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Immune Responses against a New HIV-1 p24-gp41/pCAGGS-IL-12 DNA Vaccine in Balb/c Mice

Fatemeh Roodbari[1,2], Farzaneh Sabahi[1], Mohamad Nabi Sarbolouki[3], Farzaneh Barkhordari[4], Ahmad Adeli[4], Amel Jamedar[1,5], Fereidoun Mahboudi[4*]

[1]Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, [2]Department of Cellular and Molecular Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, [3]Institute of Biochemistry and Biophysics, Tehran University of Basic Sciences, [4]Biotechnology Research Center, Pasteur Institute of Iran, Tehran, [5]Department of Microbiology and Virology, Faculty of Medicine, Mashad University of Medical Sciences, Mashad, Iran

ABSTRACT

Background: Development of an effective vaccine is highly needed in order to restrict the AIDS pandemic. DNA vaccines initiate both arms of immunity without the potential of causing disease. HIV-1 p24 and gp41 (gag and env) proteins play important roles in viral pathogenesis and are effective candidates for immune induction and vaccine design. **Objective:** In this study, new DNA vaccine candidates constructed from HIV-1 fused p24-gp41 or gp41 alone were evaluated in Balb/c mice for induction of cellular and humoral immune responses. **Methods:** Recombinant plasmids, pcDNA3.1/Hygro expression vector containing immunogenic sequences of fused p24-gp41 or gp41alone were produced. Dendrosome used as a system for carrying vectors in laboratory animals, and an IL-12 containing vector (pCAGGS-IL-12) was co-immunized with the p24-gp41 vector as a genetic adjuvant. Induction of effective immune responses against the designed vectors as DNA vaccine candidates in Balb/c mice was evaluated. Levels of total antibodies, IgG isotypes (IgG2a and IgG1); IFN-γ and IL-4 were measured by ELISA. MTT assay was used to evaluate lymphoproliferation. **Results:** The results confirmed that the immunogenic epitopes of both p24 and gp41 genes are highly effective inducers of immune responses, and administration of fused p24-gp41 alone or along with IL-12 resulted in further enhancement of immune responses. Group 4 that received fused fragments (p24-gp41) along with an IL-12 expressing vector demonstrated a significantly higher Stimulation Index (SI) and IFN-γ production ($p<0.0001$) with a significant increase in IgG2a/IgG1 ratio, indicating the stimulation of CMI towards Th1. Although gp41 containing vector (group 6) also showed significant increases in both proliferation and IFN-γ production, the responses were persistently lower than that of p24-gp41 containing vectors. Total antibody

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***Corresponding author:** Dr. Fereidoun Mahboudi, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran, Tel/Fax: (+) 98 2166480780, e-mail: mahboudi@pasteur.ac.ir

production was highest in group 6 as expected. **Conclusion:** Dendrosome proved to be an efficient carrier of recombinant plasmids constructed in this study. Further studies are necessary to evaluate these constructs as HIV vaccine candidates.

Keywords*:* Dendrosome, DNA Vaccine, gp41, HIV-1, IL-12, p24

INTRODUCTION

More than 30 million people around the world have died of AIDS-related diseases so far. In 2010, 2.7 million people were newly infected with HIV, and 1.8 million men, women and children died of AIDS-related causes. Thirty four million people around the world are now living with HIV (1). Approximately, 16,000 new infections occur daily of whom 95% are in developing countries. The first report of HIV infection in Iran was in 1987 (2,3). Based on the last report of World Health organization (WHO) in 2009, the estimated number of individuals infected with HIV is over 92000 (74000-120000) in Iran and more than 6400 (5200-8000) deaths were reported (4-6).

Antiviral drugs for HIV cannot completely clear the virus; the potential of development of drug resistance is high and many persons cannot use anti-HIV drugs based on approved guidelines due to their high cost. Control of HIV infection and AIDS will be one of the major problems in developing countries in the near future (7,8). Therefore, an effective vaccine seems to be the best way to rescue populations at risk and to control HIV epidemics (8-10).

