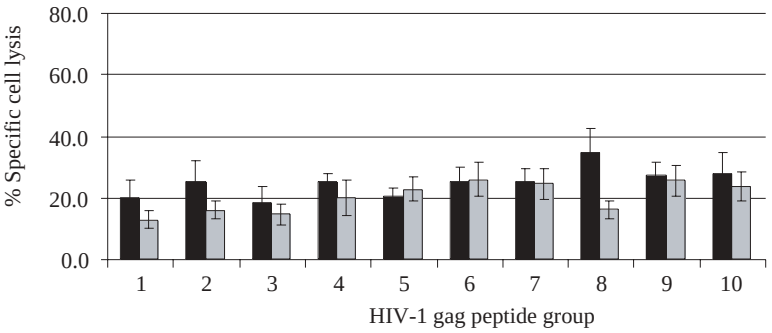

Fig 5–CTL response (percent specific lysis) after immunization of 10 mice with prime-boost regimen of rBCG/HIV-1gagE and rDIs/HIV-1gagE and restimulation after 7 months against 10 different gag peptide groups. Dark bar, subcutaneous rBCG/HIV-1gagE and intravenous rDIs/HIV-1gagE; shaded bar, intradermal rBCG/HIV-1gagE and intradermal rDIs/HIV-1gagE. Error bars represent ± standard deviation.

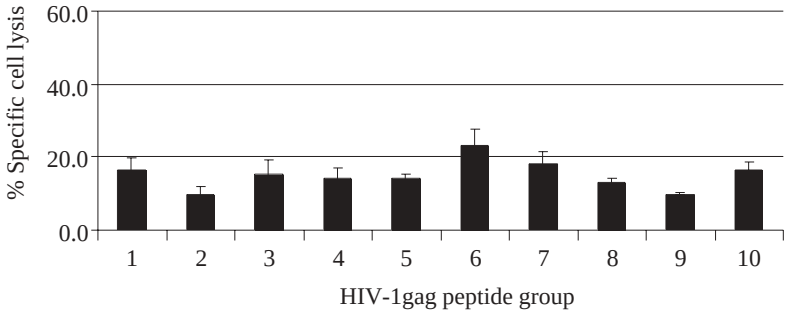

Fig 6–CTL response (percent specific lysis) after immunization of 12 mice with prime-boost regimen of intradermal rDIs/HIV-1gagE and restimulation after 2 months against 10 different gag peptide groups. Error bars represent ± standard deviation.

could be used as a live vector to introduce HIV-1 gag antigen to trigger immune induction into mice and CTL response in the form of antigen-specific cell lysis (Promkhatkaew *et al*, 2009). However, the durability of the immune response is still in doubt as it declined after a short period of time. Therefore, we used other immunization methods in order to overcome this problem and thereby leading to a more promising live HIV vaccine.

We postulated that immunity induced by a single vaccine alone might be insufficient to provide persistent protection against HIV-1 infection, so we evaluated a consecutive immunization strategy involving a prime-boost regimen comprising two live vectors, recombinant BCG, rBCG/HIV-1gagE (Promkhatkaew *et al*, 2009) and recombinant Vaccinia virus, rDIs/HIV-1gagE. When mice were primed with rBCG/HIV-1gagE and boosted with rDIs/HIV-1gagE, CTL responses were stronger than using the same immunogen for priming and boosting. Although after a single injection of rBCG/HIV-1gagE, CTL activity after 2 months was 3-fold lower than after 1 month (data not shown), results from prime-boost immunization showed that high activity remained during the same immunization duration. Immunity was mounted against almost all peptide pools representing antigenic epitopes along the gag protein sequence. Additionally, these prime-boost regimens could sustain the CTL activities over the period of 7 months of study, although some decline was observed. Since these were obtained 6 months following priming, it may be attributable to memory T-cells induced by the recombinant BCG that could persist until receiving another stimulation by recombinant Vaccinia virus to be-

come CTLs. Persistence of anti-gag memory T-cell response for 6 months elicited by a live bacterial vector based HIV-1 gag vaccine has been reported (Rayevskaya and Frankel, 2001).

When mice were primed and boosted with rDIs/HIV-1gagE alone, cell lysis activities were much lower than those obtained by a regimen employing rBCG/HIV-1gagE as the priming antigen. These may be due to the effect of anti-Vaccinia virus antibodies raised by the initial immunization, which might neutralize the subsequent virus exposure resulting in less virus immunogen left to induce CTL. Similar results have been reported indicating that repeated injections of recombinant Canarypox or Vaccinia virus might have led to anti-vector immune response and diminish the efficacy of repeated booster immunizations with the same vector (Kundig *et al,* 1993; Tartaglia *et al,* 1998). As the regimens in the present study were composed of two different live vectors, antibodies to the BCG component would have no adverse (neutralization) effect on the Vaccinia virus, and thus on the induction of HIV-specific effector or memory T-cells.

In order to induce effective immunity, the route of vaccination is also an important factor, particularly for the response in lymph nodes. Since skin contains more antigen-presenting cells, including Langerhans and dendritic cells, than muscle (Bos and Kapsenberg, 1993), and as BCG can infect macrophages, which constitute the major population of tissue-resident mononuclear phagocytes, we hypothesized HIV antigen presentation into two possibilities. One possibility is that macrophages may phagocytose BCG expressing an HIV antigen and process it into peptides for presentation with MHC class I molecules to T-lymphocytes. The other possibility is that BCG may be directly captured by dendritic cells and the protein is endocytosed and digested into peptides. Therefore, we were interested to compare the results of immunizing with recombinant BCG through the s.c. and i.d. routes. After a single injection, CTL response through i.d. route was very low compared to the s.c. route. It may be speculated that via an s.c. injection, BCG might encounter populations of cells that would be more appropriate for infection and replication than those found localizing in the epidermis through an i.d. injection. Moreover, by prime-boost immunization, s.c injection of rBCG/HIV-1gagE followed by rDIs/HIV-1gagE i.v. gave moderate to extremely higher CTL induction than those obtained i.d. for both immunogens. These results are consistent with other findings describing that Vaccinia virus delivered in mice via the i.v. route is more efficient in CD8+ CTL elevation than given intramuscularly (Hanke *et al,* 1998), intraperitonealy or by s.c. route (Gheradi *et al,* 2000).

However, after seven months the high CTL activity induced by both regimens declined to a level not different either by boosting with rDIs/HIV-1gagE i.v. or i.d. These results may imply that rBCG/HIV-1gagE given by the s.c. route might propagate rapidly into surrounding cells at the injection site and present the antigen efficiently to lymph nodes within the first 1-2 months. After that the effector CTL might be decomposed but memory T-cells either elicited by s.c. or i.d. route might still exist and expand to respond to subsequent antigen boosting via i.v. or i.d. route.

From the present study, combining the two immunogens into one immunization regimen resulted in high and more sustainable T-cell response than that induced by either immunogen alone. It also suggests that two different live vectors, such as BCG to deliver a priming antigen and Vaccinia virus for a boosting antigen via the i.d. route,

is appropriate to be used for prime-boost HIV vaccine immunization in mice. Since i.v. immunization is not practical in humans, the i.d. route for both recombinant BCG and Vaccinia virus is the method of choice for HIV vaccination.

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