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Characterization of Immune Responses Induced by Combined Clade-A HIV-1 Recombinant Adenovectors in Mice

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ABSTRACT

Background: Numerous evidences indicate that in some HIV-1 positive patients, the humoral and cellular immune responses are induced against HIV-1 proteins and this is inversely related to the progress of infection. **Objective:** The aim of this study was the evaluation of the Adenovectors containing HIV genes in induction of immune responses in mice. **Methods:** The HIV-1 genes including gag p24, rev, nef and exon-1 of tat were amplified from HIV-1 RNA (clade-A). The cDNA of each gene was cloned into a transfer vector. The transfer vector was then co-transformed into *E. coli* strain BJ5183 together with pAdenovector ΔE1/E3. The recombinant adenoviral construct was transfected into QBI-293A cells. Recombinant viruses were purified and titrated on 293 cell plates. Expression of transgenes was evaluated using western blotting. Then 10^{12} viral particles were injected into 15 groups of 5 mice and all patterns of combination of these 4 HIV-1 genes were evaluated. After 2 weeks, humoral and cellular immune responses were evaluated using ELISA, cell proliferation and ELISpot (IL-2, IL-4 and IFN- γ) assays, consecutively. **Results:** It was demonstrated that each gene was expressed. The response targets were mostly toward Th1, though several Th2 responses were also observed. Single injection in our study induced a good cellular response but the humoral responses were not as strong as the cellular ones. **Conclusion:** Considering and comparing all results and evaluating the various possible interactions revealed that simultaneous injection of tat and gag has enhanced the humoral and cellular responses.

Keywords: Adenovector, Cellular Responses, HIV Vaccine, Humoral Immune Responses

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INTRODUCTION

A number of evidences indicate that the humoral and cellular responses induced in some HIV-1 infected patients is inversely related to infection progress (1). Also in some cases it has been specified that the immune response against one HIV-1 protein may lead to long-term non-progression of the disease. Most of such reports have been published on Tat. Based upon these studies, by determining the formation of antibody response against Tat, the rate of HIV progression can also be determined in HIV infected patients (2,3). Besides Tat, similar results have been reported on Rev Protein (4). Rev and Tat are two small regulating proteins which are expressed in initiation of virus cycle before other genes and are crucial for virus pathogenesis. The two proteins have considerable amount of conserved sequences among different types of geographically distinct virus clades and strong response of immune system against them has a protective role against infection progress (5,6). Another protein with significant role in infection progress is Nef; it possesses conserved structures which have remained constant or with the least changes in different types of HIV-1, HIV-2, and SIV (7). This protein has an important role in disease pathogenesis and numerous reports indicate that a number of patients with slow infection progression rate have been infected by HIV types which either lack the *nef* gene or show some defects in this gene (8,9). Therefore, it is obvious that generation of immune response against this protein is inversely correlated to the rate of the disease progress (10,11). Similar reports have also been published about the structural protein Gag. In addition to considerable level of conservation in the sequence of this protein, it has been revealed that some patients with very slow rate of the disease progression possess strong immune response against Gag, and these responses have inverse correlation with the viral load. These responses are mainly targeted against p24 region of Gag (12).

As a group of viral vectors, adenovectors (Ad) have attracted many researchers and are capable of infecting both diving and non-diving cells. Moreover, very high titers of them can be prepared (1×10^{11} - 10^{13} viral particles/ml) and no specific side effect has been observed in these high titers. (13,14). Although it has been identified that making use of recombinant Ad5 (rAd5) along with HIV genes induces mucosal and systemic immune responses, i.e. cellular and humoral response, against these proteins(15,16), immunological efficiency of the vaccine decreases in most patients against this vector due to preexisting Ad5 immunity (17). This immunity has been one of the main reasons for stopping Merck recombinant Ad5 (rAd5) phase II proof-of-concept STEP study (18,19). Numerous strategies are followed by researchers to tackle the problem, one of which is to use Ad5 in high doses (20,21).

In the present study, we employed incompetent rAd5 lacking E1 and E3 genes in high titers and used only one injection since several injections of an adenovirus of a single serotype decreases the expression level of the transgene due to the presence of the previous response and ultimately leads to the suppression of immune response (22). Furthermore, we evaluated the immunological response in a comparative way to show the effects of all different combinations formed gag, tat, rev, and nef of HIV-1 subtype A, on inducing an immunological response.

MATERIALS AND METHODS

Mice Immunization and Sample Preparation. Six week-old female BALB/c mice were purchased from Pasteur Institute of Iran (Tehran, Iran) and kept at the animal house of the medical faculty of Tarbiat Modares University (Tehran, Iran). All experiments were conducted in accordance with NIH animal care guidelines. All mice were allowed a week of adaptation time before initiating experiments. The mice were then randomly divided into 15 groups of 5. The employed vaccine components and doses are shown in Table 1. In multi-component groups, rAd5 vectors were formulated in 1:1 or 1:1:1 and or in 1:1:1:1 ratio of the vectors. All animals were immunized by a single intramuscular injection of recombinant virus diluted in phosphate-buffered saline (PBS) while the mice in the control group were only injected with Ad ΔE1/E3 vector. A control rAd5 vector contained no gene insert. rAd5 was delivered to the mice at a dose of 10^{12} viral particles. Additionally, due to the adjuvant effects of Ad on immune system and sence one of the goals of the current study was to compare the results of the immunological response in different groups, mice in groups 1 to 13 received a specific amount of Ad, so the total number of adenoviral particles in all 15 groups was the same and equal to 4×10^{12} viral particles.

