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HIV-1 Gag p24-Nef fusion peptide induces cellular and humoral immune response in a mouse model

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Summary. – Many Human immunodeficiency virus (HIV) candidate vaccines have been tested in clinical trials, but none was sufficiently effective in the prevention of HIV infection. A HIV vaccine should induce humoral as well as cell-mediated response, the latter including the cytotoxic CD8+ T lymphocyte (CTL) response. In this study, we immunized BALB/c mice with a purified fusion peptide Gag p24-Nef and evaluated immune responses. As for the cellular responses, the adjuvanted fusion peptide induced lymphocyte proliferation, CTL response, and cytokines IFN-γ and IL-4 in the Th1 pattern. Humoral immune response to the adjuvanted fusion peptide included an increase in IgG antibodies of more IgG2a than IgG1 subtype. These results indicate that the employed HIV-1 peptide construct can elicit both cellular and humoral immune responses in mice. Further studies aimed at memory T cells and other aspects of immune responses are needed before a comprehensive assessment of this candidate vaccine could be provided.

Keywords: epitopes; fusion peptide; HIV-1 p24-Nef; immune response

Introduction

Many candidate vaccines have been tested against HIV-1 infection, but a potent and effective vaccine still remains elusive (Stambas *et al*., 2005). An effective vaccine against HIV-1 infection has to stimulate both cellular and humoral immune responses (Tritel *et al*., 2003). Many studies revealed that the specific T cell response is very important in the controlling of the HIV-1 viral infection and disease progression. Therefore, a design and development of the vaccine against HIV-1 that induces a strong CTL response could support the protection against the disease (Barouch *et al*., 2002). In fact, an effective vaccine against HIV-1 infection should induce strong CTL responses in order to control the infection and also elicit humoral immune response to clear up circulating viral particles (Rinaldo *et al*., 1995; Betts *et al*., 1999; Tritel *et al*., 2003).

HIV-1 positive patients show a strong CTL response that correlates with the decreased plasma viral load (Allen *et al*., 2000). In general, it is believed that a broadening of the CTL immune response against HIV-1 infection increases the vaccine efficacy. For this purpose, construction of the vaccine with different immunogenic epitopes derived from HIV-1 proteins could be valuable and effective in eliciting a protective response and enhancing cellular and humoral immune responses (Bolesta *et al*., 2005).

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Abbreviations: HIV-1 = Human immunodeficiency virus 1; IFN-γ = interferon γ; IL-4 = interleukin 4; CTL = cytotoxic T lymphocyte; SI = stimulation index; LDH = lactate dehydrogenase

Some regions of Gag p24 and Nef proteins of HIV-1 contain conserved epitopes that elicit a strong immune response. Gag p24 contains particular domains on its protein molecule that hold mostly conserved CTL epitopes (Bolesta *et al.*, 2005). A number of studies have confirmed that the strong CTL responses against Gag p24 may control HIV-1 infection and prevent disease progression (Zuniga *et al.*, 2006). Moreover, analysis of HIV-1 Nef sequences revealed that the sequences with highly conserved and immunogenic epitopes could stimulate immune responses (Addo *et al.*, 2003). Nef is a small regulatory protein, located in the cytoplasm and expressed in the early phase of HIV-1 replication. It is essential for efficient HIV replication and pathogenesis (Liang *et al.*, 2002). Nef is an important target of the immune response in HIV-1 infected patients and also later throughout the stages of AIDS, it is recognized by the immune system which responds to it (Addo *et al.*, 2003)

In this work, we report the construction of a HIV-1 p24-Nef fusion peptide and its use as a vaccine for mice, in which we analyzed the cellular and humoral immune responses, namely lymphocyte proliferation, CTL response, cytokines IFN-γ and IL-4, IgG antibodies, and their isotypes.

Materials and Methods

Fusion peptide. p24-Nef fusion peptide, originated from Gag p24 ($aa_{159–173}$) and Nef ($aa_{102–117}$) proteins, was synthesized according to the solid-phase method by GL Biochem Company (Shanghai, China). Purification of the peptide was performed using HPLC to obtain a peptide with the purity exceeding 95%.

