Immunogenicity and Protective Effect of a DNA Construct Encoding Certain Neutralizing Epitopes of Herpes Simplex Virus Type-1 Glycoprotein B

(HSV-1 / glycoprotein B gene / neutralizing epitopes / DNA immunization)

T. BAMDAD[1], M. H. ROOSTAEE[1], M. SADEGHIZADEH[2], F. MAHBOUDI[3], A. KAZEMNEJAD[4], H. SOLEIMANJAHI[1]

[1]Department of Virology, School of Medical Sciences, [2]Department of Genetics, School of Basic Sciences, Tarbiat Modarres University, Tehran, Iran

[3]Department of Biotechnology, Pasteur Institute, Tehran, Iran

[4]Department of Biostatistics, School of Medical Sciences, Tarbiat Modarres University, Tehran, Iran

Abstract. Much attention is presently focused on the vaccination with certain epitopes of an antigen. To further study the ability of neutralizing epitopes mapped in the first 1515 nucleotides of glycoprotein B of herpes simplex virus type-1 (gB-1) to induce neutralizing antibodies, a DNA immunization approach was employed. Vaccination of mice with a plasmid expressing the neutralizing epitopes induced humoral immune responses, although the antibody titre was significantly lower than that of antibodies induced by the full-length *gB-1* gene. Furthermore, the plasmid DNA could not protect the mice against HSV-1 lethal challenge, but could significantly prolong the survival time compared to mock-vaccinated group.

Herpes simplex virus type 1 (HSV-1) is responsible for a wide range of human diseases from the localized infection such as orolabial or corneal lesions to life-threatening encephalitis, neonatal disease and pneumonia in the immunocompromised individuals (Hwang et al., 1999; Stanberry et al., 2000; Roizman et al., 2001). Primary infection is every time followed by the establishment of a latent infection in the nervous system, where it can be reactivated, resulting in the reinfection and spreading of the virus among uninfected populations. To control the infection, the development of an effective vaccine that prevents or reduces the primary and recurrent infections would be of great importance.

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Corresponding author: Taravat Bamdad, Department of Virology, School of Medical Sciences, Tarbiat Modarres University, P.O.Box 14115-111, Tehran, Iran. Tel.: +98 21 8011001 ext 3880; Fax: +98 21 8013030; Bamdad_T@modares.ac.ir

Abbreviations: CTL – cytotoxic T lymphocyte(s), gB – glycoprotein B, HSV-1 – herpes simplex virus type 1, LD50 – lethal dose 50%, TCID50 – tissue culture infectious dose 50%.

It seems that DNA vaccination would be a promising approach to achieve an effective immunity against HSV infections (Stanbery et al., 1996; Bernstein et al., 1999). Such vaccines can be tailored to express only those epitopes involved in protection and to direct a selective immunity (Rodrigues et al., 1998; An et al., 2000). It has been shown that CTL activity, antibody and T-helper responses can be induced by appropriate epitopes (Yu et al., 1998). Moreover, DNA vaccination technology, as a discovery tool for the identification of more immunogenic epitopes of an antigen, has facilitated the development of new effective vaccines.

HSV-1 glycoprotein B is essential for infectiveness, virus penetration and cell fusion. It is also one of the main targets for neutralizing immunity (Glorioso et al., 1984). There are many reports indicating that gB-based DNA immunization has provided the protection of animals against the lethal, latent and recurrent HSV infections (Mester et al., 2000; Gaselli et al., 2001; Baghian et al., 2002). Beside the strong CD_4^+ and CD_8^+-related immunity (Manickan et al., 1995; Wallace et al., 1999), gB-1 elicits a considerable amount of neutralizing antibodies against HSV-1 (Sanchez-Pescador et al., 1992). Epitopes for neutralizing antibodies cluster in the three major domains: D1, D2 and D5a (Navarro et al., 1992). In the present study, using a DNA vaccination approach the strength of the epitopes located in the D1 and D2 domains to induce neutralizing antibodies and protect mice against HSV-1 lethal challenge has been investigated. Mammalian expression vectors carrying the full-length or a truncated derivate of the *gB-1* gene encoding D1 and D2 domains were constructed. The antibody titre induced by the gB-1 derivate was measured and compared to that of antibodies induced by the vaccine candidate encoding full-length gB-1 or the wild-type virus. Furthermore, the protection of vaccinated BALB/c mice against the lethal challenge of HSV-1 was evaluated. The results showed that the full-length

gB-1 gene could protect all the inoculated mice against the wild-type virus, while the truncated form could produce neutralizing antibody and delay the death following the HSV-1 challenge.

Material and Methods

Virus and cell line

An HSV-1 isolate was obtained from a patient with herpetic encephalitis signs. The isolated virus was confirmed to be HSV-1 by specific fluorescent mAb. The virus was propagated in HeLa cell monolayer and stored at -80˚C until further use.

