Enhanced Immune Responses against HIV-1 with Adenovector (Gag and Tat) Prime/Protein Boost Regimen and GM-CSF Injection

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ABSTRACT

Vaccines against the HIV-1 virus offer the best hope for eliminating HIV-associated mortality. Recombinant adenovector type 5 (rAd5) vaccine is a potential candidate for preventive vaccine strategies.

In this study, we evaluated the rAd5 prime/protein boost strategy in a murine model. We used rAd5 harboring single HIV-1 genes. These genes, including gag (p24) and exon1 of tat, were amplified from HIV-1 (clade A) RNA using nested PCR. Recombinant vectors were constructed, purified and then injected at 10^12 viral particles into four groups, each comprising five mice. The groups were each assigned to receive one of rAd5 prime/protein boost Gag, Tat with and without recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF), and rAd5 with and without genes. The humoral responses were evaluated using ELISA and cellular immune responses checked by cell proliferation and ELISpot assays (IL-2, IL-4 and IFN-γ).

It was shown that compared with the rAd5 injection alone, the rAd5 prime/protein boost plan increased cellular immunity (p = 0.009) as well as humoral immunity (p = 0.009). Moreover, rGM-CSF as an adjuvant enhanced cell-mediated immunity and increased IL-4 expression (p = 0.032).

The results revealed that the simultaneous use of multiple antigens and heterologous prime/boost strategy can enhance both humoral and cellular immune systems. Moreover, subcutaneous injection of rGM-CSF increases IL-4 production and shifts the immune pattern to Th2. These strategies can potentially be used to develop an efficient HIV-1 vaccine.

Keywords: Adenovirus; HIV vaccine; Multiple antigen; Prime-boost; rGM-CSF

INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) infection has reached epidemic proportions in many countries in the
world. According to the most recent data by the Joint United Nations Programme on HIV/AIDS (UNAIDS), more than 35 million people are currently infected with HIV-1 and in 2012 over 1.6 million died due to AIDS-related diseases. According to that report, developing an HIV-1 preventive vaccine is a world health priority. Gene therapy as prevention method of AIDS was introduced in 1996, by reinforcing humoral and cellular immune system. In this way, first and the second generation of vaccines had been investigated, respectively, including live attenuated vaccine, killed viruses and subunit vaccines. Some studies clearly showed that a potent HIV-1 vaccine could elicit both cellular and humoral immune responses. It is indicated that DNA vaccine expressing the HIV-1 constructs, the third generation of vaccines, elicits immune responses. A popular viral vector in vaccine production is based on Adenovector (Ad). Adenoviral vectors are a promising tool for vaccine development because of the ability to induce both proliferative and non-proliferative cells, high expression of inserted genes, good reproducibility in vitro, lack of entrance to the host genome, and their genetic and physical stability. High expression of co-stimulating molecules on the adeno-virus-infected dendritic cell surfaces lead to better antigen presentation followed by a more potent immune response. E1 and E3 regions are removed in Ad in vaccine production, which allows more regions to insert foreign genes into virus genome, thus preventing virus proliferation. Despite all these characteristics, it has been shown that only 60–90% of people have acquired immunity against adenovirus. Neutralizing reactions are common with this virus and booster doses had not provided a good result when the vector alone was injected. Several heterologous prime/boost regimens have been developed in order to overcome this problem and to induce a good response against the virus. Ad prime/protein boost is one of these strategies. The effect of heterologous prime/boost regimens on inducing a variety of antigen-processing cells as native antigen, leads to the reinforcement of antigen-specific immunity against different pathogens. One of the most important challenges in AIDS vaccine development is non-simultaneous stimulation of cellular and humoral immune responses, which may be solved by heterologous prime/boost regimens. Moreover, the immune response by this strategy is more stable. The cellular pattern of the immune system is a key point in vaccine development.

In this article, the multiple antigen strategy and recombinant adenovector type 5 (rAd5) prime/protein boost regimen with or without recombinant granulocyte-macrophage colony stimulating factor (rGM-CSF) were used simultaneously in order to enhance the immune response against HIV-1. This project was approved and supported financially by Tarbiat Modares University.