Mice. Six- to eight-week-old female inbred BALB/c mice were purchased from the Pasteur institute (Karaj, Iran) and were housed for one week before the experiment. All animal procedures were performed according to the approved protocols.

Immunization. Mice were divided into four groups containing four mice each. The first group of mice was immunized subcutaneously on day 0 with 20 μg of p24-Nef fusion peptide mixed with equal volume of complete Freund's adjuvant and on day 28 with 20 μg of p24-Nef fusion peptide mixed with incomplete Freund's adjuvant in a total volume of 200 μl. The second group of mice was injected with peptide without adjuvant and the third and fourth groups were injected with adjuvant or PBS, respectively. On day 42, the blood samples were collected by cardiac puncture and the immune sera were stored at -20°C until use.

Lymphocyte proliferation assay. At the time of blood collection, spleens from the immunized mice were removed under sterile conditions and suspended in PBS containing 2% FBS. Red blood cells were lysed and single-cell suspension was adjusted to 4×10^6 cells/ml in RPMI 1640 (Gibco BRL) supplemented with 5% FBS, 4 mmol/l L-glutamine and 25 mmol/l HEPES, 0.1 mmol/l non-essential amino acids, 1 mmol/l sodium pyruvate, streptomycin (100 μg/ml) and penicillin (100 IU/ml) and 100 μl of the cell suspension was cultivated in 96-well plate with 2 μg of peptide for 72 hrs. Phytohemagglutinin-A (5 μg/ml; Gibco) was used as a positive control. Then, the cell proliferation was measured by adding 20 μl of methabenzthiauron (MTT) to each well and the plates were further incubated at 37°C for 4 hrs. After incubation, the plates were centrifuged and the supernatant was aspirated carefully and formazan crystals were solubilized by adding 100 μl of dimethyl sulfoxide into each well. The $A_{540/620}$ of each well was determined. Stimulation Index (SI) was calculated according to the formula: SI = $A_{540/620}$ of the wells stimulated with antigen/$A_{540/620}$ of the wells containing only the cells with medium.

ELISA of cytokines. Two weeks after the second injection, a total number of 4×10^6 spleen cells were plated on each well of 24-well plate using complete RPMI 1640 and stimulated *in vitro* with 10 μg/ml of p24-Nef fusion peptide and incubated at 37°C in 5% CO_2. 72 hrs post stimulation, supernatants were removed and the concentration of IFN-γ and IL-4 was estimated by ELISA Kit (Quantikine, R&D Systems) according to the manufacturer's instruction.

CTL activity assay. Splenocyte suspensions from immunized mice were prepared as the effector cells and mouse mastocytoma P815 (H-2d) cells as the target cells. The target cells were pulsed overnight with 20 μg/ml of p24-Nef fusion peptide. Target cells in amount 20,000 cells/100 μl /well were incubated with 100 μl of effector cells at a ratio of 50/1 or 100/1 effector/target (E/T) for 4 hrs in RPMI 1640 containing 2% BSA at 37°C and 5% CO_2. After centrifugation, 100 μl supernatants of each well were transferred to the 96-well flat-bottom plates, and the cytotoxicity was determined by assaying a lactate dehydrogenase (LDH) release (Takara) measured at $A_{492/620}$. Specific lysis was calculated by the formula: Specific cytolysis (%) = [optical density (OD) of experimental LDH release – OD of spontaneous LDH release of effector cells – OD of spontaneous LDH release from target cells]/(maximum LDH release of target cells – OD of spontaneous LDH release of target cells) × 100%. All determinations were performed in triplicates. Maximum lysis was determined from supernatants of cells that were lysed by 1% Triton X-100 and spontaneous release was determined from target cells incubated with RPMI 1640 containing 2% BSA only.

ELISA of antibodies and their isotypes. The presence of specific antibodies in immune sera was determined by ELISA. After completing the standard protocol (Jamali *et al.*, 2009) color density was measured at $A_{450/600}$ nm with ELISA plate reader. For detection of specific IgG1 and IgG2a subclasses, HRP conjugated goat anti mouse IgG1 and IgG2a secondary antibodies (Sigma) were used.

