Immunological Response Analysis of BALB/c Mice to Recombinant gp41-p24 Fusion Protein from Human Immunodeficiency Virus Type 1 (HIV-1)

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Abstract—Some of the human immunodeficiency virus (HIV) proteins such as pg41 and p24 have shown can induce immunological response in animal models. A fusion recombinant protein gp41-p24 was evaluated the immunological response of this protein in mouse to find out the capability of considering as a vaccine candidate. A prime-boost method of immunization with recombinant protein gp41- p24 in Monophosphoryl lipid A and synthetic Trehalose Dicorynomycolate (MPL+TDM) adjuvant in mice was performed. The recombinant fusion protein was refolded to confirm to show the secondary structure using circular dichroism (CD) spectropolarimeter. The study shows that α-helix and β-chain structures of refolded protein, and random coil and turn structures of unfolded protein have increased significantly. The immunological response of this protein was evaluated in mice using antibody and cytokine titer. In this study we have shown that immunization with gp41-p24 in MPL+TDM adjuvant with novel formulations enhance immune responses after 2 weeks. There was significant difference between antibody response (after booster 2), lymphocyte proliferation, secreted IL-2 (after booster 1) and IFN-γ (after prime injection) with antigen or adjuvant alone.

Keywords— gp41-p24, Refolded, MPL+TDM

I. INTRODUCTION

ACQUIRED Immune Deficiency Syndrome (AIDS), caused by infection with the human immunodeficiency virus typ-1 (HIV-1), is a large and growing problem worldwide causing enormous morbidity and mortality [1]. The HIV-1 pandemic has grown to become one of the major infection disease threats to human health and social stability that world has ever encountered [2, 15]. The basic genes of the virus consist of gag, pol and env; the trans-membrane protein gp41 of HIV-1 is the most important antigen for sensitive and specific detection of anti-HIV antibody [16]. Effective anti-retroviral therapy has attenuated the expansion of the epidemic in some industrialized countries. Promising studies of candidate vaccines in animal models have provided reasons to hope that developing a safe and effective AIDS vaccine will be possible [2]. Development of a safe and effective vaccine against HIV-1 is the focus of worldwide efforts. Numerous vaccine modalities have been tested in animal models, including soluble recombinant protein [3]. The majority of vaccine candidates evaluated have included some from of the HIV-1 envelope glycoprotein [11, 12]. Beside, adjuvants have been shown to augment cellular and humoral responses to protein antigens [4, 8, 13]. Polylactide co-glycolide polymer (PLG) micro particles with entrapped gp120 have also been shown to induce Cytotoxic T Lymphocyte (CTL) activity in mice [6]. PLG micro particles and MF59 combination induced CTL activity against HIV-1 p24 gag. In baboons, the adjuvant combination significantly induced enhanced antibody titers after a single dose of gp120, but the responses were comparable with gp120 and p24 Antigens alone [1].

Recombinant antigen comprising both gag and env-coded proteins conjugated with immunomodulator polyoxodionium and the ability to elicit immune response in animals was evaluated. Animals have reported to develop strong humoral immune response to both p24 and gp41 parts of chimerical antigen [7]. Recombinant protein V3-gp41 in liposome with adjuvant MA729 gave the best antibody response [8]. Investigated the immunogenicity in mice of gp41HA, a recombinant protein containing the ectodomain of gp41 from HIV-1. Envelope glycoprotein gp41 fused to a fragment of the influenza virus HA2 hemaglutinin protein in systemic adjuvant N-acetylmuramyl-L-alanyl-D-isoglutamin with method prime/mucosal boost that induce strong humoral immune response [9, 10]. It has become increasingly clear that an effective HIV-1 vaccine will be required to induce broad immune responses, including both humoral and cell mediated immunity [1, 5]. In this study, we tried to evaluated the induce immune response in mice of a recombinant fusion protein gp41-p24 (HIV-1) consisting of the two immunologic part of HIV-1 gp41 and p24 sequences.

II. METHOD

A. Preparation of recombinant fusion protein gp41-p24

Recombinant fusion protein gp41-p24, as antigen, had been produced by cloning methods [16] To refold the recombinant fusion protein gp41-p24, the dilution method was
utilized as described previously Briefly, an appropriate volume of Hepes buffer (pH 7.0) was added gradually to the protein for reducing urea from 8 to 1 M. Consequently, the protein was dialyzed for removal of urea 1 M [16, 9]. Refolded protein was tested by electrophoresis with %12 native gel and investigation of secondary structure with circular dichroism (CD) spectropolarimeter, Jasco-810, cell length 1mm, scanning speed 200nm/min, measurement range 350-190 nm, band width 1nm [30]. Sample concentrations of refolded and unfolded protein, were determined by Bradford method using Coomassie brilliant blue G-250 (Merck, Darmsted, Germany) as previously reported (Bradford, 1976). The molecular weights of proteins were determined by electrophoresis with 12% polyacrylamid gradient gels. The immune reaction of refolded protein was evaluated by ELISA and Western blot methods [9, 16].