Construction of plasmids

Glycoprotein B gene of the KOS strain of HSV-1 was cut out from the clone pAcgB-1 (a gift from Dr. Ghiasi, UCLA, Los Angeles, CA) (Ghiasi et al., 1999) using *Bam*HI restriction enzyme. The pcgB plasmid expressing gB-1 was constructed by the insertion of the *gB-1* gene into the *Bam*HI site of the pcDNA3 vector under the control of CMV promoter. The clones were screened and the orientation of the inserted DNA was determined using *Pvu*I restriction enzyme.

To construct a vector expressing neutralizing epitopes of gB-1, a 1515 nt fragment of the gene was obtained by cutting the pAcgB-1 with *Bam*HI and *Pst*I restriction enzymes. This fragment encodes 475 amino-terminal residues of the mature protein carrying the major continuous and discontinuous neutralizing epitopes. The *Pst*I adaptor was engineered to encode a stop codon in all three reading frames. The adaptor was synthesized by GENSET SA (Paris, France) in two separate strands (5'G TAGATAGATAGT3' and 3'ACGTCATCTATCTATCAGATC5'). Formation of ds-oligomers created *Pst*I and *Xba*I overhangs at the 5' and 3' ends, respectively. The synthetic fragments were not phosphorylated at the 5'ends to eliminate the possibility of tandem formation. Thus, ligation of the fragments and the adaptors with the pcDNA3 vectors digested with *Bam*HI and *Xba*I took place in a one-step reaction. The presence of the fragment in the constructed vector (pc1500) was then determined with suitable restriction enzyme digestion, Southern blot and DNA sequence analysis.

Transfection and expression analysis

The subconfluent COS-7 cells grown on coverslips were transfected with each construct using DOTAP liposome (Roche Inc., Mannheim, Germany) according to the manufacturer's instruction. Regarding the pcgB vector 72 h after transfection, the cells were washed 3 times with PBS and fixed with absolute methanol at –20˚C for 10 min. The expression of gB-1 was analysed by indirect immunofluorescence. The cells were incubated for 45 min at 37˚C with hyperimmune human serum diluted 1/100 in PBS. The cells were then washed and incubated at 37˚C for 45 min with fluorescein isothiocyanate-conjugated antihuman IgG and counterstained with 0.01% Evans blue. The coverslips were mounted with 80% glycerol in phosphate buffer and examined by fluorescent microscopy.

To analyse the expression of the truncated form of gB-1 the same protocol was used except that for cytoplasmic detection of the secretory protein, a time course analysis was applied.

Immunization of mice

Groups of 3–4 weeks old female BALB/c mice (6/group) were each given three injections of pcgB or pc1500 plasmids, at 21 day intervals, at a dose of 90 µg per mouse intramuscularly. The control groups were injected with a sublethal dose of HSV-1 as positive control and pcDNA3 and PBS as two separate negative controls.

Antibody detection

Fourteen days after the last immunization, the sera were collected from the vaccinated mice to check for specific antibodies. The sera were heat-inactivated at 56˚C for 30 min. One hundred µl of two-fold dilutions of each serum were added to a 96-well plate and 100 µl of the virus suspension containing 100 $TCID_{50}$ of HSV-1 were then added to each well. The plate was incubated at 37˚C for 1 h. The reaction mixtures were then added to the 3×10^3 HeLa cells grown on a 96-well plate. After 1 h adsorption, 100 µl of DMEM supplied with 4% FBS were added to each well. The highest dilution of each serum that neutralized the virus in 24 h was taken as the serum titre.

Virus challenge

Three weeks after the last inoculation, all the mice were challenged with 10 LD_{50} of the virus intraperitoneally. The challenged mice were monitored for a month. To confirm that the death of the challenged mice was due to the HSV-1 infection, samples of brains and lungs of the sacrified mice were subjected to HSV-1 isolation.

Statistical analysis

For comparison of antibody titres, analysis of variance (ANOVA) and a multiple comparison test of LSD (Least Significant Differences) were used. Mice survival data were analysed for time using Kaplan-Meier survival analysis.

Results

Cloning and in vitro expression

We generated two DNA constructs; pcgB and pc1500 using the pcDNA3 expression vector. The plasmid pcgB was constructed by the insertion of the full-length *gB-1* into the *Bam*HI site of the pcDNA3 vector.

The pc1500 contained the *gB-1 Bam*HI-*Pst*I fragment encoding the 475 amino-terminal residues of the gB-1. The plasmids were characterized by restriction analysis, Southern blot detection and sequence analysis of the inserted genes. The sequenced regions were completely identical with those of the KOS strain of HSV-1. The expression of full-length *gB-1* was confirmed in the transfected COS-7 cells by immunofluorescence reaction using HSV-1 polyclonal antibody 72 h after the transfection. For cytoplasmic detection of the secretory protein expressed by pc1500, the cells were tested 20–60 h after transfection. The cytoplasmic detection of gB in transfected cells showed an increased expression in time.

