

# Immunization with cytomegalovirus gB protein produced by the Baculovirus Expression Vector System to elicit humoral immune response in BALB/c mice

Azizi Saraji A<sup>1</sup>, Asiyabi S<sup>2</sup>, Jalali F<sup>2</sup>, Aghasadeghi MR<sup>3</sup>, Shahmehri M<sup>4</sup>, Vahabpour R<sup>4\*</sup>

<sup>1</sup>Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

<sup>2</sup>Bam University of Medical Sciences, Bam, Iran.

<sup>3</sup>Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran.

<sup>4</sup>Department of Medical Lab Technology, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

## ABSTRACT

**Introduction:** Due to the role of neutralizing antibodies which can prevent human cytomegalovirus (HCMV) infection, most of the efforts have been focused on designing vaccines capable of eliciting protective humoral immunity. The aim of this study was to evaluate the antibody response of BALB/c mice to a truncated HCMV glycoprotein B produced in insect cells using Baculovirus Expression Vector System (BEVS). **Methods:** The ectodomain of HCMV gB coding sequence was synthesized and the recombinant protein was expressed in *Spodoptera frugiperda* (Sf9) insect cell line using BEVS. The expression of the recombinant HCMV gB was verified using an HRP-conjugated polyclonal antibody, specific for HCMV gB. The levels of antibody responses and characterization of the subclasses of IgG antibodies were evaluated after vaccination of the mice. **Results:** The expression of truncated HCMV gB protein (~ 70 kDa) in the infected insect cells was verified by Western blot analysis. Measurement of IgG subclasses showed the dominance of IgG1 subclass response among all of the IgG subclasses ( $P < 0.05$ ) while the titers of IgG2a and IgG2b were approximately the same. **Conclusion:** This study demonstrated that BEVS could be used as an efficient approach for the expression of this truncated protein. The results also showed the use of this recombinant protein as a subunit vaccine could induce a significant antibody response, tilted toward IgG1.

**KEYWORDS:** Human cytomegalovirus, Ectodomain of glycoprotein B, Subunit vaccine.

## INTRODUCTION

Human cytomegalovirus (HCMV) is the cause of a lifelong and persistent viral infection in the majority of humans. Whereas primary infections with HCMV are usually asymptomatic in healthy individuals, complications can be emerged in immune-compromised patients following acute CMV infection or its reactivation, presenting different clinical demonstrations [1]. The incidence of HCMV infection among newborns in the United States and Western Europe has been reported to be 0.5-0.7% and in South America, Africa and Asia is ranged 1-2% [2]. More than 10% of liveborn infants with CMV infection have symptoms such as hepatomegaly, jaundice, growth retardation, purpura, microcephaly, chorioretinitis, thrombocytopenia, seizures, or focal neurologic deficits [3-5]. On the other hand, HCMV infection is capable of causing

complications in solid organ transplant recipients and other immune-compromised individuals such as patients with HIV/AIDS [3]. Such complications include pneumonitis, gastrointestinal ulceration, retinitis, or hepatitis which sometimes can lead to more dangerous diseases; however, these complications have been decreased with the application of potent antiretroviral drugs [3, 2].

HCMV has the largest genome among the *Herpesviridae* family which is 230 kb [4]. This genome codes for nearly 200 proteins of which glycoprotein B (also known as UL55) is present in the outer envelope of the HCMV particle and is recognized as a major target for the neutralizing antibodies [3]. Glycoprotein B constitutes of 906 amino acids with transmembrane and cytoplasmic domains which are required for the viral entry into permissive cell types including placental precursor cells [6-8]. Previous studies have recognized numerous antibody-binding sites on HCMV gB from which at least two domains could induce neutralizing antibodies within natural life cycle of the virus [9-11]. The high incidence rate of human infection with HCMV and its burden to public health have led to efforts to

\*Corresponding Author: Rouhollah Vahabpour, Department of Medical Lab Technology, School of Allied Medical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Email: farhang\_v\_r@yahoo.com

Tel/Fax: (+98) 2122717504

develop more efficient treatments or prophylactic vaccines [1]. The seronegative women of childbearing age are the main target populations for these prophylactic vaccines whereas infants population stand as the second potential target because of their role in the viral dissemination [12]. Additionally, HCMV-seronegative patients who are on the transplantation lists might benefit from such vaccines, considering the life-threatening risk of HCMV infection, post-transplantation period. Current approved antiviral drugs for the treatment of HCMV infection are only partially competent and may fail due to the emergence of drug resistance mutations [6]. Hence, the production of an efficient vaccine against HCMV has always been a high priority and would provide a great medical benefit throughout the world [9, 10].

Despite noticeable progress in HCMV research and the availability of some candidate vaccines in the early phase of development, no licensed product is yet under consideration to be released to the market. At the beginning of the vaccine studies, an attenuated HCMV named "Town vaccine" was administered in kidney transplant recipients; although, the vaccination failed to prevent the infection [13]. However in 2012, a MF59-adjuvanted gB subunit vaccine was investigated in a phase II clinical trial and revealed noticeable efficacy in preventing the infection [14]. According to the previous studies, an ideal HCMV vaccine must be capable of inducing neutralizing antibodies which should efficiently prevent the infection of various cellular targets and inhibit the viral dissemination [15]. Regarding the vital role of neutralizing antibodies to prevent HCMV infection, Negro et al. isolated polyclonal antibodies from seropositive donors and observed a protective effect of these antibodies when administered in the fetus [16].

