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Expression of the Herpes Simplex Virus Type 2 Glycoprotein D in Baculovirus Expression System and Evaluation of Its Immunogenicity in Guinea Pigs

Fatemeh Fotouhi[1], Hoorieh Soleimanjahi[*1], Mohammad Hasan Roostaee[1] and Abdolhossein Dalimi Asl[2]

[1]*Dept. of Virology and* [2]*Dept. of Parasitology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran*

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ABSTRACT

Background: Herpes simplex virus type 2 (HSV-2) is highly prevalent and major cause of genital herpes in humans. The life-long nature of infection and the increasing prevalence of genital herpes imply that vaccination is the best strategy for controlling the spread of infection and limiting HSV disease. HSV glycoprotein D (gD) is one of the most important viral immunogen which has an essential role in virus infectivity and induction of immune responses. **Methods:** HSV-2 DNA was extracted and used as template in polymerase chain reactions to amplify gD2 gene. The PCR product was confirmed by restriction enzyme analysis, cloned into a cloning vector and then sequenced. The Bac-to-Bac expression system was used to express HSV-2 gD in insect cells. The expressed protein was used as subunit vaccine to immunize guinea pigs after confirmation. **Results:** The expressed protein was confirmed with SDS-PAGE and Western-blot analysis. In Western-blot analysis, two major protein bands, with approximate molecular weights of 52-55 and 41-43 kDa corresponding to the glycosylated and non-glycosylated forms of gD2 protein, were observed, respectively. Immunization with the recombinant gD2 could elicit humoral responses in guinea pigs as measured by neutralization test and ELISA, and offered high protection against induced HSV-2 genital disease. **Conclusion:** The baculovirus expression of heterologous genes permits proper folding, post-translational modification and oligomerization in manners that are often identical to those that occur in mammalian cells. Expression of proteins under the control of the strong polyhedrin promoter, allowing high level protein production, can be used as subunit vaccine. *Iran. Biomed. J. 12 (2): 59-66, 2008*

Keywords: Baculovirus, Herpes simplex virus type 2 (HSV-2), Insect cells, Protein expression, Subunit vaccine

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is the primary cause of genital herpes, a common sexually transmitted disease that occurs worldwide. Following the primary infection, the virus migrates to sensory ganglia and establishes latency in dorsal root ganglia. The latent virus undergoes periodic spontaneous reactivation resulting in recurrent clinical symptoms. In pregnant women, the infection causes unplanned abortion, premature delivery and congenital neonatal herpes [1]. It has been demonstrated that HSV-2 is also associated with an increased risk of HIV infection with a significant increase in the disease severity [2]. Considering the ability of HSV to develop latent infections, it seems that immunization remains the best strategy for prevention of HSV related diseases [3]. Many approaches to HSV vaccines including killed virus, live attenuated, subunit, vectored and DNA vaccines have been evaluated [4]. Subunit vaccines contain only the immunogenic components of a pathogen and, therefore, they are potential alternatives to the live vaccines due to their higher degree of safety [5]. HSV genome encodes at least 11 viral glycoproteins. The HSV gB and glycoprotein D (gD) are attractive proteins to include in the HSV subunit vaccines because they elicit both humoral and cell mediated immunity [6]. These two proteins are highly identical to HSV-1 and HSV-2 and, therefore, they may produce a

*Corresponding Author; Tel. (+98-21) 8828 83561; Fax: (+98-21) 8801 3030; E-mail: soleim_h@modares.ac.ir

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cross-reactive immunity against both the viruses [7]. Subunit vaccines, containing gD or a combination of gD and other glycoproteins, have been shown to be immunogenic in animals and humans [8, 9]. Recombinant gD has been expressed in prokaryotic and eukaryotic expression systems [10, 11]. Since native gD conformation is critical for immunization against the HSV infection, this protein need to be expressed in a suitable expression system [12, 13].

