

Evaluation of cross immune response in DNA based vaccinated mice against HSV-1 and HSV-2

Research Article

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Abbreviations: Dulbecco's modified eagle medium, (DMEM); fetal calf serum, (FCS); glycoprotein D, (gD); Herpes simplex virus, (HSV); lactate dehydrogenase, (LDH); Cytotoxic T Lymphocyte, (CTL)

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Summary

Herpes simplex viruses are the most widespread human viral infections which are major targets of vaccine development. An effective vaccine for HSV must be stimulating both arms of immune system. DNA immunization with HSV-gD gene has been shown to induce both humoral and cellular immune responses against HSV infections. In the present study the cross immune responses of HSV-gD1 (gD1) or HSV-gD2 (gD2) against two HSV strains were evaluated. Mice were immunized with DNA vaccine containing gD1 or gD2 gene showed considerable responses against HSV-1 and HSV-2 respectively. While gD1 immunized mice showed significant cellular and humoral cross immune responses against HSV-2 strain but, gD2 did not. Due to important role of cellular immune responses against HSV infection, it can be concluded that vaccination with gD1 is more effective compared to gD2 when used as candidate vaccine against HSV infection.

I. Introduction

Herpes simplex virus (HSV) with two closely related serotypes, HSV-1 and HSV-2, is one of the most common infectious agents in human. They cause orolabial or sexually transmitted genital and lifelong latent infections (Roizman and Knipe 2001). The incidence of herpes infections continues to increase in populations, and new strains of HSV viruses become resistant to chemotherapy (Bernstein and Stanberry 1999). Thus, prevention and control of HSV infection on a global basis is needed.

HSV glycoproteins are the major targets of vaccination studies as they are highly immunogenic and able to induce humoral and cellular responses. The glycoprotein D (gD) of HSV is a major envelope protein which also expresses in the membrane of infected cells. It plays an important role in the initial stages of viral infection and induces high titers of virus neutralizing antibodies (Fuller and Lee 1992). This glycoprotein is highly conserved and antigenically cross-reactive in HSV-1 and HSV-2. Several reports have described the use of plasmid DNA_s encoding HSV proteins to evoke a

protective immunity in animal models. DNA-based immunization elicits a significant cellular immune response and cytotoxic T lymphocytes (CTL_s) as well as humoral immunity to the expressed antigens (Manickan et al, 1995; Tengvall et al, 2005; Soleimanjahi et al, 2006).

It has been shown that immunization with gD recombinant protein induces significant protection against clinically apparent genital herpes in women who were seronegative for both HSV-1 and HSV-2 (BenMohamed et al, 2003). Immunization of animals with purified gD1 or gD2 proteins stimulates the production of neutralizing antibodies and a cross-protective immune response to lethal virus challenge. It is, therefore, a logical target for construction of recombinant vaccines against HSV infections (Heber-Katz and Dietzschold 1986). At the amino acid level, gD1 is 85% identical to gD2. They are similar in antigenic structure and thermal stability but vary in secondary structure which is measurably different (Nicola et al, 1996).

In the present study, the cross immune responses were evaluated in DNA vaccinated BALB/c mice that

immunized with gD1 or gD2 using Cytokine and CTL assay as well as antibody titration.

II. Materials and Methods

A. Mice

Six to eight week old inbred female BALB/c mice were obtained from Pasture Institute of Iran (Karaj, IRI). Given free access to food and water, mice were housed for one week before experiments, and maintained in a good standard condition. All experiments were done according to Animal Care and Use Protocol of Tarbiat Modares University.

B. Cell lines and viruses

African green monkey kidney (Vero) cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS) at 37°C, and used for preparation of virus stocks. WEHI 164 and spleen cells were grown in RPMI-1640, supplemented with 10% FCS. The KOS strain of HSV-1 and Iranian isolate of HSV-2 were propagated and subjected to titer determination on Vero cells.