B. Evaluation of fusion protein gp41-p24 by ELISA

The specificity and sensitivity of the proteins were evaluated by ELISA technique [29, 16]. First, the antigens such as refolded protein, unfolded protein and standard protein of gp41-p24 from Human Immunodeficiency Virus type 1 were coated in well plate (Greiner, Frickenhausen, Germany) with 10 µg/ml [23]. Incubated at 37°C for 2 h. The plates were washed with PBS buffer containing 0.05% Tween 20 (PBS-T) and blocked with 1% BAS in PBS buffer for 2 h at 37°C. Serum of HIV-1 positive samples and negative samples (diluted 1:100) were added to the wells (100µl per well) and the plates incubated 1h at 37° C [16]. The wells were washed five times with the wash buffer (50 mM Tris, pH 8.0; 100 mM NaCl; 0.2% Tween 20). Goat anti-human IgG antibody conjugated to Horse-radish peroxidase (Calbiochem, San Diego, CA) was diluted 1:10,000, added to each well and the strips were incubated at 37°C for 1 h. After thorough washing, 100 µl of substrate solution (0.1 M citrate monohydrate, 0.2 M disodium hydrogen phosphate, 1 mg/ml OPD and 0.3% hydrogen peroxide) was added to each well and the strips were incubated in dark at room temperature for 15 min. Enzyme reaction was stopped by adding 100 µl of 2 N H2SO4 to each well. The absorbance was recorded at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA) [30].

C. Western blot analysis of fusion protein gp41-p24

The refolded protein gp41-p24 and standard protein gp41-p24 were electrophoretically separated as indicated above and then transferred to nitrocellulose paper (Bio- Rad, Hercules, Calif ) in Tris-HCL- glycine- methanol buffer for 1 h. The membrane was blocked with 1% skim milk in PBS for 1 h in room temperature, then overnight with dilution serum (1:100 in TBST buffer). Strips were washed and incubated with a secondary antibody conjugated to horseradish peroxidase. The blot was developed by incubating the membrane in the substrate solution containing 3µl/ml H2O2 and 0.5 mg/ml 3,3’-diaminobenzidine tetrahydrochloride (Sigma) in PBS [30, 9].

D. Animals and immunization protocols

Female BALB/c (H-2d) mice 6-8 weeks of age were purchased from Pasteur Institute of Iran, Tehran, Iran, and were housed in the animal facility on standard rodent diet and allowed to acclimate for at least 1 week prior to this study. All experiments involving mice were done under strict compliance with the guidelines established by Pasteur institute of Iran. There were one case group and three control groups of mice in this study. On day 0, mice in the case group (n=30) was primed by a single intramuscular (IM) injection with 40µg recombinant protein gp41-p24 per mouse in adjuvant MPL+TDM (Sigma). On day 0, mice in control 1 group (n=30) was primed by a single IM injection with 40 µg recombinant protein gp41-p24 per mouse alone. On day 0, mice in the control 2 group (n=30) was primed by a single IM injection with 40 µg recombinant protein gp41-p24 per mouse alone. On day 0, mice in the control 3 group (n=30) was primed by a single IM injection with Hepeste 20 mM. Case group (n=10), control 1 (n=10), control 2 (n=10) and control 3 (n=10) were single boosted with the same dose given IM injection on day 14. Also, the case group (n=5), control 1 (n=5), control 2 (n=5) and control 3 (n=5) were two boosted with the same does given IM injection on day 28 [1, 9]. In total, there were six groups of mice in the study.

E. Determination of antibody responses after immunization

Micro plates (Fisher) were coated using coating buffer to gather with 10 µg/ml [23] of recombinant protein gp41-p24 and incubated overnight at 4° C. The plates were washed with PBS buffer containing 0.05% Tween 20 (PBS-T) and blocked with 1% BAS in PBS buffer for 2 h at 37°C. Serum of HIV-1 infected mice was added to the wells (100µl per well) and the plates incubated overnight at 4° C. Anti-mouse IgG (Sigma) diluted to 1:20,000 in PBS buffer containing 0.5% BSA was added (100 µl per well), and incubated for 2h at 37°C. The wells washed with PBS-T and incubated with TMB reagent (Sigma) for 15 min at room temperature. The reaction was terminated with 2 N H2SO4 and the plates were measured for absorbance at 405 nm [17].