Several prokaryotic and eukaryotic expression systems have been used for *in vitro* production of the subunit vaccines from foreign genes. Eukaryotic expression system is the first choice for production of proteins that undergo post-translational modification like glycolysation [14]. Recombinant baculoviruses have been widely used as safe vectors to express heterologous genes in the cultured insect cells and larvae. In this system, the recombinant proteins are processed, modified and targeted to appropriate cellular compartments and are immunologically and biologically similar to their authentic counterparts [15]. The generation of recombinant baculoviruses by traditional methods has several drawbacks including a low frequency of recombination and requirement for several rounds of plaque purification, whereas the use of site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*, has greatly facilitated the rapid generation of the recombinant baculoviruses [16]. In the present study, the Bac-to-Bac baculovirus expression system was used to express the HSV-2 gD protein in insect cells. The immunogenicity of the expressed protein was determined by the induction of protective immunity in a guinea pig model. To our knowledge, this is the first report of HSV-2 gD expression in insect cells using the Bac-to-Bac expression system.

MATERIALS AND METHODS

Cell culture and virus. *Spodoptra frugipdera* (Sf9) and HeLa cells were purchased from the Cell Bank (Pasture Institute of Iran, Tehran) and grown in Grace's and DMEM medium (Gibco, Canada), respectively. The media were supplemented with 10% fetal bovine serum (Gibco, Canada). HSV-2 was isolated in our lab previously [17], and confirmed using monoclonal anti-HSV-2 antibody (Dako, Germany).

Animals. Female guinea pigs (250-300 g) were obtained from Pasture Institute of Iran (Tehran) and provided unlimited access to food and water. All experiments were done according to the Animal Care and Use Protocol of Tarbiat Modares University (Tehran, Iran) in 2005.

Amplification and cloning of the HSV-2 gD. HSV-2 was propagated in HeLa cells and the viral DNA was extracted according to the standard protocols [18]. The gD2 gene was amplified by PCR using designed HSV-2 gD specific primers as described previously [17]. The PCR product was extracted from agarose gel using DNA extraction kit (Macherey-Nagel, Canada) and subsequently cloned into the *EcoR*V site of the pBlueScript plasmid (Stratagene, Canada). The recombinant vector was confirmed using restriction enzyme analysis and sequencing.

Generation of recombinant bacmid. The gD2 gene was subcloned into the *EcoR*I and *Xho*I restriction enzyme sites of pFastBacHTc donor plasmid and identified with restriction enzyme analysis. The recombinant donor plasmid was transformed into the *E. coli* DH10Bac competent cells for site-specific transposition of the gD2 DNA from the transposing vector to a bacmid DNA through lacZ gene disruption. The transformed cells were plated onto the Lauria Broth (LB) agar containing kanamycin (50 µg/ml), gentamicin (7 µg/ml), tetracycline (10 µg/ml), Bluo-gal (100 µg/ml) and isopropylthio-β-galactoside (IPTG, 40 µg/ml) and incubated at 37ºC for 48 h. The high molecular weight (MW) bacmid DNA was isolated from the overnight cultures by alkaline lysis purification according to the instructions supplied by the manufacturer (Invitrogen, Canada). Successful transposition was verified by PCR analysis using either M_{13}/pUC or gD2-specific primers.

Transfection of Sf9 cells to produce recombinant baculovirus. Sf9 cells were transfected with isolated recombinant DNA using Cellfectin for the production of the recombinant baculovirus according to the manufacturer's instructions. The transfected cells were incubated at 27ºC for 72 h, allowing baculovirus propagation and release into the culture medium. Recombinant baculovirus production was monitored daily by visualization of the cytopathic effects (CPE). The culture medium was collected, clarified by centrifugation at 1000 rpm and subjected

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for multiple rounds of virus propagation. The clarified virus stock was titrated as plaque forming unit and stored at 4ºC.