Statistical analysis. All data expressed as means ± SD are representative of two to four different experiments. All the statistical analyses were analyzed by one-way ANOVA followed by Tukey's test. In all the cases, the differences showing $P \leq 0.05$ were considered as significant.

Results

Lymphocyte proliferation assay

In this study, a lymphocyte proliferation was evaluated using MTT assay. Immunization of mice with the adjuvanted fusion peptide (Table 1) increased the lymphocyte

proliferation as compared to the other groups (P <0.002). Immunization of mice with the non-adjuvanted fusion peptide increased the proliferation responses also, but significantly enough only in comparison with control groups 3 and 4. There was no significant difference between control groups 3 and 4 (the mice immunized with adjuvant and PBS, respectively).

ELISA of cytokines

After cultivation of splenocytes and re-stimulation with peptide *in vitro,* the supernatants were assessed for the presence of IL-4 and IFN-γ. As shown in Table 1, vaccination with the adjuvanted fusion peptide significantly increased IFN-γ/IL-4 ratio as compared to the other groups (P <0.0001). Also, immunization of mice with the non-adjuvanted fusion peptide considerably increased IFN-γ/IL-4 ratio (P <0.05), when compared to the control groups. There was no significant difference between control groups 3 and 4.

CTL activity assay

Following immunization, specific cytotoxicity against p24-Nef fusion peptide was done using LDH release assay. CTL activity was significantly increased in mice vaccinated with the adjuvanted fusion peptide compared to all other groups (P <0.001). Immunization of mice with the non-adjuvanted fusion peptide in effector/target ratio of 100, considerably increased the cytotoxicity response (P <0.05), when compared to the control groups 3 and 4. There was no significant difference observed between control groups (Table 1).

ELISA of antibodies

Specific antibodies against p24-Nef fusion peptide in immune sera were determined by ELISA. Immunization of mice with the adjuvanted fusion peptide significantly (P <0.0001) enhanced a specific IgG response against fusion peptide as compared to the rest of groups (Table 1). Immunization of mice with the non-adjuvanted fusion peptide considerably (P <0.05) enhanced specific IgG response against fusion peptide compared to the control groups 3 and 4. However, there was no significant difference between control groups.

Antibody isotyping

We used ELISA for the determination of the specific isotype of IgG antibody against p24-Nef fusion peptide. Assessment of specific IgG1 and IgG2a in individual experimental groups revealed that immunization of mice with the adjuvanted p24-Nef fusion peptide significantly increased titers of IgG1 and IgG2a antibodies (P <0.001) as compared to the other groups. In this experimental group, IgG2a was raised more than IgG1, what indicated that the adjuvanted fusion peptide induced a Th1 type response (Table 1). There was no significant difference between isotype response elicited in mice with the non-adjuvanted fusion peptide and the control groups 3 and 4. Again, there was no significant difference in the specific isotype between control groups.

Discussion

Basic studies (Pal *et al.*, 2005) and results of human clinical trials (Calarota *et al.*, 2001) have indicated that broadening of the immune response against HIV-1 may increase vaccine efficacy. Therefore, the use of conserved and immunogenic sequences in multi-epitopic vaccine may be conducive to achieve an effective vaccine (Bolesta *et al.*, 2005). In this study we analyzed the immune response against a fusion peptide constructed from the majority of immunogenic and conserved sequences of

Table 1. Cellular and humoral response against the adjuvanted and non-adjuvanted p24-Nef fusion peptide