Table 1. Mouse immunization groups.

Group no.	BALB/c Mice
I.	Ad5 ΔE1/E3-tat
II.	Ad5 ΔE1/E3-rev
III.	Ad5 ΔE1/E3-nef
IV.	Ad5 ΔE1/E3-gag
V.	Ad5 ΔE1/E3-tat + Ad5 ΔE1/E3-rev
VI.	Ad5 ΔE1/E3-tat + Ad5 ΔE1/E3-nef
VII.	Ad5 ΔE1/E3-tat + Ad5 ΔE1/E3-gag
VIII.	Ad5 ΔE1/E3-rev + Ad5 ΔE1/E3-nef
IX.	Ad5 ΔE1/E3-rev + Ad5 ΔE1/E3-gag
X.	Ad5 ΔE1/E3-nef + Ad5 ΔE1/E3-gag
XI.	Ad5 ΔE1/E3-tat + Ad5 ΔE1/E3-rev + Ad ΔE1/E3-nef
XII.	Ad5 ΔE1/E3-tat + Ad5 ΔE1/E3-rev + Ad5 ΔE1/E3-gag
XIII.	Ad5 ΔE1/E3-rev + Ad5 ΔE1/E3-nef + Ad5 ΔE1/E3-gag
XIV.	Ad5 ΔE1/E3-tat + Ad5 ΔE1/E3-rev + Ad5 ΔE1/E3-nef + Ad5 ΔE1/E3-gag
XV.	Ad ΔE1/E3 vector : control

Five BALB/c mice were put into each group and a single injection of 10^{12} viral particles was performed on them. The viral dose injected to each mouse in each group was identical to that in other groups, and the final dose was 4×10^{12} viral particles. Hence in groups 1 to 13 the adenovector without transgene was injected to the mice so that the viral dose received by them would be equal. In multiple groups, adenovectors were mixed completely prior to injection and were then injected. The group 15 merely received the adenovector without transgene. Besides the mentioned 15 groups, three mice received only the injection buffer and showed no response in evaluation of immunological responses and their results are not illustrated. All injections were carried out one week after presence of mice in animal house.

Vaccine Vectors. Four replication-incompetent Ad5-HIV recombinants were used in this study. For HIV-1 genes, 200 µl of plasma from an HIV-1 infected drug user HIV/AIDS was extracted using the extraction procedure of the AccuPrep® viral RNA extraction kit (BIONEER, Korea). The HIV-1 genes, sequence of *gag p24*, *rev*, *nef* and exon 1 of *tat* were amplified from 20 µl HIV-1 RNA extract by nested polymerase chain reaction using the one step RT-PCR kit (BIONEER, Korea). First and second PCR procedures were performed as described previously (23). The two exons of *rev* gene were fused by spliced-overlapping extension to make the complete *rev* ORF (24). The nucleotide sequences of *gag p24*, *tat*, *rev* and *nef* were verified by commercial sequencing. Results of sequence analysis identified that all of the samples studied were of A subtype. The cDNA of each gene was first cloned into a transfer vector containing CMV5 promoter and poly A (Qbiogene, USA). The CMV5 promoter contains the CMV promoter itself as well as enhancer sequences and it also contains an optimized Kozak sequence. The transfer vector was then co-transformed into E. coli strain BJ5183 (Qbiogene, USA) together with pAdeno Vator ΔE1/E3 (Qbiogene, USA). In the pAdeno Vator ΔE1/E3, E1 and E3 were deleted. The recombinant adenoviral construct was afterwards transfected into QBI-293A (Qbiogene, USA) cells so as to produce viral particles. Recombinant viruses were then purified by Vira Trap™ Adenovirus maxi purification kit (Biomiga, USA) and titrated on 293 cells to determine plaque forming units (pfu) according to AdenoVator™ manual (Qbiogene, USA). Expression of each HIV-1 protein in 293 cells infected with rAd5-HIV-1 genes was evaluated by western blot. The 293 cells were infected at an MOI of 25 with rAd or Ad5 ΔE1/E3 vector as negative controls. When most of the cells exhibited a cytopathic effect, cell lysates were prepared and electrophoretically separated on an SDS-polyacrylamide gel and then blotted onto a nitrocellulose membrane (Amersham, Bioscience) overnight at room temperature (RT). The membrane was blocked in 5% fat-free dry milk in TBS (20 mM Tris-HCl, pH 7.5, 0.2 % Tween 20) for 3h and then incubated with mouse monoclonal antibodies anti-Nef, anti-Tat, anti-Gag p24 or anti-Rev, all diluted 1:1000 for 3h at RT. Peroxidase-labeled goat anti-mouse antibody(Abcam, France) was used as a secondary antibody(1:4000, 2h, RT) followed by detection with insoluble Tetramethyl benzidine (TMB) (Sigma).

Sample Collection. Mice were sacrificed 2 weeks after the immunization. Blood samples were collected by cardiac puncture and serum were prepared and frozen. Spleens from immunized animals were removed aseptically and unicellular suspensions were then prepared by passing the spleens through a metal mesh. Splenocytes were isolated by Ficoll (Sigma) gradient centrifugation and resuspended in R-10 medium (RPMI-1640 containing 10% heat-inactivated FCS, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin). Cells were stained with Trypan blue and immediately counted and used freshly in all assays.