MATERIALS AND METHODS

Mice

Female BALB/c mice (6 weeks old) were purchased from the Pasteur Institute of Iran and kept on a 12 hour light/dark cycle with controlled humidity (60–65%) and temperature (22° ± 2°C) under specific pathogen-free conditions in Tarbiat Modares University (Tehran, Iran) animal house. Food and water were supplied ad libitum. All of the animal experiments were performed according to the guidelines of the Institute for Laboratory Animal Research. Mice were kept in the animal house to adapt for a week and then were divided into four groups.

Adenovector

Two replication–incompetent Ad5-HIV recombinants were used in the present study. For HIV-1 genes, 200 μl of plasma from an HIV-1 infected drug user was extracted using AccuPrep viral RNA extraction kit (BIONEER, Korea). The HIV-1 genes, a sequence of the gag P24 and Exon 1 of the tat gene, were amplified from the HIV-1 RNA sample by nested reverse transcriptase PCR. The nucleotide sequence of tat and gag were verified by commercial sequencing as being subtype A (BIONEER, Korea). Recombinant adenovector type 5 (rAd5) ∆E1/E3-tat, rAd5 ∆E1/E3-gag were constructed, produced and purified according to Adenovator manual (Qbiogene, USA) and the expression of each HIV-1 protein in 293 cell line infected with rAd5-HIV-1 genes was evaluated by western blot. All procedures for adenovector HIV-1 construction have been described previously.

Immunization Strategy

BALB/c mice were divided into 4 groups (five in each group) and immunized by intramuscular (femoral muscle) injection of rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag (1:1) at day 0. Injected construct volume was 10^12 viral particles in 150 μl buffer. First group only
received gag and tat gene inserted rAd5. In the second group, 14 days after rAd5 injection, Gag and Tat recombinant HIV-1 NM proteins (Trinity Biotech Bray, Ireland) (100 μg/mouse) were injected subcutaneously (SC). Protein purity was more than 99%. In the third group, rGM-CSF (1 μg/mouse, R&D Systems Inc. Minneapolis, MN 55413 USA) as an adjuvant was SC injected into mice 30 minutes before protein injection on the 14th day. The fourth group was the control group which received rAd5 without the gene. The employed vector components are shown in Table 1.

Table 1. Mouse immunization schedule of Adenovector Prime/Protein Boost (with/without GM-CSF) vaccination strategies for HIV-1 virus.

<table>
<thead>
<tr>
<th>Group Name</th>
<th>Day 0 (rAd5 Prime)</th>
<th>Day 14 (Protein Boost)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rAd5 ΔE1/Eγ-tat, rAd5 ΔE1/Eγ-gag</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>rAd5 ΔE1/Eγ-tat, rAd5 ΔE1/Eγ-gag</td>
<td>Gag and Tat Protein</td>
</tr>
<tr>
<td>3</td>
<td>rAd5 ΔE1/Eγ-tat, rAd5 ΔE1/Eγ-gag</td>
<td>Gag and Tat protein+ GM-CSF</td>
</tr>
<tr>
<td>4</td>
<td>rAd5 ΔE1/E3 vector without gene(Control)</td>
<td>--</td>
</tr>
</tbody>
</table>

Mice were sacrificed by intraperitoneal (IP) injection of ketamine/xylazine (Sigma-Aldrich, St. Louis, MO, USA) on day 14 (control group and group 1) and on day 24 (groups 2 and 3) then spleens were aseptically removed. Spleen mononuclear cells were separated and transferred to R10 media [Roswell Park Memorial Institute(RPMI) cell culture media containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/ml penicillin and 100 μg/ml streptomycin]. Live cells were counted in the cell suspension using Trypan blue dyes (Sigma -Aldrich, St. Louis, MO, USA).