Humoral responses

To evaluate the humoral immune responses induced by the neutralizing epitopes, the antibody titres induced by pcgB, pc1500 and the live HSV-1 were compared in the vaccinated mice. The sera were assayed for specific antibody by the virus neutralization test. Table 1 shows the average of $\log_{10}$ titres of neutralizing antibody in each group. All the test and positive control groups showed antibody responses; however, the mice in the HSV-1 group had a higher antibody level than the other groups. Compared to the live HSV-1 immunized group, the average $\log_{10}$ of the antibody level of the mice inoculated with pcgB and pc1500 were about 1.3 and 3.8 fold lower, respectively. However, the mean antibody titre of the pc1500 group was higher than those of the mock-infected animals. All differences among the antibody titres of the test groups and also among the test groups and the control groups were statistically significant ($P < 0.05$).

Protection against HSV-1 lethal challenge

A challenge dose of $10LD_{50}$ was used to assess the protection induced by the *gB-1* gene or its derivate. All the groups of mice were challenged by i.p. injection of HSV-1 and monitored daily for clinical signs and mortality (Fig. 1). Similar to those of HSV-1-vaccinated animals, the survival rate of the mice receiving pcgB was 100%, although one of them showed the sign of transient paralysis in its legs. The protection rate in the pc1500-injected animals against mortality was restricted to 33% of the cases, but the median survival time was prolonged in comparison with the pcDNA3- and PBS-injected groups. While 83% of the mock-vaccinated mice died within 8 days, in the pc1500 group the median survival time was 15 days after the challenge (Table 2).

Table 1. Antibody titres in the mice after immunization

Vaccine group	No. of responders (%)	HSV-1-specific antibody titres*
pcgB	6 (100)	1.76 ± 0.22
pc1500	6 (100)	0.6 ± 0.33
Live HSV-1	6 (100)	2.3 ± 0.15
pcDNA3	0	0
PBS	0	0

* mean ± SEM ($\log_{10}$)

The mice were immunized with various immunogens 3 times, at 21 day intervals. On day 14 after the last immunization, serum samples were collected and titered for specific antibody by the neutralization test. The data presented in the Table are the mean of $\log_{10}$ antibody titres in each group ± SEM. The LSD test showed significant differences among the titres ($P < 0.05$).

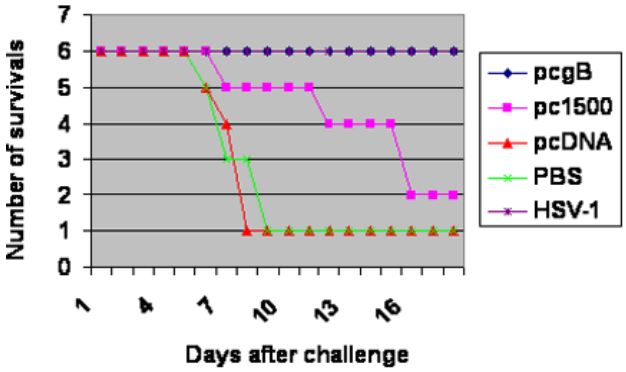

Fig. 1. The effect of immunization with pcgB and pc1500 vectors on the survival of the mice infected with 10 LD_{50} of HSV-1. Groups of mice injected with pcDNA3, PBS and sublethal dose of HSV-1 were used as control groups. The mice (6 mice /group) were immunized 3 times, at 21 day intervals intramuscularly. On day 21 post-immunization, mice were challenged with HSV-1 and monitored daily for mortality.

Table 2. Resistance to HSV-1 i.p. challenge in the immunized mice

Vaccine group	No. of survived mice (%)	Median time of survival (days)	Mean time of survival ± SEM (days)
pcgB	6 (100)	ND	ND
pc1500	2 (33)	16	14.17 ± 1.48
Live HSV-1	6 (100)	ND	ND
pcDNA3	1 (17)	8	9 ± 1.49
PBS	1 (17)	7	9.17 ± 1.5

The mice were immunized with various immunogens 3 times, at 21 day intervals. On day 28 after the last immunization, the mice were challenged with 10 LD_{50} of HSV-1. The mice were monitored daily for survival.
Median time of survival shows that 50% of animals had survived at the indicated time.
ND – no death was detected.

Discussion

DNA immunization is a promising approach to developing effective vaccines. In addition, compared to other recombinant vaccination methods, the advantages of DNA immunization as a discovery tool provide a rapid way to identify more immunogenic epitopes of an

antigen and their capacity to elicit effective immunity. Definition of these epitopes will provide a rationale for tailoring recombinant vaccines.