Enzyme-linked Immunosorbent Assay (ELISA). Serum from immunized and control mice were evaluated by ELISA for binding antibodies to HIV-1 Gag p24, HIV-1 Nef, HIV-1 Tat and HIV-1 Rev proteins. All recombinant HIV-1 MN proteins and antibodies used for ELISA and western blotting were purchased from Abcam (France) or Trinity Biotech (Bray, Ireland) and used under the reaction conditions recommended by the vendors. ELISA microplates (Polysorp, Nunc, Denmark) were coated overnight with 100 µl HIV-1 P24 recombinant protein (4 µg/ml), HIV-1 Nef recombinant protein (2 µg/ml), HIV-1 recombinant Tat protein (1 µg/ml) in 0.1 M carbonate buffer (pH 9.6) and HIV-1 recombinant Rev protein (1 µg/ml) in PBS buffer (pH 7.4). Plates were blocked for 1h in 3% bovine serum albumin at room temperature. 100 µl 1:10 diluted

serum samples were subsequently added to the wells in duplicate for 1h at 37°C. The plates were washed four times with PBS containing 0.05 Tween 20 and then horseradish peroxidase-conjugated anti-mouse IgG, anti-mouse IgG1 or anti-IgG2a was added for 1h at 37°C. After washing, the color of wells were developed with TMB and after termination of reaction by sulfuric acid addition, Optical Density (OD) of wells was read at 460 nm using a Stat Fax 2100 Micro Plate Reader (USA) using 630 nm as the references wavelength. Anti Tat, Nef, Rev or Gag p24 monoclonal antibodies served as positive controls. Cut-off values of samples were defined as optical densities greater than the mean value for negative samples plus 3×SDs.

Cell Proliferation Assay. Cell proliferative responses were studied through culturing 5×10^5 spleen cells in 200 µl R10 medium in the presence of 5 µg of HIV proteins in 96-well, tissue culture grade, flat-bottom microplates (Nunc, Denmark) for 5 days at 37°C and 5% CO₂. HIV antigens used as stimulator included: HIV-1 MN recombinant protein Gag p24, Nef, Tat and Rev. In this stage, we used cell proliferation kit (MTT), a colorimetric method for non-radioactive quantification of cell proliferation (Roche Diagnostic GmbH, Roche applied Science, Germany). Based on manufacturer's protocol, after the incubation period, 20 µl MTT labeling reagent (with a final concentration of 0.5 mg/ml) was added to each well and incubated for 4h at 37°C and 5% CO₂. Then 100 µl of the solubilization solution was added to each well and incubated overnight at 37°C and 5% CO₂. Finally, the absorbance of the formazan product was measured at 550 nm by ELISA reader while the reference wavelength was 690 nm.

Enzyme-linked Immunospot (ELISpot) Assay. An ELISpot assay was used to detect HIV-1 proteins Tat, Rev, Nef and Gag p24 specific IFN-γ, IL-2 and IL-4 producing T cells from mice splenocytes which were previously stimulated by rAd5 based specific groups. The ELISpot assay was performed in different groups in such a way that, in the first four groups which only had a single type of recombinant vector, only the same type of recombinant protein was utilized. For instance, in the first group that rAd5-tat was injected to mice, the immunogen used in the assay was recombinant Tat Protein, and similarly in the second group where mice were injected by rAd5-rev, recombinant Rev Protein was applied as immunogen. Similar procedures were carried out for groups 3 and 4. Assay was performed in duplicate, once for the first response protein and another time for the second response protein. For example, in the fifth group where the mice received rAd5-tat + rAd5-rev, the response was measured once against recombinant Tat protein and then against recombinant Rev protein. Fresh cells were utilized in each test to enable the comparison of results and interactions among responses. The murine ELISpot assay was run according to the manufacturer's protocols R&D systems kits (Minneapolis, US) in duplicates. In brief, mouse fresh splenocytes were used at concentration of 5×10^5 cells per 100 µl 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, Rev, Nef or Gag p24 proteins were used as immunogens. The concentration and the response time of recombinant proteins had been titrated before. Following 48h of incubation at 37°C in 5% CO₂, the cells were removed and the wells were washed and incubated with the detection antibody overnight followed by the addition of streptavidin-AP and BCIP/NBT chromogen. The color reaction was then stopped by washing with distilled water. Subsequently, the plates were air dried and spots were counted visually using an inverted microscope. Responses were considered to be positive if number of

spot-forming cells (SFC) were greater than double the number in negative control wells. The results were reported as the number of spots per 10^6 cells per well. Background spots were subtracted from each immunogen.

Statistical Analysis. Data obtained in this study was analyzed using SPSS statistical software version 11.5 Descriptive data was calculated as mean, median, and standard deviation (S.D.) in different groups. Also with the purpose of performing analytical and descriptive analysis in the groups under study, non-parametric Mann-Whitney U test was utilized and the values less than 0.05 was considered to be a significant parameter.

RESULTS

Verification of Transgene Expression. As was mentioned in the previous section, rAd5 was constructed containing *tat*, *nef*, *gag p24*, or *rev*. In order to independently evaluate the effect of each of these genes, these rAd5 were synthesized separately. Since these four genes produce the best response in their native state as was explained in the introduction section, their native sequences were used. It has been identified that exon 1 has the major role in performing the task of *tat*. Therefore, this exon was utilized. On the other hand, because *rev* requires both exons for accomplishing its task, both of them were used in *rev* case. As a result, we found out that each of the genes had detectable expression levels (Figure 1). We found out that all of the genes belonged to the A subtype. Subtype A is likely to be the dominant HIV-1 subtype in Iran (25).