C. Preparation of Recombinant plasmid

DNA fragments containing gD1 or gD2 genes were subcloned into pcDNA3 under the control of the CMV immediate early promoter. Preparation of competent cells, transformation of bacteria, DNA preparation, and electrophoresis in agarose gels were performed according to the standard protocols (Sambrook and Russell 2001). Maxipreparation of the interest clones were done using endotoxin free plasmid isolation kit (Macherey-Nagel) and transfected into COS-7 using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The recombinant proteins expression in mammalian cells were examined by indirect Immuno Fluorescence Test (Fotouhi et al, 2005; Soleimanjahi et al, 2006)

D. Study design

The mice were put in eight groups (10 mice per group). The first and second groups of BALB/c mice were immunized three times intramuscularly with 100 µg of pcDNA3 gD1 per mouse into the left and right quadriceps muscles on days 0, 14, and 21. The other two groups of mice were immunized with 100 µg of pcDNA3 gD2 per mouse in the same manner. The fifth and the sixth groups of mice received 100 µg of the pcDNA3 per mouse as null vector. Finally the two last control groups of mice were inoculated intraperitoneally three times with 100 µl of inoculums containing 10⁵ pfu of HSV-1 or 10⁴ pfu of HSV-2 (sub lethal doses). The serum samples were obtained before and 21 days after the last inoculation and subjected to viral neutralization test against HSV-1 and HSV-2 separately. The splenocytes from these subjects were stimulated in vitro with HSV-1 or HSV-2 and subjected to evaluation of CTL responses.

E. Viral neutralizing test

The mice were bled by retro-orbital puncture 21 days after the final vaccination. The sera were separated, heat inactivated at 56° C for 30 min and stored individually for serological analyses. Serial twofold dilutions of serum samples were prepared in duplicate in DMEM containing 5% FCS and then mixed with 100 TCID₅₀ of HSV viruses separately and incubated for 1 hour at 37° C. One hundred microliter of the incubated mixture were added on monolayer Vero cells and then incubated at 37° C for 3 days. The presence of replicating virus in cells was scored by cytopathic assays. The neutralizing titre was defined as the reciprocal of the highest serum dilution at which no viral plaque was detected. In assay complete cytopathic effect was seen in virus control wells.

F. Cytokine assays

Three weeks after the last immunization, spleens of individual mice were removed aseptically and homogenized in RPMI 1640 (Gibco-BRL) supplemented with 10% FCS and antibiotics. Erythrocyte-depleted spleen cell suspensions were prepared by treatment with potassium carbonate-buffered NH₄Cl solution and plated at a concentration of 2×10⁶ viable cells/ well in 24 well plates. The cells were stimulated with HSV-1 or HSV-2 and incubated in a humidified 5% CO₂ atmosphere. After 48 h later, supernatants were removed and kept at -80 ° C for evaluation of secreted IL-2 level.

The concentration of IL-2 in the supernatants was estimated using ELISA kit (Bender Medsystems), according to the manufacturer's instructions by comparing the optical densities of the unknowns to those of the standards and presented as mean concentration (pg/ml) ± standard error.

G. CTL assay

Three weeks after the last immunization, splenocyte single cell suspensions were prepared without in vitro stimulation. The WEHI 164 target cells were infected with 5 MOI HSV-1(KOS) or HSV-2 for 4 h and washed three times with assay medium. The CTL activity was measured by the lactate dehydrogenase (LDH) release assay in 96-well round-bottom plates. Target cells (2 × 10⁴ cells/well) in a 100-µl volume were incubated with 100 µl of effector cells at various effector/ target ratios for 4 h in phenol red-free RPMI 1640 containing 3% FCS. After centrifugation, the supernatants (50 µl/well) were transferred to the 96-well flat-bottom plates, and lyses of target cells was determined by measuring LDH release using the LDH assay kit (Takara Company) according to the manufacture's instruction. Blank PBS buffer and a 0.1% Triton X100 solution in PBS buffer were used as controls. The LDH-mediated conversion of the tetrazolium salt into red formazan product measured at 490 nm after incubation at room temperature for 30 min. The percentage of specific cytolysis was determined by the following formula:

$$\text{the specific cytolysis (\%)} = \frac{(\text{optical density [OD] of experimental LDH release} - \text{OD of spontaneous LDH release of effector cells} - \text{OD of spontaneous LDH release of target cells})}{(\text{maximum LDH release of target cells} - \text{OD of spontaneous LDH release of target cells})} \times 100\%.$$

All determinations were performed in triplicate.