F. Lymphocyte proliferation assay

Lymphocytes were isolated from spleens (of mice) by passing tissues through a sterile mesh. Cells were suspended in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, Gibco-BRL, Gaithersburg, MD), penicillin (100 U/ml), streptomycin (100 mg/ml), and 2 mM L-glutamine and 10 mM 2-mercaptopoethanol. Erythrocytes in spleen cell preparation were lysed with 0.1 M ammonium chloride. Splenocytes were cultured in triplicate (10^6 cells/well) in 96-well flat-bottom microtiter plates. Stimulated with purified recombinant protein gp41-p24 (12µg/ml) or concanaavalin A (1 µg/ml; Sigma), or medium alone and incubated at 37°C under 5% CO2 [25]. Supernatants were collected after 72-h of culture and tested by ELISA for IFN-γ and IL-2 with commercial anti-cytokine antibody pair (R&D Systems, Minneapolis, MN) as described in the protocols provided by the manufacturer. Alternatively, after 5 days, the culrures were pulsed with 1µCi of [3H] thymidine (5 ci/mmol; Amersham, Arlington Heights, Ill). For 18h and harvested and
cell proliferation was evaluated by liquid scintillation counting. Results are expressed as mean counts per minute (cpm) of stimulated less that of medium control cell cultures (Acpm), and stimulation index (SI, the ratio of the mean counts per minute of antigen-stimulated to that of medium control cell cultures. [20, 25].

G. Determination of cytokine levels (IL-2 and IFN-γ) by ELISA after immunization

Cytokine levels in culture supernatants were determined by ELISA assay. We used detection kit (R&D Systems, Minneapolis, MN,) for assaying cytokine levels IL-2 and IFN-γ [18]. Briefly, 96-well flat-bottom plates were coated with 100 μl of anti-cytokine antibodies diluted in buffer, as specified by the manufacturer, and incubated overnight at 4-C. The wells were washed with PBS plus 0.05% Tween 20 (PBS-T) and blacked at 37°C for 2h with PBS 1% bovine serum albumin. Serial two fold dilution of supernatants appropriate dilutions of standard cytokines were added in duplicate and incubated at 37°C for 1 to 2 h. The wells were washed with PBS-T and incubated with the specific biotinylated anti-cytokine antibody diluted in PBS-T plus 1% BSA for 1 to 2h. After 3 or 4 washes, the wells were incubated with horseradish peroxides-conjugated streptavidin at 37 o C for 15 min and developed with TMB reagent (Sigma). The reaction was terminated with 2N H2SO4 and the absorbance values were measured at 450 nm [8, 25].

H. Statistics

Statistical analyses were performed with Sigma Stat, SPSS 12, 2005 and Prism 2, 2003. Data for lesion progressions were analyzed with analysis of variance (ANOVA) for group comparison and t tests for other analyses. A P <0.005 was considered significant.

III. RESULTS

A. Preparation recombinant protein gp41-p24

In this study, we refolded the protein successfully and observed protein refolding by native gel. The results show that molecular weight of these proteins was 32 KD MW with purity more than 98% (data not shown) Using spectrophotometer and Bradford test, it was shown that the concentration of this protein was 3.2 mg/ml. The analysis by CD spectropolarmetere determined that α-helix and β-chain structure of refolded protein was more than unfolded protein and also random coil and turn structures of refolded protein were less than unfolded protein.

To make sure of refolding process, the recombinant gp41-p24 fusion proteins in the form of refolded and unfolded was coated in the microtiter ELISA plate and tested against human HIV positive sera. It is shown that the immune reaction in the presence of refolded form is significantly higher than unfolded form. The results showed that with p<0. 05 there is no significant difference between standard protein and refolded protein but indicated that with p<0. 05 there is significant difference between standard and unfolded protein.

B. Antibody secretion after immunization

The average level of antibody production against recombinant protein gp41-p24 was measured by ELISA method in different groups of BALA/c mice was estimated a with 95% confidence interval after 2 and 4 weeks. It is shown that the difference of average level of antibody secretion in mice by ELISA method after 2 weeks injection was not significant compared with 4 weeks injection. This means that the maximum secretion level of antibody after second boosters in 2 weeks was similar to 4 weeks in the control group. Therefore, our results were based on the 2 weeks study groups. In all groups, the maximum level of antibody secretion was observed after receiving the secondary booster (p<0.05). On the other hand, the antibody secretion was the highest in the test group followed by control 2 group. Control 1 and control 3 had the minimum level of antibody secretion. The test group (Injected with recombinant fusion protein gp41-p24+MPL+TDM), Control group 1 (Injected with MPL+TDM), Control group 2 (Injected with recombin fusion protein gp41-p24) and Control group 3 (Injected with Hapes 20 mM).