Assay	Adjuvanted p24-Nef	Non-adjuvanted p24-Nef	Controls	
			Adjuvant	PBS
Lymphocyte proliferation (SI)	2.47 ± 0.475	1.38 ± 0.208	0.978 ± 0.181	0.982 ± 0.187
IFN-γ/IL-4	8.1 ± 1.4	3.0 ± 0.5	1.21 ± 0.34	1.33 ± 0.21
CTL activity (%)				
E/T = 100	43.8 ± 5.51	25 ± 6.51	15.4 ± 2.26	14.12 ± 3.88
E/T = 50	24.03 ± 6.7	9.0 ± 2.1	5.7 ± 1.79	6.3 ± 1.89
Antibodies (A_{450})				
Total-IgG	0.618 ± 0.0328	0.21 ± 0.02	0.114 ± 0.035	0.073 ± 0.017
IgG2a	0.315 ± 0.037	0.12 ± 0.0047	0.107 ± 0.0036	0.0985 ± 0.0051
IgG1	0.268 ± 0.044	0.114 ± 0.014	0.1097 ± 0.0082	0.11 ± 0.009

HIV-1 proteins (Currier *et al.*, 2006). Studies directed at these sequences revealed a good deal of flexibility to bind a wide range of major histocompatibility complex (MHC) molecules in humans, mice, and non-human primates (Addo *et al.*, 2003; Thakar *et al.*, 2005; Currier *et al.*, 2006). In the present study, the p24-Nef fusion peptide was used as an immunogen. Analysis of the immune response produced against each of these proteins alone showed, that they could stimulate both humoral and cellular immune responses (Couillin *et al.*, 2001; Coleman *et al.*, 2005; Thakar *et al.*, 2005). This is the first work that employed sequences of Gag p24 and Nef proteins in a fusion peptide. Nevertheless, other works used Gag p24 and Nef in conjunction with other epitopes (Gomez *et al.*, 2007; Gudmundsdotter *et al.*, 2008).

In this study, evaluation of the immune response against adjuvanted p24-Nef fusion peptide showed a significant increase in lymphocyte proliferation response as compared to the other immunization groups. This increase indicated that candidate vaccine could successfully induce cellular immune response. In addition, CTL activity of mice vaccinated with the adjuvanted fusion peptide significantly increased as compared to the other groups. Considering the important role of CTL response in HIV-1 infection, (Hamajima *et al.*, 1997; Spearman *et al.*, 2009) the findings of this work pointed to the potential significance of this construct as a HIV-1 vaccine candidate. Studies of the natural infection in HIV-1 positive patients and Simian immunodeficiency virus (SIV) infection in non-human primate model suggested that CD8+ T cell response had a protective effect in HIV-1 infection and depletion of this population during SIV infection of macaques resulted in the increase of viral load (Couillin *et al.*, 2001; Bolesta *et al.*, 2005; Thakar *et al.*, 2005).

The shift of the cytokine pattern is also an important factor in the induction of a protective response to HIV-1 infection. Cytokine profile analysis of this candidate vaccine showed that the adjuvanted fusion peptide induced an eightfold increase in the IFN-γ/IL-4 ratio leading to a Th1 cytokine pattern compared to the other groups. The shift to a Th1 immune response plays an important role in viral infections such as HIV-1 and this profile facilitates the induction of cellular immunity against viral pathogens (Cristillo *et al.*, 2006; Pajot *et al.*, 2007; Koopman *et al.*, 2008). Humoral immune response and antibody subtypes specific to p24-Nef fusion peptide were also examined. The results indicated that immunization of mice with the adjuvanted fusion peptide induced specific IgG and shifted humoral immune response to IgG2a isotype. IgG1 is a Th2 antibody and IgG2a is a Th1 antibody marker (Jamali *et al.*, 2009). It was observed that the ratio of IgG2a/IgG1 increased in the adjuvanted fusion peptide immunization group, what confirmed the Th1 profile of immune response in this group. Taken together, results of this work demonstrated that the candidate vaccine conferred a strong immunogenicity and induced both cellular and humoral immune responses. The results also showed that immunization with the non-adjuvanted fusion peptide conferred undetectable or weak immune response, what generally confirmed important role of adjuvants in the vaccinations.

In conclusion, this work showed that p24-Nef fusion peptide has a significant role in the induction of the immune response in a non-target species. Further studies considering the cellular aspects of the response including memory T cell response and effect of adjuvants are necessary to pursue the evaluation of this novel candidate vaccine.

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