The use of an attenuated vaccine is not safe for the development of HIV vaccines, due to high mutation rate and tendency of recombination in HIV genome. The most common approach to make HIV vaccines is the use of viral subunits as immunogens or complete genome sequences capable of encoding a whole protein (11). The use of DNA vaccines is an important technology for vaccination and is commonly used in various studies to control incurable infectious diseases (12,13).

DNA vaccines can significantly induce cellular immune responses because the antigens are produced inside the cells, therefore they are processed and presented through MHC-I pathway. The secreted proteins can also be presented to antigen presenting cells (APCs) and the MHC-II pathway is activated (8).

Production of a high level of neutralizing antibodies as well as induction of cytolytic T lymphocytes (CTLs) have been demonstrated as valuable immunologic tools in controlling and/or preventing of HIV infection or declining the rate of progression to AIDS (13-16). An accurately designed DNA vaccine can achieve all of these goals.

Dendrosomes are a new family of circular particles that are non-toxic, natural, covalent or self-assembled, and their synthesis is cost worthy and simple. In addition, their use is easier in comparison to some of the other transporters of synthetic DNA such as cationic lipids and liposomes. Many experiments have shown their efficacy in presenting genes to target cells (14,17).

p24 (core antigen encoded by gag region) and gp41 (envelope protein encoded by env region) of HIV-1 play important roles in host-virus interaction and viral pathogenesis. Studies have shown that neutralizing antibodies that are specific for a wide range of viral isolates can identify epitopes within gp41 protein (18,19). On the other hand, products of gag region are important targets for CTL induction in HIV infected patients, and T helper and CTL-specific anti-gag responses are associated with lower viral load. Therefore, the use of immunogenic peptides derived from p24 and gp41 would be an invaluable combination as an HIV vaccine candidate (20-22).
A number of research groups have shown the potential of combined immunization of viral DNA vaccines with plasmids encoding cytokines or co-stimulatory molecules (23). Among various factors, IL-12 is shown to be a critical cytokine; promoting Th1-biased cellular immune responses (23,24).

MATERIALS AND METHODS

Plasmids. A construct containing immunogenic fragments of gp41 (nucleotides 202-309) and p24 (nucleotides 1-693) of HIV-1 was prepared and cloned in pcDNA3.1Hygro. The resulting construct was named pcDNA3.1/hygro (p24-gp41).Each of the considered fragments were first multiplied separately by PCR a method. The multiplied units were then fused by SOE technique (25,26).
Another construct was also prepared by subcloning one of the fused proteins (gp41) in pcDNA3.1Hygro and used as a control in this study. Both pcDNA3.1/hygro (p24-gp41) and pcDNA3.1Hygro (gp41) were confirmed by restriction enzyme analysis and sequencing. pcDNA3.1Hygro, pcDNA3.1Hygro (p24-gp41), pcDNA3.1Hygro(gp41), pCAGGS and pCAGGS-IL-12 (the last two plasmids were kind gifts of professor Masanori Matsui, PhD from Department of Microbiology, Saitama Medical School, Japan) (27), were all transformed independently into *E. coli* TOP10 F'. Large scale purification of the vectors was performed using Endo Free Plasmid Mega kit (Qiagen, Germany) based on the manufacturer's recommendations. In order to assess the quality and the quantity of the resulted DNA, the products were run on agarose gel and evaluated by spectrophotometery. Purified plasmids were dissolved in water and stored at -20°C until use.
Dendrosome 123 (Den 123). Dendrosome 123 was synthesized under sterile condition by professor Sarbolouki at the Institute of Biophysics and Biochemistry of Tehran University as published before (14,28).
It was mixed in PBS and incubated at room temperature for 15 minutes. The suspension was then filtered through a 0.2 μm filter and kept at 4°C (17).
Experimental Animals. Female Balb/c mice of 6-8 weeks of age were used (Pasteur Institute of Iran, Karaj, Iran) and taken care of based on animal care guidelines. Blood was collected retro-orbitally from each mouse and incubated at 37°C for 90 minutes. Sera were separated by centrifugation at 8,000 rpm for 1 minute and stored at -20°C. Seventy mice were divided into 7 groups of 10 as follows:

Table 1. Various groups of animals and the component they received in this study.