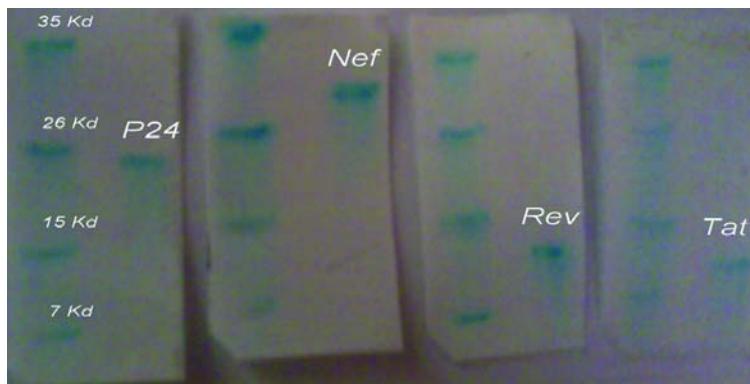


Figure 1. HIV-1 protein expression from the rAd5 was confirmed by western blot. Detection was performed on 293 cells infected with the constructs (A) rAd5-p24, (B) rAd5-nef, (C) rAd5-rev and (D) rAd5-gag tat. Two days post infection; the cells were washed and lysed. The cell lysates were then loaded onto a gradient of polyacrylamide gel and transferred to a nitrocellulose membrane. Expression was detected with mouse monoclonal antibodies independently. The mouse immunoglobulins were detected using a horseradish peroxidase (HRP)-conjugated anti-mouse mAb. The blots were developed using insoluble Tetramethyl benzidine. The 293 cells were infected with Ad5 Δ E1/E3 vector as a negative control.

Antigen-specific Humoral Immune Responses. A great deal of research has been carried out on these four genes previously. However, our goal was to perform a comparative study and provide an evaluation of the response caused by different combinations of the four genes. The results were thus classified into four different groups which were suitable for comparison and the study of the effects that an immunogen exerts in response to other immunogens. As shown in Figure 2, this classification was done based upon the presence of all responses with one common gene. In other words, in all the mice which had received e.g. rAd5-tat, the amount of antibody against Tat was evaluated while in all the mice which had received rAd5 –rev, the amount of antibody against Rev was assessed.

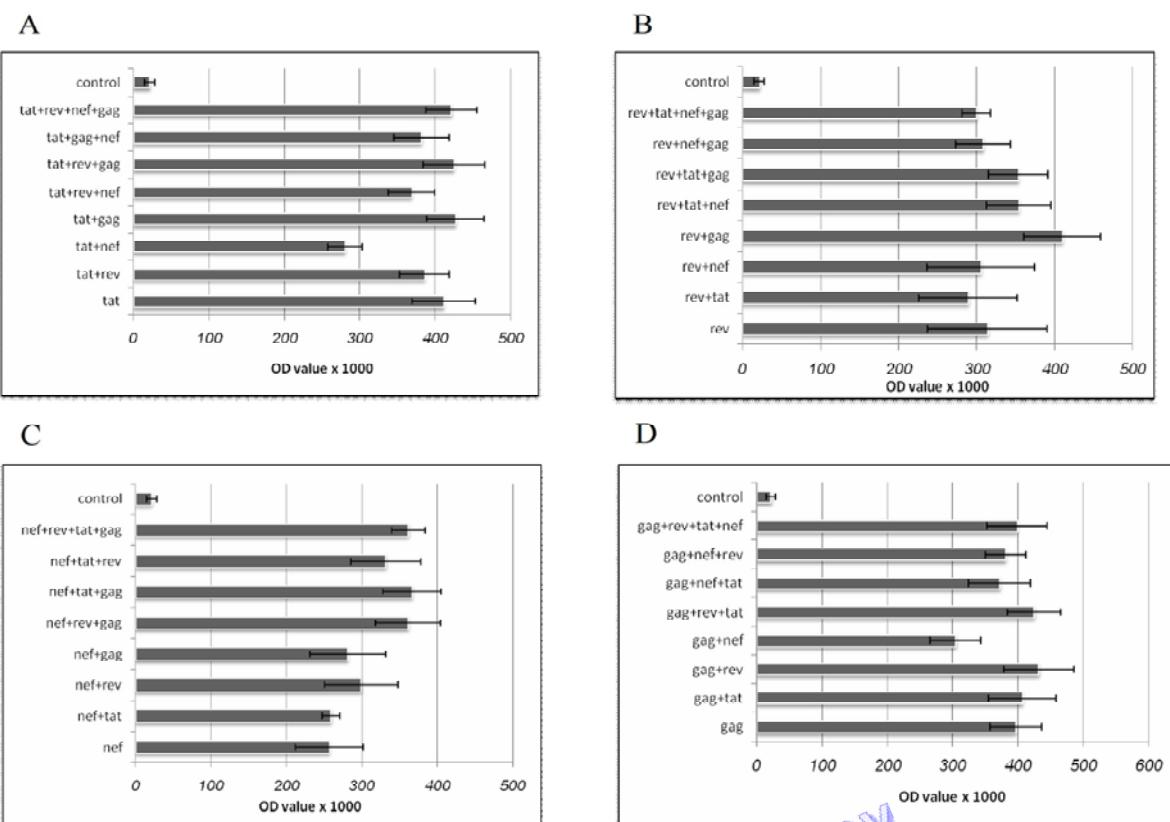


Figure 2. In each group, the antibody response is assayed against one of the main genes. Figures 2A to 2D demonstrate the antibody response against Tat, Rev, Nef, and Gag, respectively. Two weeks after injection of rAd5-transgene to the mice, their blood serum was extracted and placed in ELISA plates, coated with recombinant antigen and ELISA test was performed. The control animals in this test had received only empty adenovectors. Monoclonal antibody against the recombinant protein in ELISA test was considered as a positive control indicating the validity of the test. The error bars depict the standard error of the mean.