Expression of recombinant protein. The overnight cultured Sf9 cells were inoculated with recombinant baculovirus at a multiplicity of infection (MOI) of 10 and incubated at 27ºC for 72 h. The clarified culture medium (supernatant) and the cells (pellet) were collected after centrifugation. To prepare cell lysate, the pelleted cells were washed three times with cold PBS, resuspended in cell lysis buffer (50 mM Tris-HCl, pH 8.5, 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM phenylmethyl sulfonyl fluoride) and sonicated three times for 10 s by 3 min intervals, using ultrasonicator at 50% power (Helscher, UP 400 s, Germany). After centrifugation at 9000 ×g at 4ºC for 15 min, the clarified supernatant was stored at -20ºC for further analysis.

Determination of gD2 protein. The culture medium and cell lysate of the Sf9 infected cells with recombinant baculovirus were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) and stained with Coomassie brilliant blue. For further characterization, the separated proteins on SDS-PAGE were electrotransferred into nitrocellulose membranes using electro-blotting system (Bio-Rad, Germany) according to the standard protocols [18]. Briefly, the membranes were blocked at room temperature for 1.5 h with 1% bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.4) containing 0.1% Tween 20 (TBS-T). The blotted proteins were reacted with C65019M mouse anti-gD2 monoclonal antibody (Biodesign, Japan) diluted 1:50 in TBS-T at room temperature for 1.5 h. The appropriate secondary antibody, rabbit anti-mouse IgG conjugated with HRP (Tebsun, Iran) diluted 1:2000 in TBS-T, was added and incubated at room temperature for 1.5 h. The protein bounds were visualized by staining the membranes with diaminobenzidine (Sigma, Germany).

Immunization schemes. Infected Sf9 cells with recombinant baculovirus were collected, suspended in cold PBS, freeze-thawed three times and clarified by low speed centrifugation and the supernatant were used to immunize animals [10]. Female guinea pigs (n = 21) were put into three groups (7 animals/group). The first group was inoculated twice with supernatant from 1×10^6 infected Sf9 cells subcutaneously using Friends' complete or incomplete adjuvant on day 0 and 14, respectively. Sham-immunized guinea pigs were inoculated with supernatant from 1×10^6 uninfected Sf9 cells in the same manner. The last group received wild HSV-2 (10^4 Pfu) intraperitoneally and served as positive control. All animals were left to bleed under anaesthesia (Ketamine) at 20 days after the second vaccination. The collected sera were assayed for analysing of antibody responses.

Neutralizing antibody assays. Serum antibody titers were determined with some modification using methods described previously [19]. Briefly, sera were inactivated at 56°C for 30 min prior to serial dilution in DMEM. One hundred microliter of each dilution was mixed with 100 µl of 100 Pfu of live HSV-2. The mixtures were incubated at 37°C for 1 h and then inoculated in duplicate onto Vero cell monolayer in 96-well plates. Virus was absorbed for 1 h (at 37°C) and then 100 µl DMEM/2% FBS was added to each well and incubated at 37°C for 3 days. Virus and medium controls were included in each assay. Titers of sera were defined as the $\log_{10}$ of the final serum dilution that produced a >50% reduction in the number of viral plaques, when compared to the virus controls.

ELISA. Sera were assayed for HSV-specific IgG responses in ELISA using a modified method described elsewhere [20]. Briefly, 96-well plates (Nunc, Germany) were coated overnight at 4°C with prepared recombinant gD2 at a concentration of 0.5 µg per well in carbonate buffer (pH 9.5). Other incubations were carried out in 100 µl volume at room temperature for 1 h and the plates were washed four times with PBS (pH 7.2) between steps. Bovine serum albumin (1%) in PBS-T was used as a blocking agent and for serial dilution of serum samples. It was developed using peroxidase-conjugated anti-guinea pig IgG (Biodesign, Japan) and O-Phenylenediamine hydrochloride. The end-point titer was defined as the $\log_{10}$ of the serum dilution resulting in an OD_{490}>0.1 and greater than twice of the background.