Groups	Components
1	Pc DNA3.1/Hygro & Den123
2	Pc DNA3.1/Hygro (p24-gp41) & Den123
3	PBS
4	Pc DNA3.1/Hygro (p24-gp41) & pCAGGS-IL 12 & Den 123
5	pCAGGS & Den 123
6	Pc DNA3.1/Hygro (gp41) & Den123
7	Den 123

Four groups served as controls and received pcDNA3.1Hygro, PBS, pCAGGS or sterile Den123 (groups 1, 3, 5, and 7). The other 3 groups received pcDNA3.1Hygro P24-gp41 (group 2), pcDNA3.1Hygro P24-gp41 along with the pCAGGS-IL-12 (group 4) or pcDNA3.1Hygro gp41 alone (groups 6).

Injection Protocol. The first injection was done at time zero followed by the next injection at week 4 (booster dose). The animals were sacrificed after 4 weeks of the second injection. In each injection, 100 µg of purified plasmid along with Dendrosome (5mg/ml) with a final volume of 100 µl was used. Injection included of 50 µl into two muscles of the four corners of the legs for each mouse.

Measurement of Lymphocyte Proliferation. Using MTT method, spleen cells were evaluated for their proliferation capacity towards the specific antigen. Spleens were taken out by an aseptic technique, separately homogenized and after removal of red blood cells with a lysis buffer, washed and kept in a special medium containing RPMI-1640 (Gibco, Germany), 10% inactivated FCS, 1% L-glutamine, 1% Hepes, 0.1% 2ME, and penicillin-streptomycin (all from Gibco, Germany). Cells with a concentration of $3\text{-}5\times10^5$ were added to wells of 96-well microtiter plates (Grainer, Germany). Five µg/ml of purified p24-gp41 protein (Pasteur Institute of Iran, Karaj, Iran) (29) was added to positive control wells while negative control wells contained no protein. PHA (Gibco, USA) was added to cells as a positive control. The plates were incubated for 3 days at 37°C in a 5% CO2 humidified incubator. After 3 days, 25 µl of a filtered solution of 3-(4,5-dimethyl tiazol-2)-2,5 Dimethyl Tetrazolium Bromide (MTT, Sigma) at 5mg/ml concentration was added to each well and the microplates were incubated at 37°C in 5% CO2 and a humid environment for 4 hrs. The supernatant was carefully removed and the purple precipitate (formazan) was dissolved in 100 µl of dimethylsulfoxide (DMSO). The absorbances were read at 570 nm.

Evaluation of Cytokine Production in Vitro. This test was performed as described for MTT with the difference that the experiment was carried out in 24 well plates containing 1-3×10^6 cells and the final volume in each well was 1000 µl. The amount of purified antigen was also adjusted based on the higher volumes in 24 well plates. The supernatants were removed after 3 days and kept at -70°C until use. The level of IFN-γ and IL-4 were measured using a commercial sandwich ELISA kit (R&D, USA), based on manufacturer's recommendations.

Total Antibody Measurement by ELISA. ELISA was used to measure p24-gp41 specific antibody in the animal sera. An indirect ELISA was set up after checker board titration. In summary, 100 µl volumes of 10 µg/ml purified P24-gp41 in carbonate-bicarbonate buffer, was added to 96 microtiter plates (High binding ELISA strip plates, Grainer, Germany), coated with adhesive films and incubated at 4°C overnight. Plates were washed with 300 µl wash buffer (PBS containing 0.5% Tween 20) 3 times and incubated with a blocking buffer (PBS and 1% BSA) for 2.5 hrs at room temperature. 100 µl of sera from each group were diluted to 1/50 and added to each well. The plates were incubated at 37°C for 1 hr and washed again. Then, 100 µl of HRP rabbit anti-mouse IgG conjugate (RAZI Fara Teb, Iran) was diluted 1/4,000 and added to each plate, followed by incubation at 37°C for 1 hr. After washing, 100 µl of 3, 3', 5, 5'-tetramethylbenzidine or TMB (RAZI Fara Teb, Iran) was added to each well. ELISA reaction was stopped by adding 2N H_2SO_4. Absorbances were measured at 450 nm with an ELISA reader (17,29).