**Enzyme-linked Immunosorbet Assay (ELISA)**

Sera from immunized and control mice were evaluated by ELISA for binding IgG2a and IgG1 antibody isotypes to HIV-1 Gag (P24) and HIV-1 Tat proteins. Mouse serum at 1:10 dilution was prepared. Optical Density (OD) values are compared in three groups but in our study, the type and quality, but not the quantity, of antibody is considered. For the better representation, the mean OD in each group was multiplied by one thousand. The IgG2a/IgG1 ratio was calculated for each group. The recombinant HIV-1 Gag, Tat proteins, and antibodies (the monoclonal antibody anti-IgG1 and anti-IgG2a) for ELISA were purchased from Abcam (France) or Trinity Biotech (Bray, Ireland) and used under the reaction conditions recommended by the vendors as described in a previous study.

**Cell Proliferation Assay**

For cell proliferative responses, we used the colorimetric cell viability assay (MTT) for the non-radioactive quantification of cell proliferation (Roche Diagnostic GmbH, Roche applied Science, Germany). Results of duplicate determinations were expressed as the stimulation index (SI), defined as OD in the presence of test specific antigen relative to OD in the presence of cells alone.

**ELISpot Assay**

ELISpot assay was used to detect HIV-1 proteins Tat and Gag-specific IFN-γ, IL-2 and IL-4 producing T cells in mice spleen mononuclear cells in immunized and control groups. The murine ELISpot assay was run according to the manufacturer’s protocols (Minneapolis, USA). In brief, mouse fresh spleen mononuclear cells were used at a concentration of 5x10^5 cells per 100µl of the R-10 medium, which were transferred to 96-well plates coated with anti-IFN-γ, anti-IL-2 or anti-IL-4. Cells were stimulated with concanavalin A at a concentration of 0.5 µg/well serving as a positive control while R-10 medium alone was used as a negative control. 5 µg of the specific antigens, i.e. recombinant tat, or gag (p24) proteins were used as immunogens. The concentration and the response time of recombinant proteins had been titrated before. Subsequently, the plates were air dried and spots were counted visually using an inverted microscope with high magnification. Spots were fully countable and did not overlap.

**Statistical Analysis**

Data obtained in this research was analyzed using SPSS statistical software version 11.5 (SPSS Inc, Chicago Illinois, USA). Descriptive data were calculated.
as the mean, median, and standard deviation (SD) in different groups. In order to perform analytical and descriptive analysis in the groups under research, the non-parametric Mann-Whitney U test was utilized, a $p$-value less than 0.05 was considered as being significant.

**RESULTS**

**Antigen Specific Humoral Immune Response**

Antibody titers against gag and tat were measured in all groups. The antibody titer in rAd5 prime/protein boost receiving mice were significantly higher compared with rAd5-only injected mice ($p=0.009$). Moreover, gag and tat antibody titers in the mice receiving cytokines were significantly higher than the rAd5 prime/protein boost receiving group ($p=0.047$) and the rAd5 group ($p=0.009$). Details of the results are depicted in Figure 1. The antibody types were also analyzed and the IgG2a/IgG1 ratio was calculated for each group (Figure 2).

![Figure 1](image1.png)

Figure 1. Specific antibody response assay against HIV-1 vaccine of Adenovector Prime/Protein Boost (with/without GM-CSF) in immunized mice; ELISA test for control group (rAd5 ∆E1/E3 Vector without gene) and group 1 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag) was done 14 days after treatment; however, specific antibody response for group2 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag + Gag and Tat Protein) and group3 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag + Gag and Tat Protein + GM-CSF) was assayed in the 24th day of experiment. Mean of OD Value × 1000 of Ad only, Prime/Boost, Prime/Boost+GM-CSF groups were 834±48.76, 2206.6±258.57, 2570.4±169.14, respectively and the $p$-value of all groups was significant compared to control group ($p<0.05$). Significant differences are indicated by * $p<0.05$. The error bars depict the standard error of the mean.