H. Statistical analysis

The SPSS version 13 was used for statistical analysis. Antibody titer, CTL responses (LDH assay) and IL-2 production were analyzed by one-way analysis of variance (ANOVA). Results were considered to be statistically significant when the P value was less than 0.05.

III. Results

A. Induction of HSV neutralizing antibody in immunized mice

Three weeks after the last immunization, sera were collected from immunized mice, and the neutralizing titers were determined using Virus Neutralization Test (VNT) as shown in **Table 1**. The result showed both gD1 and gD2 based DNA vaccine induce significant level of antibody in comparison with pcDNA3 vector groups. Furthermore the results showed that both gD1 and gD2 could induce high level of cross antibody against HSV-2 or HSV-1 respectively, but the differences are not statistically significant.

B. Cytokine assay

Three weeks after the last immunization, cellular immunity was evaluated by measuring IL-2 levels as an indicator of Th1 cell responses. Following *in vitro* restimulation of splenocytes, a predominant IL-2 secretion was found *in vitro*. As shown in **Figure 1**, KOS or HSV-2 immunized mice showed large amounts of IL-2 secretion. Immunized mice with gD1 and gD2 vaccines showed significant level of IL-2 induction, compared to the mock-

immunized groups ($p < 0.0001$). Furthermore gD1 showed suitable levels of IL-2 production, but no significant differences were shown between restimulation by HSV-1 or HSV-2.

In contrast, supernatants from gD2 group which is restimulated by HSV-1 could not produced significant amount of IL-2 in comparison with restimulation by HSV-2. The lymphocytes from pcDNA3 mock-immunized mice secreted only very small amounts of IL-2.

Table 1. Neutralizing antibody titers in immunized mice

Groups	Against	Neutralizing antibody titer
gD1	HSV-1	64 ± 11.6
gD1	HSV-2	33.6 ± 5.5
gD2	HSV-1	22.4 ± 2.6
gD2	HSV-2	35.2 ± 5.2
HSV-2	HSV-2	268.8 ± 44.5
KOS	HSV-1	288 ± 53.3
pcDNA3	HSV-1	8 ± 1.46
pcDNA3	HSV-2	6.8 ± 1.2

Mice were immunized three times with 100 µg of the DNA containing gD-1/gD2(i.m) or HSV-1/ HSV-2 (i.p) as described in Material and Methods. Ten mice per group were bled 3 weeks after the third immunization and the neutralizing antibody titer was evaluated (ANOVA test). The indicated results represent the average of the titers from 10 serum samples ± standard error.