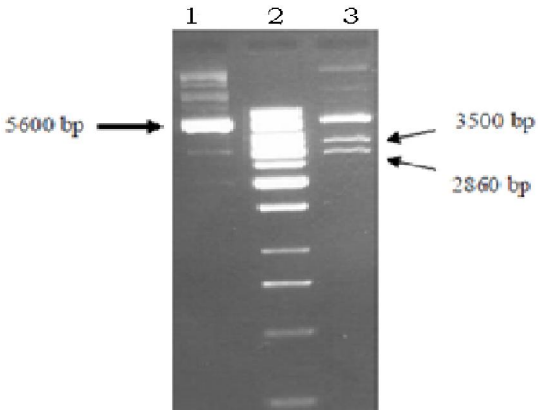

Figure 1. Results of enzymatic digestion of pcDNA3.1Hygro p24-gp41 for confirming the presence of p24-gp41. Lane 1, undigested plasmid, lane 2, 1kb DNA marker, lane 3, plasmid digested with *NdeI.*

IgG Isotyping. The basis of this test is the same as that for the ELISA of total serum IgG. To determine levels of IgG1 and IgG2a, a mouse antibody isotyping reagent kit (Saint Louis, Missouri, USA) was used according to manufacturer's instructions.

Statistical Analysis. After the raw data collection, normality test was done using Kolmogorov-Smirnov test. For general comparison between groups, one way ANOVA with

95% confidence limit and for comparison between each group, Tukey test in SPSS software, version 13, was used. The data was reported using mean ± standard deviation.

RESULTS

Cloning and Expression of Recombinant Vectors. Enzymatic digestion of positive recombinant vector pcDNA3.1/hygro (p24-gp41) with *NdeI* revealed two bands of 2860 bps and 3500 bps in electrophoresis (Figure 1).
Enzymatic digestion of pcDNA3.1Hygro (gp41) with *KpnI* and *XhoI* revealed appropriate 126 bp and 5600 bp bands (Figure 2) as expected for the recombinant vectors. Sequencing was performed on new recombinant vectors.
Lymphoproliferation Assay. Animal groups that received test vectors, showed significant proliferation in comparison to control groups. Lymphocyte proliferation was significantly higher in mice immunized with pcDNA3.1Hygro (p24-gp41) or group 2, and the SI values were higher compared to mice immunized with pcDNA3.1Hygro gp41 or group 6 alone (4.02 ± 0.74 vs. 2.65 ± 0.144), respectively. The highest SI belonged to group 4 of mice that received both pcDNA3.1Hygro (p24-gp41) and pCAGGS-IL-12 (6.55 ± 0.69).

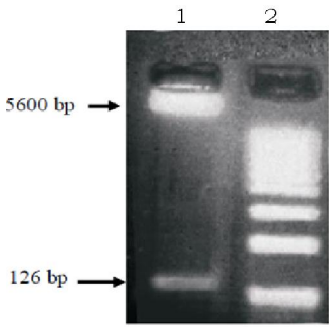

Figure 2. Results of enzymatic digestion of pcDNA3.1Hygro (gp41) with *KpnI* and *XhoI* revealed appropriate 126 bp and 5600 bp bands. Lane 1, plamid digested with *KpnI* and *XhoI*, lane 2, 100 bps DNA marker.

Comparison between SI of group 4 with those of other groups demonstrated a highly significant difference ($p<0.0001$). The second highest value of SI was for group 2 ($p<0.001$). In addition, group 6 had a significantly higher SI than those of other control groups ($p<0.05$). Significant differences existed between values obtained for groups 6 and those of groups 2 and 4, respectively. Control groups (1, 3, 5 and 7) did not show lymphocyte proliferation. There was no significant difference between SI of various negative control groups (Figure 3).