Virus challenge. Three weeks after the second vaccination, all guinea pigs were challenged with HSV-2 using a modified procedure described by others [21, 22]. The anesthetized animals were inoculated intravaginally using cotton swabs

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immersed in 100 µl of HSV-2 (10^5 Pfu ~10 ID_{50}). The swab was inserted into vagina, twisted back and forth and left for 30 min, then removed and wiped over the external genitalia. The guinea pigs were examined daily for more than 2 weeks and the severity of primary genital skin disease was quantified using a lesion score scale described elsewhere [20].

RESULTS

Amplification and characterization of the gD2 gene. The full length sequence of HSV-2 gD gene was amplified by PCR using the designed specific primers and verified by restriction enzyme analysis using *Sal*I, which created 450 and 750 fragments as expected (Fig. 1). The correctness of the gD2 ORF was confirmed by sequencing. Analysis of sequencing was accomplished by chromas software (version 1.45-Australia) and revealed about more than 99% homology with sequences that are presented in GenBank (NCBI). The nucleotide sequence data was deposited in GenBank database under the accession number: "AY 517492".

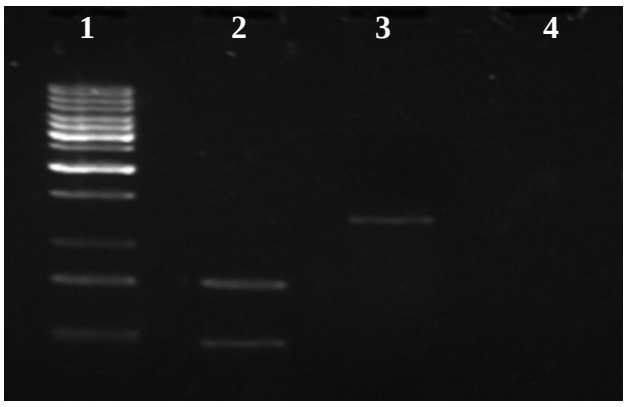


Fig. 1. Agarose gel electrophoresis of the amplified gD2 by PCR. Lane 1, DNA size marker (1 kb ladder); Lane 2, PCR product /*Sal*I; Lane 3, 1.2 kb PCR product and Lane 4, negative control.

Construction of the recombinant bacmid DNA. The gD2 gene was subcloned into pFastBacHTc plasmid in frame with the N-terminal 6xHis tag and verified by restriction endonuclease digestion. Following the transformation and plating of the cells on agar, the transposed colonies were visible as large white colonies among the blue ones harboring the unaltered bacmid. The selected white colonies were restricted onto LB agar to ensure if they have true white phenotype. Since verification of the high

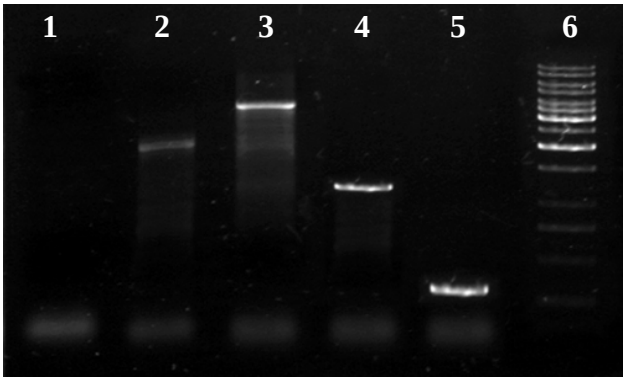


Fig. 2. Identification of recombinant bacmid DNA by PCR. Lane 1, PCR negative control; Lane 2, DNA of 1.8-kb PCR product using gD2.specific forward and M_{13}/pUC reverse primers; Lane 3, DNA of 3.6 kb PCR product using M_{13}/pUC forward and reverse primers; Lane 4, DNA of 1.2 kb PCR product using gD2-specific primers; Lane 5, DNA of 0.3 kb PCR product of non-recombinant bacmid using M_{13}/pUC primers; Lane 6, DNA size marker (1 kb ladder).