The similar procedure was also carried out in the cases of rAd5-nef and rAd5-gag p24. As can be observed, although the titer of antibodies is low, antibody response was induced against all these transgenes. In case of Tat, when the mice which had received Tat also received Nef, antibody response reduced significantly ($p=0.008$). This difference

was not significant in other groups. The presence of Nef in this group has decreased antibody titer, while the similar effect was not observed in other groups. In case of Rev, although the amount of antibody in *rev+gag* group is more than other groups, this effect is seen only in this group ($p=0.151$). In those groups where gag had been injected simultaneously with nef, a considerable increase was induced in antibody titer compared to the main group (nef). This increase is not significant in *nef+gag* group ($p=0.310$) while the effect is significant in other groups including *nef+rev+gag*, *nef+tat+gag*, and *nef+rev+tat+gag* ($p=0.008$, 0.008 , and 0.016 , respectively). Also simultaneous injection of nef with other proteins did not decrease the antibody titer compared to nef alone, while in the case of gag, simultaneous injection of nef and gag reduced antibody titer compared to gag alone ($p=0.016$). Comparing the four proteins, the greatest response was obtained from Tat and Gag, followed by Rev and Nef.

Since the type of antibody obtained in individual groups determined the target response towards Th1 or Th2, type of antibody was identified and the ratio of IgG2a to IgG1 for each group was illustrated in Figure 3. The targets responses were mostly toward Th1, though several Th2 responses were also observed. It is noteworthy to deserve that nef has induced the least IgG2a response in comparison with other groups and exerts its suppressive effects on other groups more than more.

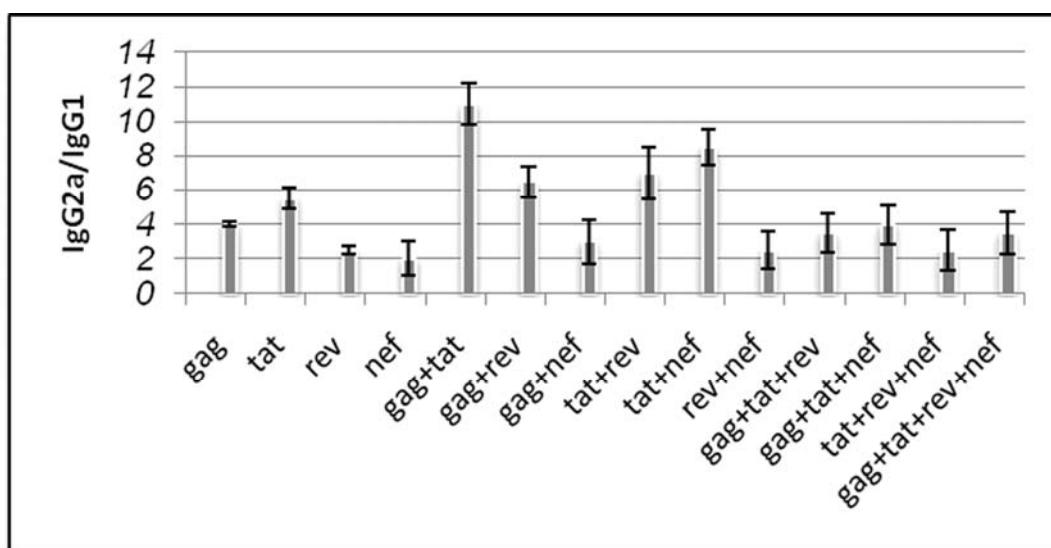


Figure 3. Type of IgG antibody yielded in each group: Two weeks after injection, serum of mice was extracted and put in ELISA microplates coated with four protein and studied using the antibody conjugated with the enzyme peroxidase anti-IgG1 or anti-IgG2a (1:10000). The standard ELISA procedure was performed as explained under materials and methods. OD of IgG2a antibody was divided by that of IgG1 antibody to represent the ratio of these two antibodies. The error bars demonstrate the standard error of the mean.

Antigen-specific Cellular Immune Responses. To evaluate the amount of cell proliferation, the splenocytes were treated with 5 µg of each HIV-1 recombinant proteins Nef, Rev, Tat, and Gag p24. Cell proliferation induces are depicted in Table 2. The cells were selected only from the group which had received a single immunogen, and due to type of method applied and its sensitivity, other groups were not evaluated.

Table 2. Level of cell proliferation: 5×10^5 spleen cells from mice groups 1 to 4 were treated with 5 μ g each recombinant protein for 5 days. Subsequent to adding MTT-labeled reagent and the solubilization solution, final OD of formazan product was measured. Control splenocytes were treated only with R10 medium.

immunogen	stimulation index
<i>Tat</i>	8.2
<i>Rev</i>	6.77
<i>Nef</i>	11.21
<i>gag p24</i>	12.49

Stimulation index was defined as:

$$SI = \frac{\text{Absorbance [A550nm-A690nm] of the splenocytes of the immunized mice + HIV-1 rec proteins}}{\text{Absorbance [A550nm-A690nm] of the splenocytes of the immunized mice + R10 medium}}$$

The splenic cells of group 15 which had received only empty adenovector were put in treatment of each of the above mentioned recombinant proteins similar to the standard process, and the obtained SI was lower than 2.