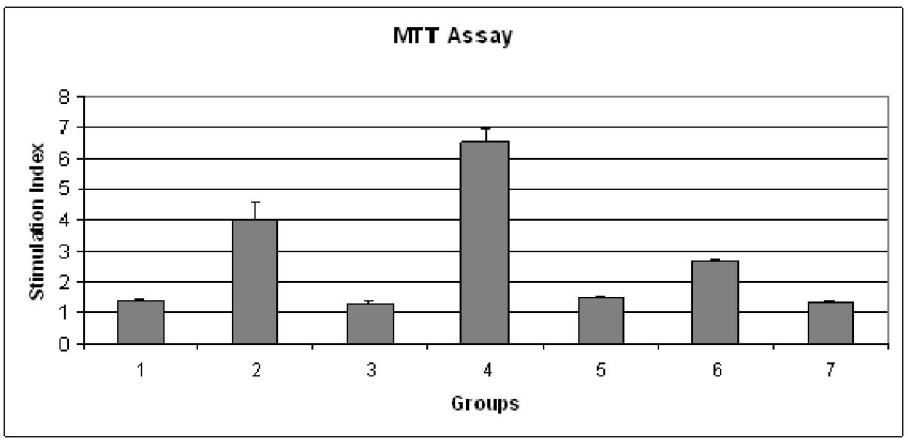

Figure 3. Mean of SI in MTT assay of various groups of mice before and after injection of a booster dose. Group 1: Negative control injected with pcDNA3.1. Group 2: Mice injected with pcDNA3.1Hygro p24-gp41. Group 3: Negative control injected with PBS. Group 4: Mice injected with pcDNA3.1 Hygro P24-gp41 along with the pCAGGS-IL-12. Group 5: Negative controls injected with pCAGGS. Group 6: Mice injected with pcDNA3.1 Hygro gp41 alone. Group 7: Mice injected with sterile Den123.

Interferon Gamma. The level of IFN-γ was assessed in supernatants of spleen cells stimulated by p24-gp41 antigen. In groups two and four, significant differences were seen in comparison with those of controls. The highest level of IFN-γ production belonged to group 4 ($p<0.0001$). The next highest value of IFN-γ was observed in groups 2 and 6.

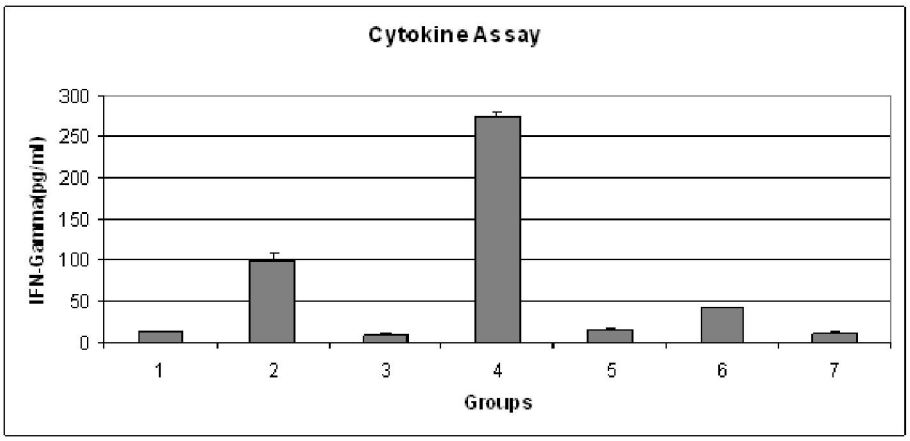

Figure 4. Mean of IFN-γ production in the supernatants of antigen stimulated cultured spleen cells of various groups of mice. Group 1: Negative controls injected with pcDNA3.1. Group 2: Mice injected with pcDNA3.1Hygro p24-gp41. Group 3: Negative controls injected with PBS. Group 4: Mice injected with pcDNA3.1Hygro P24-gp41 along with the pCAGGS-IL-12. Group 5: Negative controls injected with pCAGGS. Group 6: Mice injected with pcDNA3.1Hygro gp41 alone. Group 7: Mice injected with sterile Den123.