Some of the viral envelope glycoproteins have been used as immunogens in recombinant vaccines. Due to the role of gB and gD in HSV infectiveness, they have received the highest attention. We chose gB-1 because it contains well-characterized epitopes for induction of humoral and cellular immunity. Although there are some reports of identification of the neutralizing epitopes of gB-1, little is known about the quantitative antibody responses induced by these epitopes. Regarding the earlier studies, the truncated form of gB-1 (707 aa), which lacks the transmembrane and cytoplasmic domains, could be an effective vaccine in the induction of humoral immune responses (McClements et al., 1996). The aim of the present study is to further characterize the gB-1 neutralizing epitopes in a DNA vaccination approach and also to evaluate the strength of the epitopes located on the first 475 amino acids of gB-1 (D1 and D2 domains) to induce antibody responses. Furthermore, we wished to determine the ability of these epitopes to protect against the lethal challenge with HSV-1. Based on the previous works done on the epitope map of gB-1(Navarro et al., 1992), we chose the first 1515 nt of the *gB-1* gene as the major neutralizing epitopes. It has been shown that the gB-1 neutralizing activity depends on the three major domains, D1, D2 and D5a. These epitopes are recognized by the complement-dependent and -independent neutralizing antibodies (Navarro et al., 1992). D1 and D2 domains contain continuous and discontinuous epitopes. Continuous neutralizing epitopes are mapped between amino acids 32 and 47 and discontinuous residues are located between amino acids 273 to 298, but additional residues are required to assemble these discontinuous epitopes (Qadri et al., 1999). At least 457 amino-terminal residues are required to react antibodies with the discontinuous epitopes (Pereira et al., 1989). Other discontinuous epitopes have also been mapped in these domains (Highlander et al., 1991). It has been shown that similarly to the native glycoprotein, the 1-475 amino acid derivate of gB-1 translocates to the cell surface (Navarro et al., 1991). The amino acids 1-475 were chosen in this study to provide both the major neutralizing epitopes and the proper translocation of the protein. Although D5a contains a cluster of important discontinuous epitopes, they are only active in the dimer form of gB (Navarro et al., 1992), so it was not possible to study the D5a domain as a separate region. We constructed the vectors containing the full-length or a truncated form of the *gB-1* gene encoding intact gB or epitopes of D1 and D2 domains. Beside measuring the neutralizing antibodies induced by the vectors, a lethal challenge model was used to compare the protective effect of each vector in protection of the vaccinated mice. Our data showed that immunization with both vectors induced immune responses in the mice. Although the antibody titre in the pcgB-injected group was significantly lower than that of the HSV-1-vaccinated group, pcgB was as effective as HSV-1 in protection of mice. In comparison with pcgB, pc1500 generated lower antibody responses and provided only minimal protection. However, it is of note that the pc1500 significantly prolonged the survival time as compared with negative controls.

It is difficult to interpret the effect of pc1500 in immunization of mice. It is clear that full-length gB-1 bears additional epitopes that induce neutralizing antibodies (600-690 amino acids). Besides, it is likely that the folding of the truncated form of gB-1 used in this experiment has been altered in comparison with the native protein (Del Val et al., 1991; Higgins et al., 2000) or, more probably, the processing of this secretory protein has been altered from that of the wild-type gB and the protein was released from the cells more slowly (Qadri et al., 1999). The presence of CTL and T-helper inducing epitopes in D1 and D2 domains are not completely determined. Several studies have demonstrated H-2d-restricted CTL (Hanke et al., 1991) and T-helper epitopes in the N-terminus of gB, but it seems that they are not the dominant epitopes for induction of cellular immunity (Wallace et al., 1999). Our findings showed that pc1500 could induce $CD4^+$ T-cell response, but the stimulation index has been decreased in the pc1500-immunized mice compared to those receiving pcgB (data not shown). Beside the existence of neutralizing epitopes there are some antibody-dependent cellular cytotoxicity (ADCC)-related epitopes in these domains, but lack of effective cell-mediated immunity in the pc1500 vaccinee may be the main reason for the lower protection induced by pc1500. In the context of protection, regarding the neutralizing activity of pc1500 and with respect to the minimal cell-mediated immunity induced by the vector, it is not surprising that pc1500 could not protect the animals against lethal challenge. Neutralizing antibodies do not inhibit HSV replication especially in the neurons, although they are among the immune factors that determine the virus load and severity of the infection (Mikloska et al., 1999). ADCC and neutralizing antibodies have been shown to affect the outcome of HSV infection (Sanchez-Pescador et al., 1992). These findings are in accordance with our data in which the antibodies induced by pc1500 postponed the death following challenge with the wild-type virus.

In conclusion, the results of this study might improve our knowledge in the field of modern vaccinology using molecular tailoring of antigens toward a desired direction of immunity and development of more effective recombinant vaccines.

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