For assessing the cellular response, ELISpot test was used to assay the production of IFN- γ and IL-2 as indicates of Th1 response, and that of IL-4 as an indication of Th2 response (Figure 4).

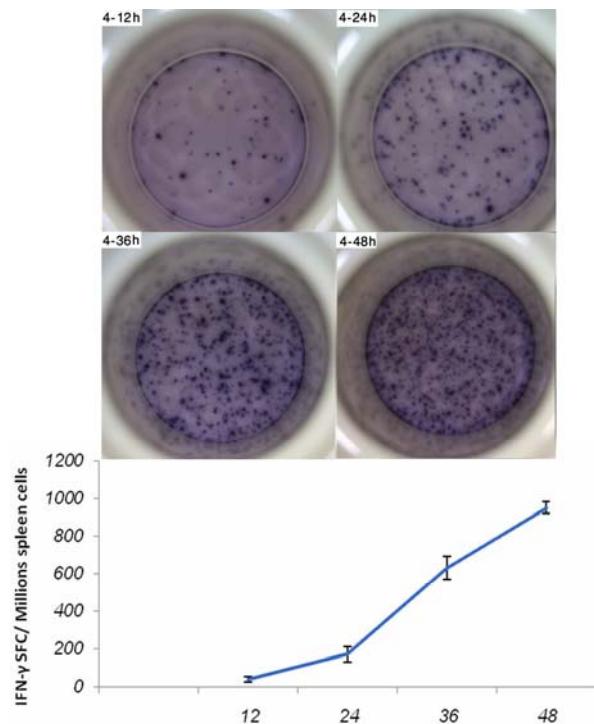


Figure 4. ELISpot assay was used to detect the production of IFN- γ and IL-2. Selection of time point for reading the assay was based on a titration over time.

In the first stage of ELISpot assay, a 48-hour incubation period and a concentration of 5 μ g were chosen for each individual immunogen, and the results are demonstrated in Figure 5.

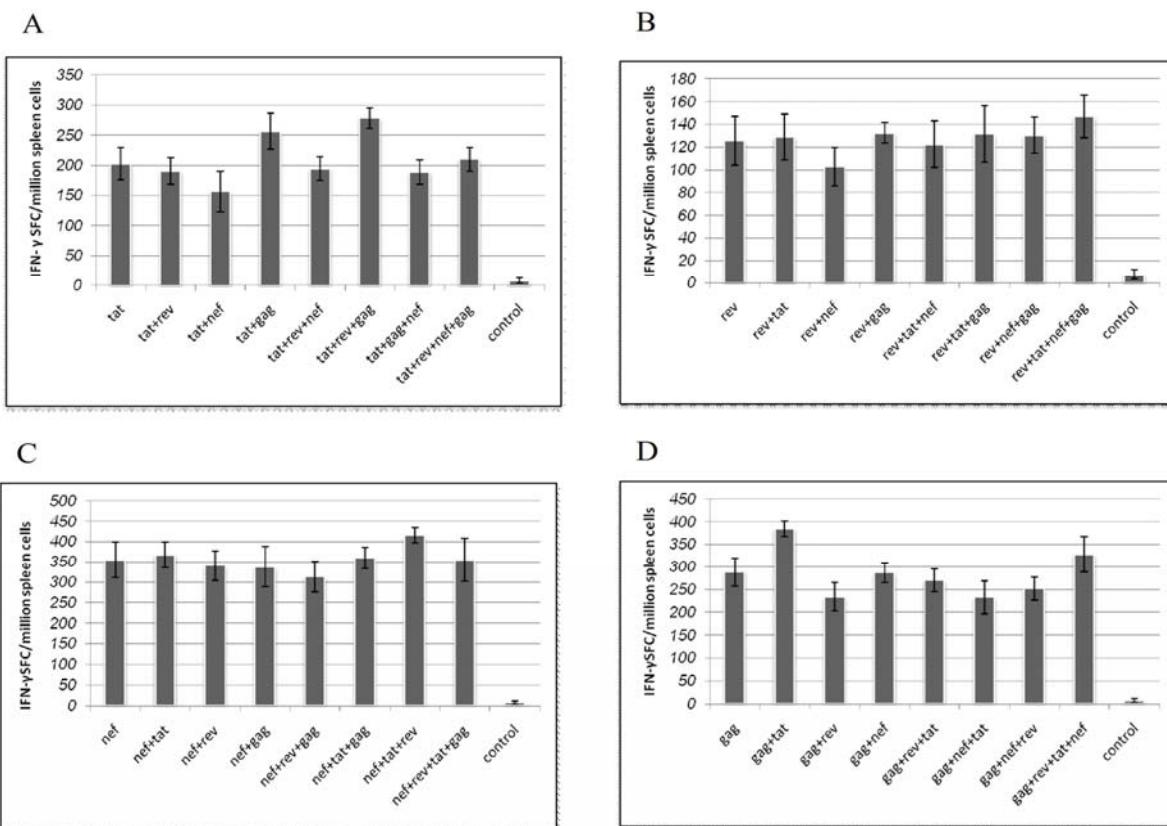


Figure 5. The results of IFN- γ ELISpot assay of groups depicted in Table 1: Two weeks after a single injection of the recombinant adenovector with a dose of 10^{12} viral particles, 5×10^5 splenic cells were extracted from immunized mice of Table 1 were treated with recombinant protein HIV-1 MN. The test protocol was carried out 48 hours later according to the above text, and the results are demonstrated as comparison with one of the major immunogens. In Figures 5A to 5D, the results have been studied compared to Tat, Rev, Nef, and Gag as the major proteins, respectively. The control group had only received empty adenovector. The error bars demonstrate the standard error of the mean. Numbers of SFCs are shown after subtracting the background.