MW recombinant bacmid DNA by digestion is not convenient, PCR was performed using M_{13}/pUC and gD2-specific primers to ensure proper transposition of the target gene in the recombinant bacmid. The bacmid DNA contains M_{13} forward and reverse priming sites flanking the mini-att Tn7 site within the LacZ α-complementation region. PCR was done using M_{13}/pUC primers for 3.6 Kb PCR product, gD2 specific primers for 1.2 Kb PCR product and gD2-forward and M_{13}/pUC-reverse primers for 1.8 Kb PCR products. Amplification of the non-recombinant bacmid using M_{13}/pUC primers generated a 0.3-Kb band (Fig. 2).

Transfection of Sf9 cells. The Sf9 cells were transfected with the isolated recombinant bacmid DNA using Cellfectin. The transfected cells displayed typical CPE, i.e. low cell density, division stop, enlarged cells and poor adherence to the substrate, indicating that the virus production was taking place. The mock-transfected Sf9 cultures continued to divide and form a confluent normal cell monolayer (Fig. 3).

SDS-PAGE and Western-blot analysis. The effects of various MOI and harvesting time in the production of recombinant gD2 were examined by SDS-PAGE analysis. The Sf9 cells were infected with recombinant baculoviruses with different MOI (1, 5, 10) and harvested in different times (24, 48, 72, 96 h, p.i.) and then subjected to SDS-PAGE. Maximal expression levels of the recombinant gD2 were obtained at 72 h post infection with a MOI

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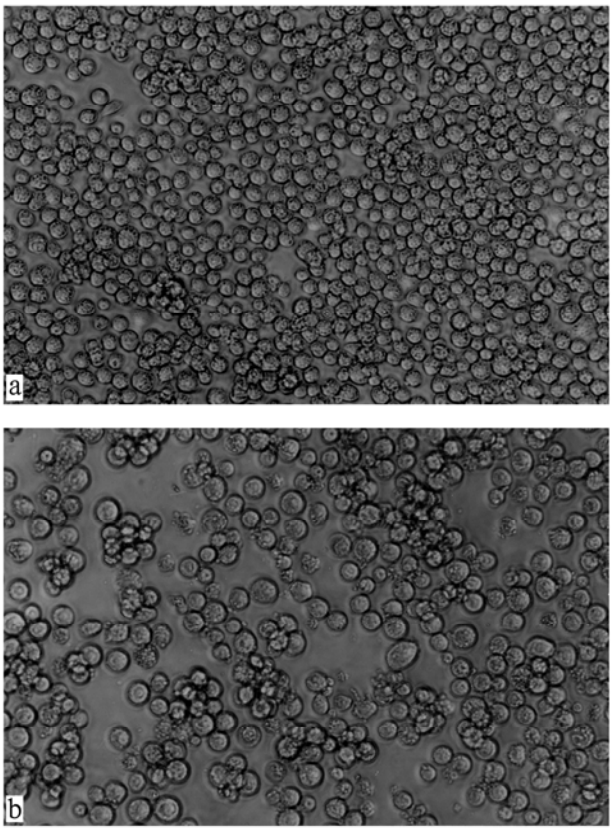


Fig. 3. **(a)** Transfected Sf9 cells with recombinant bacmid DNA and **(b)** mock-transfected Sf9 cells (× 200).

of 10 (data not shown). To analyze the gD2 recombinant protein, the Sf9 cells were infected with recombinant baculovirus with a desired MOI and harvested after 72 h. Total protein of the cell lysates