IFN-γ concentration was significantly higher in groups 2 and 6 in comparison with those of other groups. Nevertheless, the level of IFN-γ in the control groups was not significant (Figure 4).

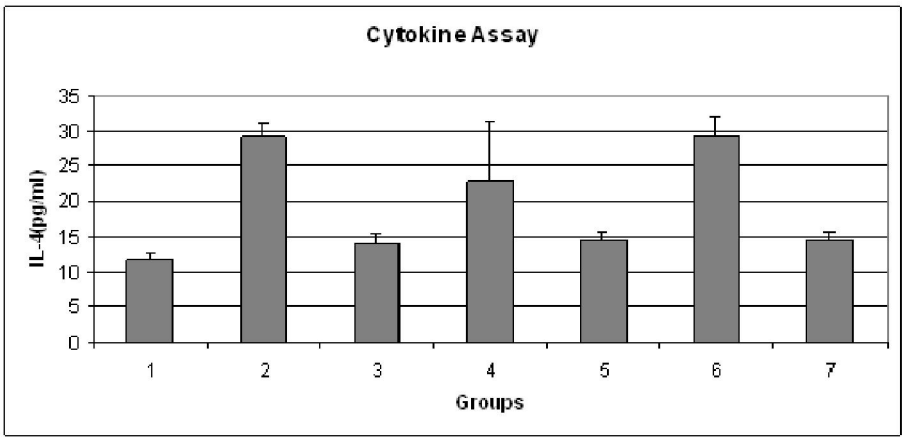

Figure 5. Mean IL-4 production in the supernatants of antigen stimulated cultured spleen cells of various groups of mice. Group 1: Negative control injected with pcDNA3.1. Group 2: Mice injected with pcDNA3.1 Hygro p24-gp41. Group 3: Negative control injected with PBS. Group 4: Mice injected with pcDNA3.1 Hygro P24-gp41 along with the pCAGGS-IL-12. Group 5: Negative control injected with pCAGGS Group 6: Mice injected with pcDNA3.1 Hygro gp41 alone. Group 7: Mice injected with sterile Den123.

Interleukin 4. The level of IL-4 was assessed in supernatants of spleen cells stimulated by p24-gp41 antigen after injection of a booster dose. In groups 6, 2 and 4, a significant change relative to the control groups was observed ($p<0.05$) (Figure 5).

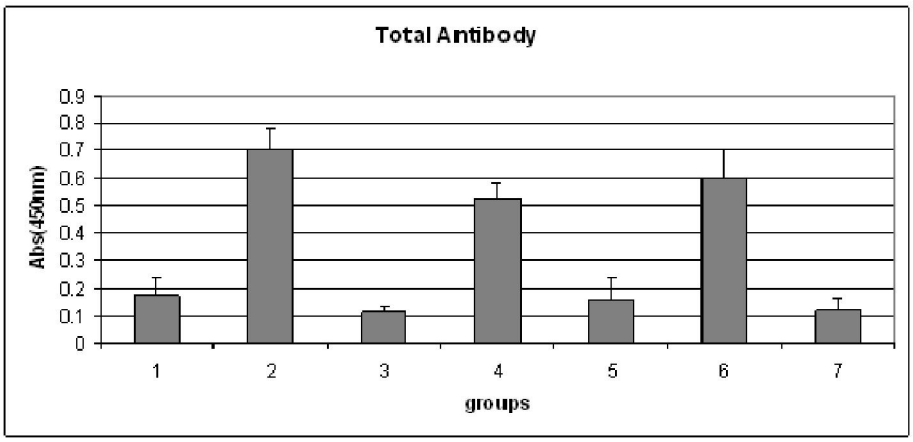

Figure 6. Mean of absorbances determined by designed ELISA for the determination of total antibody level in sera of various groups of mice. Group 1: Negative control injected with pcDNA3.1.Group 2: Mice injected with pcDNA3.1 Hygro p24-gp41. Group 3: Negative control injected with PBS. Group 4: Mice injected with pcDNA3.1 Hygro P24-gp41 along with pCAGGS-IL-12. Group 5: Negative control injected with pCAGGS Group 6: Mice injected with pcDNA3.1 Hygro gp41 alone. Group 7: Mice injected with sterile Den123.