As was mentioned in "Materials and Methods" section, evaluation of cytokine production responses was carried out in such a way that comparison of responses would be possible. These results are illustrated in Figure 6. Considering responses against Tat, simultaneous injection of *tat* with *nef* decreased the amount of IFN- γ ($p=0.056$) while simultaneous injection of *tat* with *gag* and also with *rev+gag* increased the response ($p=0.008$ and 0.032 , respectively). Responses against Rev were similar to humoral responses and have induced the weakest responses; most of the responses are analogous to each other. Response against Nef have induced the strongest responses, only the group *nef+tat+rev* has induced a significantly different response ($p=0.032$). Also, simultaneous injection of

tat+rev+gag and *nef+tat+gag* resulted in significant differences ($p=0.032$ and 0.032 , for each groups).

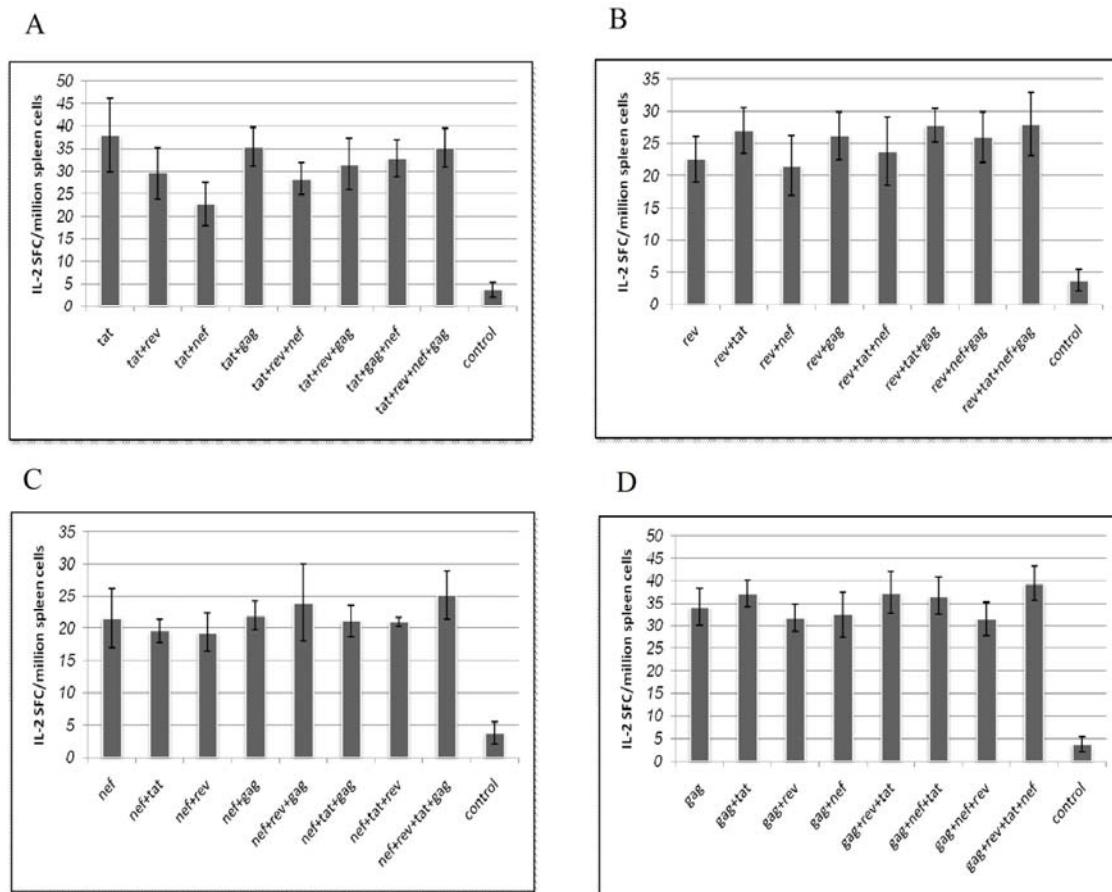


Figure 6. Results of IL-2 ELISpot in the groups reported in Table 1: Two weeks after a single injection of r Ad5 with a dose of 10^{12} viral particles, 5×10^5 splenic cells were extracted from immunized mice of Table 1 and were treated with recombinant protein HIV-1 MN. The test procedure was approved out 48 hours later. In Figures 6A to 6D, the results have been studied compared to Tat, Rev, Nef, and Gag as the major proteins, respectively. The control group had only received empty adenovector. The error bars display the standard error of the mean. Numbers of SFCs are shown after subtracting the background.

Indeed, IL-2 production represents memory cells (26). Although the responses were weak, they indicate that IL-2 is produced against all immunogens. In IL-2 responses, only a significant difference was observed in one group. Simultaneous injection of *tat* and *nef* has reduced the number of spots considerably ($p=0.06$). In comparison of the groups regarding cellular immune response, Rev has shown the lowest and Nef has demonstrated the highest immunogenicity; Tat and Gag showed higher values compared to Rev. In most of the cases, the responses due to IL-4 were almost equal to the negative control and could not be evaluated.

DISCUSSION

In the current study, we applied each of the above mentioned proteins in their wild type sequence to evaluate the amount of response induced against them. We also avoided formation of point mutations or deletion of some parts of a sequence since, as was explained in the introduction section, the immunological response against these genes in their native form results in a notable decrease in virus proliferation and also long-term non-progression of infection in the patient. Codon-optimization has effects on the expression rates of proteins by multiple mechanisms and in mice, it was shown that codon-optimization of rev and nef genes do not affect their immunogenicity (27).