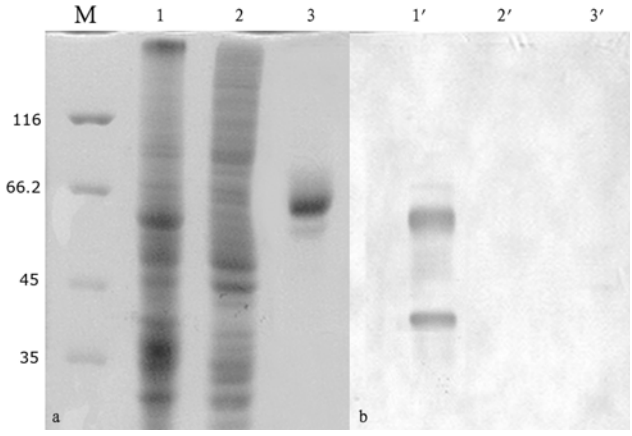


Fig. 4. **(a)** SDS-PAGE and **(b)** Western-blot analysis of HSV-2 gD expressed in Sf9 cells. Lane M, protein marker with relative molecular weight indicated; Lane 1 and 1′, the lysates from the Sf9 cells infected with recombinant baculovirus; Lane 2 and 2′, the lysate from the uninfected cells; Lane 3 and 3′, the culture supernatants from the Sf9 cells infected with recombinant baculovirus.

was visualized on 12% polyacrylamide gel and analyzed by Western-blotting using monoclonal anti-gD2 antibody. SDS-PAGE analysis demonstrated two exact polypeptide bands with predicted MW of about 52-55 and 41-43 KDa representing the fully glycosylated and non-glycosylated forms of gD2, respectively (Fig. 4). Analysis of the recombinant infected cell medium showed no detectable band. In SDS-PAGE, there was only one strong band corresponding to the bovine serum albumin, which was presented in the cell media. This suggests that the gD2 is retained in the cell or cell membrane and is not secreted into the medium as was expected.

Table 1. Antibody responses in guinea pigs immunized with recombinant gD2 or HSV-2 or sham-immunized with uninfected Sf9 cells.

Immunized -group	NT[a]	ELISA[a]
gD2	2.66 ± 0.20	3.58 ± 0.23
HSV-2	2.88 ± 0.16	3.79 ± 0.14
Sf9 cells	0.30 ± 0.00	1.30 ± 0.00

[a]Data are shown as mean ($\log_{10}$) ELISA or neutralizing antibody titers.

Humoral responses. The guinea pig model was used to confirm the *in vivo* activity of prepared recombinant gD2 and to assay its immunogenicity. The guinea pigs were immunized with baculovirus-derived gD2 or wild HSV-2 as described in Materials and Methods. A negative control group of guinea pigs was sham-immunized with PBS. Three weeks after the second immunization, the sera were analysed for anti gD2 -specific antibodies by ELISA and HSV-2 neutralization titre using plaque reduction assays. The results are shown in 1. The sham-immunized animals had no detectable antibody titre. The results indicated that the recombinant gD2 induced humoral responses as mush as wild HSV-2, which is not statistically significant.

Protection against intravaginal challenge. Three weeks after the second immunization, all animals were challenged intravaginally with HSV-2 (10^6 Pfu) as described in Materials and Methods and monitored daily for signs of genital herpes disease. Mean lesion scores for each day were calculated by dividing the sum of group lesion scores by the number of animals per group. The course of the primary disease on days 1-17 is summarized in Figure 5. The HSV-2 immunized group were

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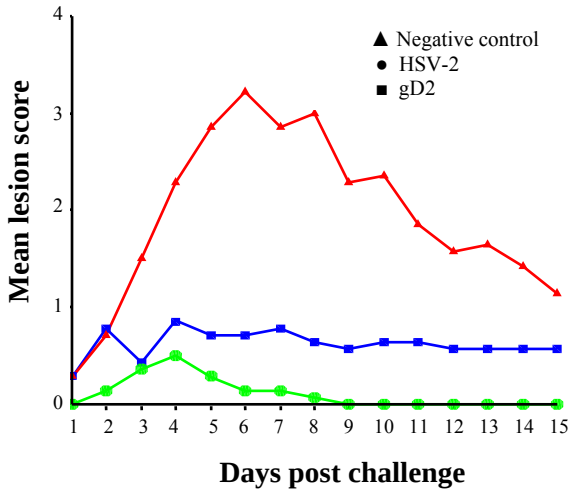