In a previous study, we constructed a DNA-based vaccine, encoding truncated HCMV gB protein fused with green fluorescent protein (GFP) to elicit the humoral immune response in BALB/c mice. The levels of elicited antibodies were then evaluated in mice using different prime-boost strategies (18). In the current study, we described the production process of HCMV gB subunit in *Spodoptera frugiperda* (Sf9) insect cells using Baculovirus Expression Vector System (BEVS). Furthermore, we conducted preliminary ELISA-based serological assays on HCMV gB-immunized BALB/c mice, using a synthetic peptide of HCMV gB, as a capture antigen.

## MATERIALS and METHODS

### Generation of the recombinant baculovirus

The DNA sequence coding for ectodomain of HCMV glycoprotein B (GenBank accession number: EF531305) was commercially synthesized (BIOMATIK, Canada) with codon-usage adaptation for expression in Sf9 insect cells with *Bam*HI/*Hind*III restriction sites at two ends inserted into pUC57 vector. The vector containing HCMV gB coding sequence was double digested using *Bam*HI and *Hind*III enzymes and the gel-extracted fragment was sub-cloned in a similarly double digested pFastBac™ HTA donor plasmid, downstream of the polyhedrin promoter of pFastBac™ HTA donor plasmid. The recombinant pFastBac HTA containing ectodomain of HCMV gB (pFastBac HTA-CMV gB) was transformed into DH-10Bac™ competent cells. The day after the transformation, white colonies with possible accurate transposition were isolated to verify correct insertion into the bacmid, using PCR

analysis with M13 universal primers according to the manufacturer's instructions (Bac-to-Bac® BEVS, Invitrogen, USA) [15]. To isolate the recombinant bacmid, PCR products were analyzed by gel-electrophoresis. Subsequently, Sf9 cells were transfected with purified recombinant bacmid, using Cellfectin® II reagent (Invitrogen, USA) [15]. The transfected Sf9 cells were cultured in Grace's Insect Medium (Sigma, USA) to produce recombinant baculovirus. Six days after the transfection, supernatants of the insect cells containing the baculovirus were collected and clarified by centrifugation to remove the cell debris. The recombinant baculovirus stock was stored at 4°C for two weeks.

### Protein Expression Optimization

The recombinant baculovirus expressing ectodomain of HCMV gB was amplified by three consecutive rounds of Sf9 cells infection. The titer of the viral stock was measured using Reed and Muench method [9]. To obtain the proper multiplicity of infection (MOI), for an efficient baculovirus protein expression, we applied three different MOI ranges, namely 0.1, 1, 10, to transduce the Sf9 cells. The cells were harvested three days after the infection by the recombinant baculovirus (MOI = 5). Subsequently, the insect cells pellet was lysed in Radioimmunoprecipitation (RIPA) buffer (Sigma-Aldrich, USA), supplemented with protease inhibitor (cOmplete, Mini, EDTA-free tablets, Sigma, USA). The cell lysate was then clarified by centrifugation at 10,000 rpm for 15 min at 4°C.

### Detection and purification of recombinant HCMV gB protein

The separated protein bands were transferred to nitrocellulose membranes (Millipore, USA) using semi-dry electroblot system (Bio-Rad, Germany). The membranes were blocked with 1% bovine serum albumin (BSA) and then were incubated with 1:5000 dilution of goat polyclonal HRP conjugated anti-Cytomegalovirus Glycoprotein B antibody (Abcam, USA). The band was visualized using DAB (3, 3'-diaminobenzidine tetrahydrochloride) solution (Bio-Rad, USA).

The clear supernatant was poured into Ni-NTA column (Qiagen) to purify His-tagged HCMV gB protein by affinity chromatography using native purification protocol. The purification of the recombinant protein was completed using dialysis against PBS (Cut off: 3500 Da) (Spectrum, USA). To examine the quality and quantity of HCMV gB protein, the purified recombinant protein was subjected to SDS-PAGE (15% acrylamide).

### Immunization groups

Female BALB/c mice (6-9 weeks old, 20 g) were purchased from Pasteur Institute of Iran. The animals were divided into three groups of 6 mice. All procedures were performed following the Declaration of Helsinki (version 2000). Mice were immunized subcutaneously on days 0, 14 and 21, using the purified recombinant HCMV gB protein and the control group received sterile phosphate buffered saline (PBS) injection.

### Serological analysis

To evaluate the humoral immune response of the immunized mice, ELISA with HCMV gB synthetic peptide "DSYHFSSAKMTATFLSKKQEVN" (Biomatik, Canada), corresponding to amino acids 361-383 (encompassing an epitope of antigenic domain II) as the coating antigen was used (10 µg/ml). The mice were bled on day 30 and 60 after the last immunization, and their total IgG, IgG1 and IgG2a antibodies were measured against the coated antigen by ELISA.