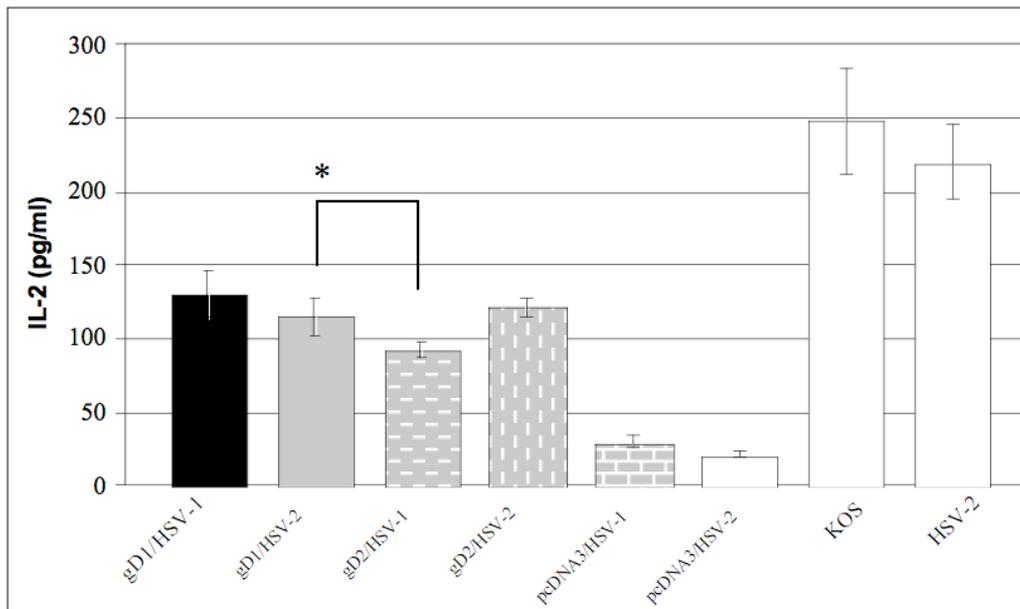


Figure 1. IL-2 production by immunized mice splenocytes. BALB/c mice were immunized three times as described in Materials and Methods. Three weeks after the final immunization, mice were euthanized; spleens from three mice per group were harvested. The single-cell suspensions were prepared and stimulated *in vitro* for 48 h with heat-inactivated HSV-1 strain KOS or HSV-2. The concentrations of IL-2 in the supernatants were measured by mouse IL-2 ELISA Kit. Each bar represents the mean of the titers from three experiments ± standard error (error bar). Mice immunized with gD1 showed suitable levels of IL-2 production but no significant differences were shown between restimulation by HSV-1 or HSV-2. In contrast, supernatants from gD2 group which is restimulated by HSV-1 failed to produce as high amount of IL-2 in comparison with restimulation by HSV-2 (* $P = 0.012$).

C. CTL assay

The CTL response in immunized mice was examined in this study by the lactate dehydrogenase (LDH) release assay in 96 well plates. The mice were immunized three times, and the CTL activity was measured as described in Materials and Methods. In contrast to gD1, gD2 immunized mice showed significant difference CTL activity when exposed to HSV-1 or HSV-2 infected target cells. The pcDNA3 mock-immunized mice did not exhibit any detectable CTL response (**Figure 2**). Based on the results, the cross CTL activities were much stronger in gD1 immunized mice as compared with those in gD2 once.

IV. Discussion

Herpes simplex virus infections are major public health problems worldwide with significant morbidity in genital herpes simplex virus type 1 and 2 (Bettahi et al, 2006). The prevalence of HSV infections has increased in the past two decades in developing countries. The magnitude of the public health problem posed by HSV-2 infection in immunocompromised individuals and failure of antiviral drugs to prevent drug resistant HSV spread, points to a clear need for a safe and effective therapeutic vaccine to elicit virus specific cellular immune responses (Hosken 2005).

During the last decade, numerous vaccines that provide protection against HSV infection have been developed. They have primarily focused on various forms of recombinantly expressed glycoproteins (Nicola et al, 1996). Among the 11 known HSV glycoproteins, HSV glycoproteins D (gD1 and gD2) are the most highly

conserved glycoproteins which have high structural and functional homology (Watson et al, 1982). Several study point to gD being a major target of HSV immune clearance due to its highly conserve and antigenically cross-reactive between HSV-1 and HSV-2 (Nesburn et al, 2005). They would engender cross - protective immune responses that would afford protection against HSV-1 and HSV-2 (Grammer et al, 1990; BenMohamed et al, 2003). Glycoprotein D has emerged as an excellent candidate antigen for inducing a protective immunity in animal models against ocular and genital infections with both types of HSV-1 and HSV-2 (Bourne et al, 2003). DNA vaccine expressing HSV gD is inducer of both humoral and cell mediated immune responses and can provide highly protective responses against disease following virus challenge (Nass et al, 2001). It was shown that antibody may help to prevent the initial infection but is usually insufficient to control and resolve HSV infection and disease. Significant benefit in blocking of HSV infection was achieved when pooled human HSV immunoglobulins was used (Keadle et al, 1997; Dalai et al, 2002). An effective vaccine against HSV should be able to generate a Th1-type T cell response for limiting the progression of disease. A Th1 type of response is important and sufficient to control the progression of infection. Studies by Heber-Katz et al have shown that the T cells which respond to HSV-1 gD₁₋₂₃ do not cross reactive with HSV-2 gD₁₋₂₃ due to the amino acid differences at positions 7 and 21 (Heber-Katz et al, 1985). Ben Mohamed and colleagues showed in 2003 that the epitopes between amino acid 332-358 including V epitope, which induced strong Th1 cytokine, had an important role in secretion of IL-2 and IFN- γ , as