It should be noted that it is not correct to state that the induced immune response is very strong in patients with slow rate of infection progress; rather, these responses have been induced against key components of the virus. For instance, among asymptomatic HIV-infected patients, a low antibody titer is related to long-term non-progression (3,28,29). Furthermore, numerous indications exist to show that when applying several appropriate immunogens simultaneously, a better response is induced and the viral escape will be much less (30,3). In the present study, we used a single injection of a high dose of non-competent rAd5 so that the least interference would occur between the host immune system and the vector components. To best of our knowledge, this concentration of Ad has been the highest concentration used so far, and although we did not evaluate it in the current research, the use of a high dose of Ad is among the ways to solve the problem of preexisting immunity against Ad, and it leads to no problems in the immunized mice regarding their weight, mobility, and food intake subsequent to injection (data not shown). This is in agreement with other investigations (20).

For all of the 4 constructs, the induced responses, especially the humoral response, were relatively weak. Nonetheless, considering a single injection and comparing the results with that of other studies related to Ads, it is predicted that booster injection using protein, viral. No antibody response has been detected in this range of viral particle concentration, and the cellular immune response has been shown to be weak (32,33). It was specified that the single injection in our study induces strong cellular and antibody responses, especially, when the Rev containing construct was used. Antibody response against Rev had hardly been observed in other investigations. Although, the antibody titers are generally low, these non-neutralizing antibodies are important because based on recent reports these antibodies might have protective results (34,35).

Comparing all of the results, it is revealed that simultaneous injection of *tat* and *gagp24* has enhanced the humoral and cellular responses and also increased the response compared to *tat* and *gag p24* separately. This effect is very obvious in duplicate groups and this simultaneous injection has led to IgG2a production with a considerable difference. It has been previously identified that Tat exhibits adjuvant characteristics in some cases and causes maturation of dendritic cells as well as activation of T cells and improves vaccine protection (36). Therefore, it is probable that making use of this property results in increased response; however, such an effect was not observed with other proteins such as Nef and Rev. Probably we can say that, since Gag p24 is a structural protein it does not show any specific immunological function, while Nef has considerable functions as an inducer or a suppressor and exerts its function by interaction with other proteins and show different effects when simultaneously injected with Tat. These functions have been less identified in the case of Rev and most of the studies on Rev at this point have investigated its role in the transfer of viral RNA; therefore, it is observed that the

influence of Tat and Nef on other proteins is the highest among other HIV-1 proteins. The next rank belongs to Rev, and the effect of Gag is the lowest among them. This should be investigated in more depth and requires more evidence. It is noteworthy to mention that, Tat and Nef also exist outside the nucleus as well as outside the cell, while Rev is mostly located inside the nucleus(6).

Among the results obtained, rAd5-*tat* is an appropriate choice and what has been observed in the current study in most of the groups indicates the stimulating properties of the responses. Also, rAd5-*gag p24* is a suitable choice and its effect is observed in the results of antibody titer and ELISpot results, especially in duplicate groups where the role of proteins can be better studied. Simultaneous injection of this protein with other proteins has increased the response. Even it has been shown in a study that using replication-incompetent Ad-SIV-*gag* alone induces a strong response in immunized monkeys and it results in protection and viremia control (16). Accordingly as was shown in the current investigation, it seems that one of the best vectors for induction of cellular immune response by Gag is the rAd along with this protein; this has been confirmed by other vectors in other studies (37). Additionally in a clinical trial pertaining to Merck it has been shown that using rAd5-*gag* induced an appropriate cellular response in 82% of the patients who had received the vaccine (38). The fact that Gag p24 has not induced a much superior immunological response in our study may be due to its lower expression and that it is not an optimized codon, while sequences of *rev* and *nef* are more stable in comparison with that of *gag p24*.

Regarding the agreement of responses, antibody titer and cytokine response are in concordance in some groups. For instance, in the group of response against Tat, the presence of Nef has led to a decrease in antibody titer (to the lowest titer) as well as a reduction in IFN- γ production (to the lowest number of spots). In general, in duplicates the presence of Nef has decreased Tat response and also reduced Rev response, however, it has not decreased Gag response. Considering that suppressing role has been identified for most of non-structural HIV proteins (39) and all these proteins have numerous tasks maybe it can be stated that these proteins function in favor of disrupting any natural function in host's body against HIV-1. This function sometimes leads to an increase in parameters of immune system regarding the environmental conditions, while in some cases it results in a decrease and the suppression of these parameters; therefore, numerous contradictory results based on the function of these proteins are observed. This hypothesis requires specific evidence which should be scrutinized more in an appropriate host.

Another observation of significance is that in groups 10 to 14, the increase of immunogens has not resulted in a considerable decrease in immunological reactions. In none of the mice which had received the four vaccines the immunological response has reduced compared to the main gene. This evidence indicates the feasibility of using multiple vaccine combinations and is currently under the great attention of researchers.

What is shown in the present study is that, the combination of *gag-tat* along with Ad generates a proper humoral and strong cellular response. Combination of 3 or 4 immunogens are not usually suggested since they contain genes which, due to their interaction caused by unknown reasons, may decrease some of the responses. We can only claim that a combination like *gag-tat* can be useful in comparison with other combinations. While our study demonstrates that the co-immunization with these four genes could result in generation of proper and specific immune responses.

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