Total Antibody. Humoral immune responses in mice immunized with the above vectors were evaluated by measuring total antibody levels using ELISA. Following the injection of the booster dose, the highest OD values belonged to groups 2, 4 and 6. These 3 groups had a significant difference compared to the control groups ($p<0.05$), but did not show any significant difference among themselves (Figure 6).

IgG Isotyping. The ratio of IgG2a to IgG1 was calculated. As it is shown in Figure 7, among the test groups, the highest ratio was observed for group 4 and the lowest ratio belonged to group 6. Group 2 was injected with pcDNA3.1/Hygro(p24-gp41) and group 4 received pcDNA3.1/Hygro(p24-gp41) plus pCAGGS-IL12, while group 6 only received pcDNA3.1/Hygro(gp41). This can be justified by the fact that groups 2 and 4 mice were immunized by plasmids that contained p24 consisting of CMI enhancing epitopes. IL-12 in group 4 directs the CMI towards Th1.

DISCUSSION

The World Health Organization has announced AIDS as the deadliest infectious disease and the fourth cause of death worldwide. Approximately, 34 million people are currently infected with HIV-1 in the world and many are progressing towards AIDS. The global spread of HIV, emergence of drug resistance mutants and difficulties in following instructions for recommended antiretroviral regimens in underdeveloped countries, encourage researchers to consider the development of a preventive or therapeutic vaccine for the control of HIV pandemic (1,19). An effective AIDS vaccine can prevent 16,000 new infections daily. Moreover, a considerable amount of money could be saved in developing countries (30,31).

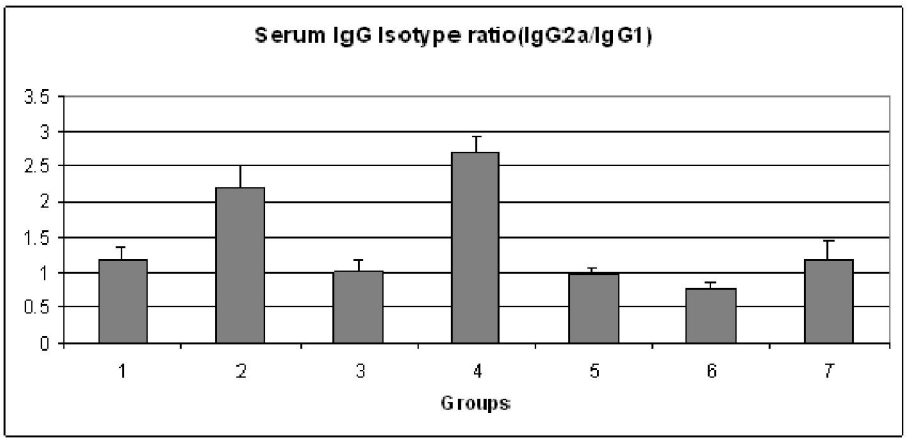

Figure 7. Mean of the relative absorbance of IgG2a/IgG1 determined by ELISA in sera of various groups of mice. Group 1: Negative control injected with pcDNA3.1. Group 2: Mice injected with pcDNA3.1 Hygro p24-gp41. Group 3: Negative control injected with PBS. Group 4: Mice injected with pcDNA3.1 Hygro P24-gp41 along with the pCAGGS-IL-12. Group 5: Negative control injected with pCAGGS. Group 6: Mice injected with pcDNA3.1 Hygro gp41 alone. Group 7: Mice injected with sterile Den123.