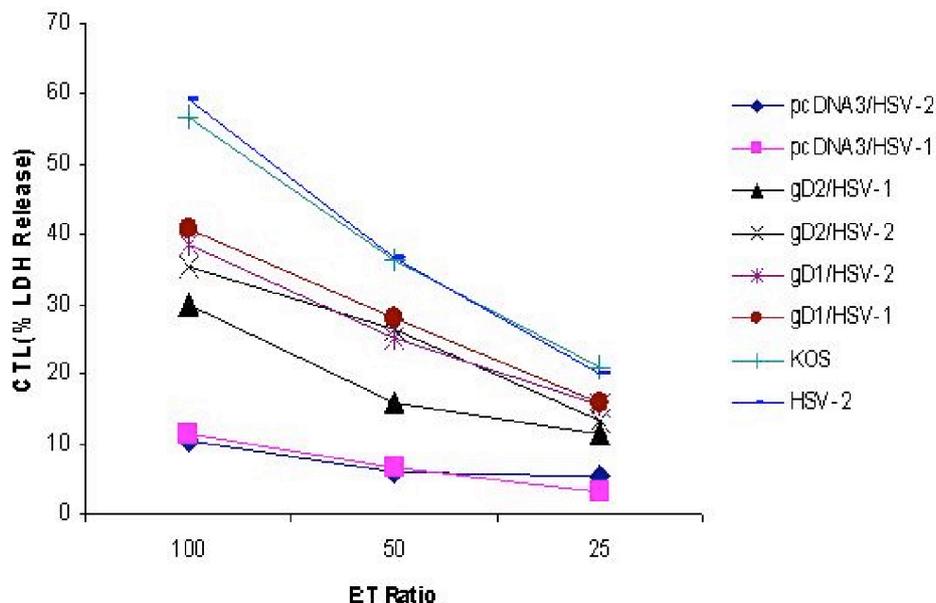


Figure 2. CTL activity in immunized mice. Splenocytes from immunized and mock-immunized mice were prepared as described in Materials and Methods. LDH release assays were performed in triplicate with splenocytes as effector cells and HSV-1(KOS strain) and HSV-2 infected WEHI 164 cells as target cells. Each point represents mean \pm standard error of three experiments. The mice immunized with gD1 and then exposed to HSV-1 or HSV-2 infected target cells not showed significant difference, in comparison with gD2 groups (*P = 0.028 compared with the gD2/HSV-2).

E: T ratio, effector-to-target cell ratio.

indicators of cellular immunity and in restricting HSV replication (Ben Mohamed et al 2003; Goel et al, 2003; Ghaemi et al, 2007).

The published data showed that gD2 vaccines provided cross-protection against disease resulting from genital HSV-1 challenge, and also gD2 has an immunomodulatory effect on HSV-1 infected mice (Bourne et al, 2003). The present study showed that, vaccination with vector containing gD1 significantly induced cellular immune response versus gD2 in challenge experiments using both HSV-1 and HSV-2 ($P < 0.05$). Although DNA vaccine containing gD1 or gD2 could induce humoral and cellular immunity against HSV-1 and HSV-2 respectively, but gD1 based vaccine was able to induce stronger cellular response compared to gD2 against both types of herpes simplex viruses. These results support the idea that the gD1 structure may constitute ability for eliciting a cellular immune response to both HSV-1 and HSV-2 when used as DNA vaccine.

In conclusion, the induction of optimal humoral and CMI responses against HSV infection is a critical determinant in the development of an efficient vaccine. Based on the data obtained in the current study gD1 DNA vaccine can use as a preferred target antigen in HSV vaccine development. It seems that the more effectiveness of gD1 in this study may be due to the existence of its specific epitopes on gD1. Since modulation of T helper subsets as effector cell populations is responsible for protective immunity, therefore, directing immune responses is needed for considerable protection. In this study we considered cross immune responses between gD DNA vaccines and the cross protection of serum from KOS infected mice as a live vaccine against HSV-2 and vice versa should be performed to compared their efficacy to gD DNA vaccine in future. By focusing specifically on high induction of cell-mediated immunity, specific HSV-gD1 based epitopes vaccines could have possible clinical applications against HSV-1 and HSV-2 in the future.

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