![Figure 2](image2.png)

Figure 2. Th1/Th2 shift in Immune response against HIV-1 vaccine of Adenovector Prime/Protein Boost (with/without GM-CSF) in immunized mice; IgG2a and IgG1 immunoglobulin isotypes were considered as markers for Th1 and Th2 immune responses respectively. Two separate ELISA tests were done for IgG1 and IgG2a antibody titer. The ratio of IgG2a versus IgG1 was calculated for each mouse. Mean of IgG2a/IgG1 ratio of Ad only, Prime/Boost, Prime/Boost+GM-CSF groups were 11.46±1.71, 12.14±2.11, 10.34±3.27 respectively, and the $p$ value of all groups was significant compared to control group ($p<0.05$). Significant differences are indicated by * $p<0.05$. The error bars depict the standard error of the mean.
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Figure 3. Level of Spleen mononuclear cells proliferation against HIV-1 vaccine of Adenovector Prime/Protein Boost (with/without GM-CSF) in immunized mice; Spleen mononuclear cells proliferation was done based on manufacturer’s protocol (Roche Diagnostic GmbH, Roche applied Science, Germany) and then SI was calculated. Mean of SI of Ad only, Prime/Boost, Prime/Boost+GM-CSF groups was $6.73\pm1.02$, $10.12\pm1.33$, $22.90\pm4.92$ respectively, and $p$-value of all groups was significant compared to control group ($p<0.05$). Significant differences are indicated by $^*p<0.05$. The error bars depict the standard error of the mean.

**Antigen Specific Cellular Immune Response**

**Cell Proliferation Assay**

Spleen mononuclear cells in each well were mixed with $5\ \mu g$ recombinant HIV-1 MN (Gag and Tat) protein, after which the SI were calculated for each group. $^25$ SI in the rAd5 prime/protein boost-treated group was significantly higher compared with the rAd5 receiving group ($p=0.009$). On the other hand, cells from the rAd5 prime/protein boost + GM-CSF treated group showed the highest SI compared with both the rAd5 prime/protein boost group ($p=0.047$) and rAd5 treated group ($p=0.009$). Details of the results are depicted in Figure 3.

Figure 4. Results of IFN-γ ELISpot assay against HIV-1 vaccine of Adenovector Prime/Protein Boost (with/without GM-CSF) in immunized mice; IFN-γ production after heterologous prime/boost strategy, was assayed in 14 and 24 day-intervals for group 1 (gag and tat rAd5) vs. control group (∆E1/E3 vector without gene) and group 2 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag + Gag and Tat protein) vs. group 3 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag + Gag and Tat protein + GM-CSF), respectively. Tests were performed according to the manufacturer’s instruction (R&D Systems Inc. Minneapolis, USA). Mean of IFN-γ spots/millions spleen cells of Ad only, Prime/Boost, Prime/Boost+GM-CSF groups was $641\pm53.27$, $1418.2\pm95.98$, $1510.6\pm123.16$ respectively and the $p$-value of all groups was significant compared with control group ($p<0.05$). Mean of IFN-γ/millions spleen cells of group3 compared to group2 was not significant. Significant differences are indicated by $^*p<0.05$. The error bars depict the standard error of the mean.
Figure 5. Results of IL-2 ELISpot assay against HIV-1 vaccine of Adeno vector Prime/Protein Boost (with/without GM-CSF) in immunized mice; IL-2 production after heterologous prime/boost strategy, was assayed at 14 and 24 day-intervals for group 1 (gag and tat rAd5) vs. control group (∆E1/E3 Vector without gene) and group 2 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag + Gag and Tat protein) vs. group 3 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag + Gag and Tat protein + GM-CSF) respectively. Tests were performed according to the manufacturer’s instruction (R&D Systems Inc. Minneapolis, USA). Mean of IL-2/millions spleen cells of Ad only, Prime/Boost, Prime/Boost+GM-CSF groups was 72.40±3.97, 177.80±18.64, 185.20±12.21 respectively and the p-value of all groups was significant compared with control group (p<0.05). Mean of IL-2/millions spleen cells of group3 compared to group2 was not significant. Significant differences are indicated by *p< 0.05. The error bars depict the standard error of the mean.