p24 and gp41 of HIV play important roles in viral pathogenesis and disease progression (19,20); hence, studies focused on these HIV proteins are of particular importance.
Various investigations have shown that despite the presence of B cell epitopes in p24, this protein induces lower levels of antibody responses in comparison with other HIV proteins; however, it is suggested that addition of envelope proteins to p24 would lead to a more effective induction of immunity (30). Active research is taking place to develop vaccines capable of inducing both humoral and cellular immunities (31).
In this study, a construct made of fused immunogenic fragments of p24 and gp41 was used for the first time. The peptides used in this study contained a number of stimulatory epitopes of B cells and specially T cells; the immune stimulatory capacity of some epitopes was proven in previous studies (15,16,31,32).
Many studies have proven the beneficial use of immune stimulatory molecules to increase or guide immune responses towards a specialized path (23,33). IL12 has a wide range of effects on T cells, such as helping to promote expansion of Th1 lymphocytes. Therefore, CD_4+ Th1 lymphocytes can further produce type 1 cytokines which in turn help in proliferation of CD8+ T cells and expression of cytotoxic mediators such as granzyme B/perforin and IFN-γ as well as more IgG2a production.
Roles of endogenous IL-12 in resistance against infection and induction of Th1-biased memory response have encouraged researchers to use this interleukin as a therapeutic factor or as an adjuvant in vaccination strategies. Recent studies have shown that either P35 or P40 chains of IL-12 can be used as an alternative for whole IL-12 in order to deliver vaccines into animal models (34). In this study, a vector containing both chains of IL-12 (pCAGGS-IL-12) which has proven effects was used (27,35,36).
A group of Balb/c mice that received pcDNA3.1Hygro containing both p24 and gp41 (group 2) showed significant differences in lymphocyte proliferation and IFN-γ compared to all other groups but not with group 6 which had received pcDNA3.1/Hygro (gp41). The significant difference can be due to the presence of various stimulatory T cell epitopes in p24-gp41. Although gp41 containing vector (group 6) also showed significant increase in both proliferation and IFN-γ tests, the responses were persistently lower than that of p24-gp41 containing vector. The difference was most obvious with IFN-γ production assay. This is probably due to a lower number of immunogenic epitopes, especially T cell stimulatory epitopes in gp41 vector. Evaluation of the ratio of IgG2a to IgG1 production, exhibited that fused fragments (p24-gp41) induced Th1 immunity. However, using gp41 alone stimulated Th2 type immune responses. These can also justify the level of difference between SI and IFN-γ in groups 2 and 6, respectively. As reported in previous studies (15,22,23,34,36,37,38), co-injection of IL-12 expressing vectors along with a DNA vaccine is expected to increase Th1 cellular immune responses including high IFN-γ production. This was the case in this study. Group 4 that received fused fragments (p24-gp41) along with an IL-12 expressing vector demonstrated a significantly higher SI and IFN-γ production with a significant increase in IgG2a/IgG ratio.
The result showed that the titer of total antibody in group 4 was slightly less than group 2 that received IL-12 containing vector (39,40). Considering the function of IL-12 which causes an increase in cellular immunity and a decrease in humoral immunity, the above

results had been quite predictable. However, this result might not be the same for all antigens. In this regard, Moore et al have reported the importance of using various genetic adjuvants based on antigen-driven profile seen after DNA vaccine immunization (36).
The highest level of IL-4 production was shown in group 6 followed by groups 2 and 4. Although the level of IL-4 has not been high in this study, it is higher in comparison with results of a similar research performed on gag gene with an IL-4 level of less than 5pg/ml. This can be due to the presence of gag and gp41 together (40).
It is noteworthy that the presence of Dendrosome has some effects on stimulation of immune response (14,17,41,42). In this study, Dendrosome was used as a suitable delivery system for a successful transfection rather than as an adjuvant.
The use of 2 immunogenic fragments of gag and env genes fused together as a DNA vaccine candidate has been the main goal of this study. The results have clearly demonstrated the higher potential of these two fragments together in the induction of both arms of immune responses towards a Th1 type response (10,19,38). Therefore, further studies on optimization of this construct will be useful as an approach for making a more optimal HIV-1 DNA vaccine.

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