Fig. 5. Effect of recombinant gD2 and HSV-2 immunization on primary genital herpes in guinea pigs. Immunized animals were challenged as described in materials and methods and evaluated daily to quantify the severity of genital disease using the following scale: 0, no disease; 1, redness or swelling; 2, several small vesicles; 3, several large vesicles; 4, large ulcers, paralysis or death. In the case of death, the final score assigned to that animal was carried through to the end of the observation period.

completely protected against HSV-2 challenge. In contrast to the negative control group in which all animals exhibited signs of severe genital herpes, only one gD2 immunized animal had signs of severe genital disease and died at day 10 post challenge. The overall severity of the disease as measured by the means of all lesion scores using repeated measurement test was significantly (P<0.001) lower in gD2-immunizied group than sham-immunized group (0.624 vs. 1.933) but was not statistically distinguishable from the HSV-2 immunized control group (P = 0.28). Thus, protection from the disease in gD2-immunized animals was as much as that achieved in wild HSV-2 immunized ones.

DISCUSSION

Several research groups have reported on the expression of the full-length and truncated HSV-2 gD in baculovirus-infected insect cells. In 1993, Landolfi *et al.* [11] constructed a recombinant baculovirus which expressed HSV-2 gD in insect cells and showed that it was immunogenic and protective against lethal HSV challenge in mice. Nicola *et al*. [23] used baculovirus derived gD2 to analyze its structural function as a viral protein which is required for postattachment entry of virus into cells.

The expression of eukaryotic genes using baculovirus expression vectors takes advantages of their protein synthesis machinery and facilitates proper folding and post-translational modifications including glycosylation, acylation, oligomerization and proteolysis [24]. In addition, the insect cytoplasmic environment provides appropriate disulfide bridge assembly. These features are important in the case of HSV-2 gD, as its structure is critical for its efficacy as a vaccine. The three N-linked and two potential o-linked oligosaccharide sites are important for the maintenance of antigenic structure and the three disulfide bonds of gD are necessary for protein stability [23].

In the present study, the Bac-to-Bac expression system was chosen because it is a valid and multi-purpose system for the production of recombinant proteins. The generation of recombinant baculoviruses by traditional methods has several drawbacks including a low frequency of recombination and requirement for several rounds of plaque purification, whereas the use of site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*, has greatly facilitated the rapid generation of the recombinant baculoviruses. Using this system, the isolated recombinant virus DNA from the selected colonies, is not mixed with the parental non-recombinant viruses, therefore eliminates the need for plaque purification and consequently reduces the time of identification and construction of the recombinant virus. Several groups have reported using of this system to successfully express the desired proteins in large quantity [16]. To our knowledge this is the first report of a full-length HSV-2 gD expression in insect cells using the Bac-to-Bac expression system. Moreover, this report is the first description about recombinant Baculovirus construction in Iran.

The gD2 is retained in the cell or cell membrane and is not secreted into the medium and reacted specifically with an anti-gD2 monoclonal antibody in Western-blot analysis, indicating that the protein is properly folded and perhaps maintains its biological functions without impairing its antigenic properties. The existence of a protein with a MW of approximately 41-43 kDa corresponding to the non-glycosylated gD polypeptide with a predicted MW of the 43.179 kDa, and one larger band with a MW of about 50-52 kDa represent fully-glycosylated gD2.

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The ability of the recombinant gD2 to elicit protective immunity against induced HSV-2 diseases was evaluated in a genital guinea pig model. Immunization with the recombinant gD2 resulted in induction of high level humoral immune responses against HSV-2, suggesting that baculovirus-derived gD2 reserved its functional structure and immunological features.

The results of challenge study indicated that immunization by gD2 could protect guinea pigs from induced HSV-2 genital disease as much as wild HSV-2. The productivity and flexibility of the insect baculovirus expression vector system and the large capacity of the baculovirus based on vectors to incorporate large amounts of foreign DNA have permitted this system to be used for the expression of target genes [25]. The application of recombinant baculovirus containing mammalian target immunogens will undoubtedly prove to be a useful tool for gene delivery and protein expression in mammalian cells for immunotherapy.

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