ELISpot Assay

Our data showed that following both the protein injection and rAd5 inoculation, IFN-γ (p=0.009), IL-4 (p=0.008) and IL-2 (p=0.009) secretion increased. Moreover, GM-CSF co-injection with protein after rAd5 inoculation increased IFN-γ (p=0.009), IL-4 (p=0.008) and IL-2 (p=0.009) secretion more than rAd5 inoculation alone. However, compared to rAd5 alone, rAd5 prime/protein boost + GM-CSF regimen caused a significant increase only in IL-4 secretion (p=0.032). Results are shown in more detail in Figures 4, 5 and 6.

Figure 6. Results of IL-4 ELISpot assay against HIV-1 vaccine of Adeno vector Prime/Protein Boost (with/without GM-CSF) in immunized mice; IL-4 production after heterologous prime/boost strategy, was assayed at 14 and 24 day-intervals for group 1 (gag and tat rAd5) vs. control group (∆E1/E3 Vector without gene) and group 2 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag + Gag and Tat protein) vs. group 3 (rAd5 ∆E1/E3-tat, rAd5 ∆E1/E3-gag + Gag and Tat protein + GM-CSF) respectively. Tests were performed according to the manufacturer’s instruction (R&D Systems Inc. Minneapolis, USA). Mean of IL-4/millions spleen cells of Ad only, Prime/Boost, Prime/Boost+GM-CSF groups was 0.6±0.89, 51.40±15.72, 74.20±6.05 respectively and the p-value of all groups was significant compared with control group (p<0.05). Mean of IL-4/millions spleen cells of group2 and group3 compared with group 1 was significant. Moreover, group3 compared to group2 was significant. Significant differences are indicated by *p< 0.05. The error bars depict the standard error of the mean.
**DISCUSSION**

In the present study, the multiple antigen strategy and rAd5 prime/protein boost regimen were used simultaneously in order to enhance the immune response against HIV-1. The rAd5 by itself can stimulate cell mediated immunity very well, but this effect is not significant for humoral immunity. Previous reports have shown a high level of gag-specific cytotoxic T lymphocyte (CTL) activity in the peripheral blood mononuclear cell (PBMC) if HIV-1 infected disease, which suggests the presence of antigenic epitopes that is identified by CTL and enhanced cellular immune system. The HIV-1 Gag is one of the most HIV-1 conserved proteins. The greatest CTL response is against the P24 antigen in the Gag protein. The HIV-1 Tat is another important protein, which acts like hydrophobic antigens and is presented to CD8 T cells through MHC-1. Experimental data showed that when the tat is taken up by dendritic cells, over-expression of MHC and co-stimulating molecules will occur, in turn leading to a higher production of Th1 cytokines and β-chemokine. Antigen presentation will increase and a more potent immune response will be induced. Therefore, the tat gene can be used as an effective adjuvant for T-cell induction.

It has been demonstrated that the tat protein alone cannot be used as an HIV-1 vaccine, and it should be used in conjunction with other effective components. In this study, multiple antigen strategies were used including both Tat and Gag proteins. It is well documented that simultaneous injection of gag and tat harboring vectors can enhance both humoral and cellular immune responses more effectively compared with other proteins. By using gag and tat genes along with Gag and Tat proteins, we can investigate the rAd5 prime/protein boost strategy. The cytokine assay section of this study was designed to measure antigen-specific IFN-γ (the best single marker for a Th1 phenotype) and IL-4 (the best single marker for a Th2 phenotype) responses after HIV immunization to better characterize Th1/Th2 responses induced by Prime/boost (rAd5 ΔE1/E3-tat, rAd5 ΔE1/E3-gag + protein gag and tat) strategy along with SC injection of GM-CSF. Our results showed that heterologous prime/boost strategy can significantly increase IL-4, IL-2 and IFN-γ production as well as cell proliferation. By using HIV recombinant peptide and different vaccination formula, the antigen-specific IFN-γ response appeared to occur early and subsequently followed by IL-4 responses to the antigens. This timing of the Th1/Th2 response was concomitant with the overall antibody responses. Antibody production is also increased by this method. As is shown in our results, this method significantly modified the IgG2a/IgG1 ratio and shifted the cellular pattern to Th1 which confirms the efficacy of rAd5 prime/protein boost strategy.