C. Proliferation of cells after immunization

The cell proliferation assay was performed in all groups of BALB/c mice as mentioned in materials and methods after 2 and 4 weeks. Our results also indicated that the difference of average level of cell proliferation in mice by LTT method after performing 2 weeks time assay was not significant with after 4 weeks time. It means that the maximum level of cell proliferation can be seen in 2 weeks after injection and with single booster. In all groups, the maximum level of cell proliferation was assayed after single boost after 2 weeks of injection (p<0.05).

The proliferation of total cells from spleen showed the highest in the test group that received recombinant fusion protein with MPL and TDM. It was shown that the cell proliferation also is high after the first boost as well as the second boost but significantly difference with those that were injected only once.

The second group, control 2, that were injected with recombinant fusion protein gp41-p24 alone in two or three times also showed high proliferation with significant difference with those that were not received any recombinant proteins, control 1 and 3, the group that received only.

D. Cytokines secretion (IL-2) after immunization

The average level of IL-2 secretion after injection with recombinant protein gp41-p24 in different groups of BALB/c mice (p<0.05) after 2 and 4 weeks was calculated. The results showed that the maximum level of IL-2 secreted was in 2 weeks after injection with single booster. Also the maximum level of IL-2 secreted was in test group followed by control 2. The minimum level was observed in control 1 and control 3.

E. Cytokines secretion (IFN-γ) after immunization

The average level of IFN-γ secretion after immunization with recombinant protein gp41-p24 with adjuvants and without in different groups of BALB/c mice (p<0.05) after 2
and 4 weeks after injection was calculated. The results showed that the maximum level of IFN-γ secretion was in the test group and then in the control 2, while there was no production in the control 1 and control 2 groups. On the other hand, it is important to notice that the maximum level of IFN-γ secretion was obtained after the first injection.

IV. DISCUSSION

The HIV recombinant protein can be as an immunogenic and inducer of humoral and cellular immune systems [9, 11, 14, 17]. In several studies, it has been described that HIV recombinant fusion proteins, specially ectodomain of gp41 from HIV-1 in different adjuvants with different doses and proper time using prime/boost method approved for use in mice induced potent immune responses (both cellular and humoral) in animals [1, 8, 9].

Results from the ELISA and western blot assays showed that refolded protein gp41-p24 before immunization of mice had immune reaction ability with the antibody using the serum of HIV positive control; different research groups obtained similar results [9, 16]. The analysis by CD spectropolarimetre determined that α-helix and β-chain structure of refolded protein gp41-p24 was more than in the unfolded protein gp41-p24 and also random coil and turn structures of refolded protein was less than in the unfolded protein [30]. This study examined recombinant fusion protein gp41-p24 containing two immunogen parts of HIV-1, gp41 and p24 sequences in MPL+TDM adjuvant that induced potent antibody responses and secretion of the Th1 cytokine (IL-2& IFN-γ) after immunization in mice [18, 19, 20].

The work described here highlights the potential of MPL+TDM as an adjuvant for a recombinant protein gp41-p24 vaccine against HIV-1. Adjuvant with entrapped recombinant protein induced potent antibody responses in mice [9, 21, 22]. The strong antibody responses were induced with recombinant protein gp41-p24 in 2 weeks after injection and with second booster immunization. Similar results obtained for 4 weeks after injection. Therefore, it was an important observation that the boosters to increase humoral immune system had an important role, as described previously [9, 24, 25]. Results from the LTT assay of the lymphocyte proliferation responses showed induced high levels of lymphocyte proliferation respectively in groups that receive recombinant protein gp41-p24 in MPL+TDM adjuvant was more in groups that receive only gp41-p24, as described previously for other antigens [8]. So the proliferation in 2 weeks after injection with booster immunization had similar result to 4 weeks after injection.

Results from the ELISA assay of IFN-γ secreting cells showed high level of IFN-γ production in response to HIV recombinant protein after injection without booster immunization. We found that IFN-γ secretion, as well as antibody production, lymphocyte proliferation and IL-2 secretion had the best production level in two weeks after injection. In addition, secretion of cytokine, IL-2 and IFN-γ, antibody secretion and cell proliferation respectively in groups that receive recombinant protein gp41-p24 in MPL+TDM adjuvant was more in groups that receive only gp41-p24, as described previously for other antigens [18, 26, 27, 28]. These result showed that gp41-p24 with different formulations according to adjuvant type, stage, dose and injection had the ability of induce cellular and humoral immune system [7, 9]. We found that prime/boost method with gp41-p24 induce secretion of the Th1 cytokine (IL-2 and IFN-γ), antibody secretion and cell proliferation in 2 weeks after injection and we need second boosters only for antibody secretion in serum. The main goal of future research to develop this protein (gp41-p24) and some HIV recombinant fusion proteins vaccine would be applying these strategies to humans in such away as to design a safe and efficacious vaccine.

REFERENCES