Amara et al. showed that heterologous prime/boost strategy in rhesus macaque controlled the viremia of SIV. It has also been shown that heterologous prime/boost acts as an amplifier for both humoral and cellular immune systems. Heterologous prime/boost strategy has also been used for other pathogens. Research results showed that a DNA prime/Ad boost strategy can induce a long-lasting immunity against anthrax. Using this strategy, specific antibody production, cellular immunity and IFN-γ level have increased. It has been shown that higher antibody production occurred when the protein was injected into Ad-receiving mice compared with gene inoculation. In the current study, it was also demonstrated that Ad prime/protein boost strategy, like other types of heterologous prime/boost strategies, elicited an immune response effectively. The amount of immune stimulation was dependent on antigen type and host species. Cytokines, when used as an adjuvant, lead to an increase in antigen immunogenicity and are also considered as an effective factor in immune system shift. Some cytokines such as GM-CSF have been shown to amplify the immunogenicity induced by heterologous prime/boost strategy. An effective HIV-1 vaccine strongly requires the stimulation of all immune system components especially, Th1 response. GM-CSF injections given in this study increased IL-4 levels but did not significantly affect is not significant for humoral immunity.
increase IL-2 or IFN-γ or shift the cellular pattern to Th1. However, in another study, we observed that IL-4 production significantly increased in the presence of GM-CSF (data is not published).

GM-CSF can stimulate a general broad spectrum of immune responses including Th1, Th2 and CTL. The strategy of injection (route, time, nature of antigen) impacts on immune system patterns, the intensity, and direction of the immune response. On the other hand, administration routes affect the quality and quantity of immune responses. SC injection of rGM-CSF leads to the attraction and activation of many dendritic cells, such as skin Langerhans cells, which in turn increases the number of resident processing cells and higher antigen processing. Previous reports indicated that rGM-CSF protein injection shows better results than plasmid-encoding GM-CSF inoculation. In addition to induction of proliferation, differentiation, and migration of dendritic cells, GM-CSF can shift cytokine response patterns to both Th1 and Th2. Moreover, GM-CSF is also known as a chemo-attractant for antigen-presenting cells (APCs) SC injection of GM-CSF induces a depot effect and gradual release of cytokines.

It is documented that an intramuscular (IM) injection injection of bicistronic HIV-1 DNA vaccine, expressing glycoprotein 120 (gp120) and GM-CSF along with one promoter, can enhance cellular immune systems, especially CD4 cells, more effectively than a separate induction of this construct. However, Simmons et al. showed that SC injection of GM-CSF along with melanoma-associated peptide vaccination did not enhance the human immune system significantly. They also showed that dose and injection duration of GM-CSF plays a critical role in immune response induction.

In another study, Kusakabe et al. injected GM-CSF containing plasmid for three times: once before, once after and once simultaneously with the vaccine injection. Their results showed that injection of GM-CSF before vaccine injection shifts the cellular pattern to Th2 and increased IL-4 production. Simultaneous injection increased Th1 and Th2 responses as well as IgG, IgG2a and IgG1 production. GM-CSF injection after vaccination shifted the cellular pattern to Th1 and increased CTL, DTH and IFN-γ production.

In conclusion, the simultaneous use of multiple antigens and heterologous prime/boost strategy can enhance both humoral and cellular immune systems effectively. These strategies can be used to develop an efficient HIV-1 vaccine. Moreover, we showed that SC injection of rGM-CSF increases IL-4 production and shifts the immune pattern to Th2. Other strategies for administration of GM-CSF must be studied. Since the immunological pattern and induction of humoral immunity are very important in vaccine design, we recommend that the multi-antigen prime-boost strategies along with different types of vaccines and different routes of vaccine administration should be considered in future studies.

REFERENCES