To measure IgG subclasses, goat anti-mouse IgG1 or IgG2a conjugated with Biotin (Abcam, USA), diluted 1:2000 in 1% blocking buffer and was added into the micro plates to be incubated at 37°C for 2 h. The 96-well plate were washed and incubated with 1:1000 dilution of streptavidin-horseradish peroxidase (Abcam, USA) at 37°C for 45 min. After the last washing step, detection was done using O-Phenylenediamine (Sigma-Aldrich, USA) as the substrate. After 30 min incubation in dark, the reaction was stopped by 0.5 M sulfuric acid and the absorbance was measured at 490 nm.

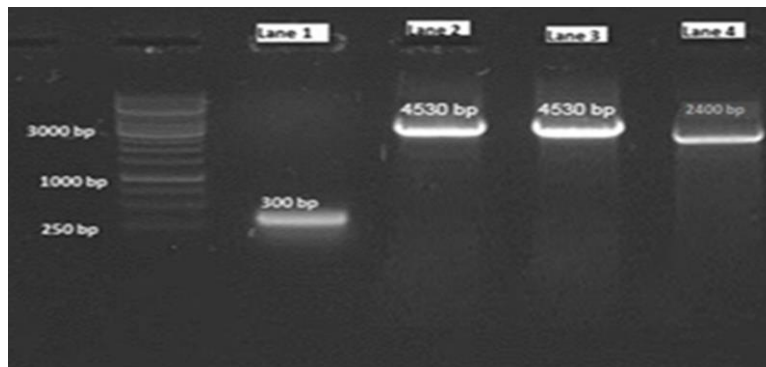
#### Statistical analyses

Statistical analysis was performed using Prism 5.0 software (GraphPad Software Inc., USA). One-way ANOVA was performed to analyze humoral immune responses. Data were represented as mean  $\pm$  standard deviation (SD). For all comparisons,  $P < 0.05$  was considered statistically significant.

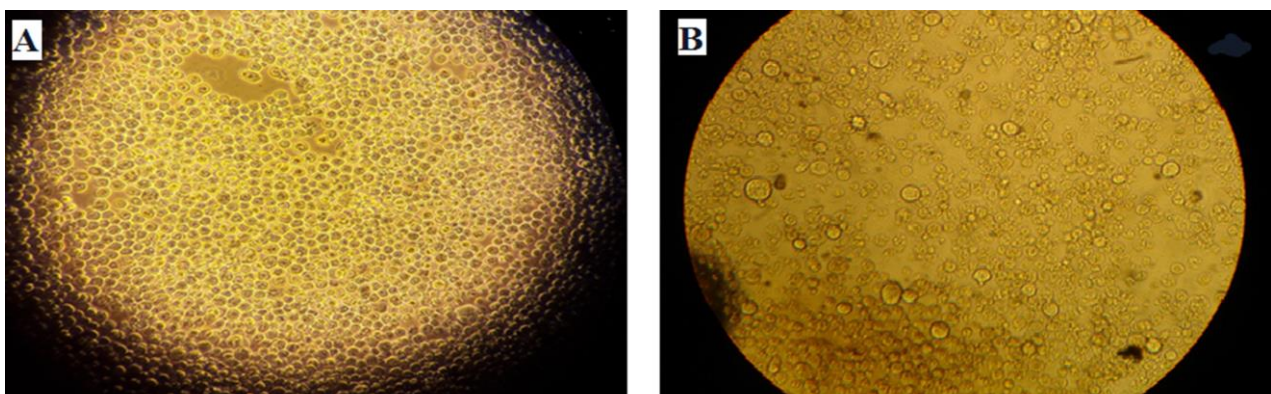
## RESULTS

### Construction of recombinant baculovirus

The synthesized HCMV glycoprotein B coding, sub-cloned into the fastback HTA (donor vector) and transformed into the DH10-Bac competent cells were verified by colony-touch PCR using M13 specific primers. The PCR product of two white colonies were verified to encompass the transgene (ectodomain of CMV gB with 2,080 bp) with about 4500 bp length in comparison with the band corresponding to the non-recombinant bacmid which was isolated from a blue colony with 2430 bp in length as a control (Fig.1). Five days after



**Fig. 1.** Gel electrophoresis of colony-touch PCR experiment for verification of correct transposition of the insert into the bacmid. Lane1: PCR band of blue colony containing the bacmid alone; Lane 2 and 3: These 4530 bp bands corresponded to the bacmids transposed with recombinant pFastBac HTA containing HCMVgB (2430 bp+ 2100 bp) which were extracted from two white colonies; Lane 4: A 2430-bp band of the bacmid transposed with an empty donor plasmid (pFastBac HTA).



**Fig. 2.** Images of Sf9 insect cells by inverted phase microscopy. A: Normal Sf9 cells before the viral infection; B: Recombinant baculovirus-infected Sf9 cells with typical cytopathic effects including increased cell diameter, vesicular appearance and detachment.

transfection of Sf9 cells with the verified recombinant bacmid, the cytopathic effects of viral infection were observed clearly (Fig. 2) and supernatant containing the recombinant baculoviruses were collected.

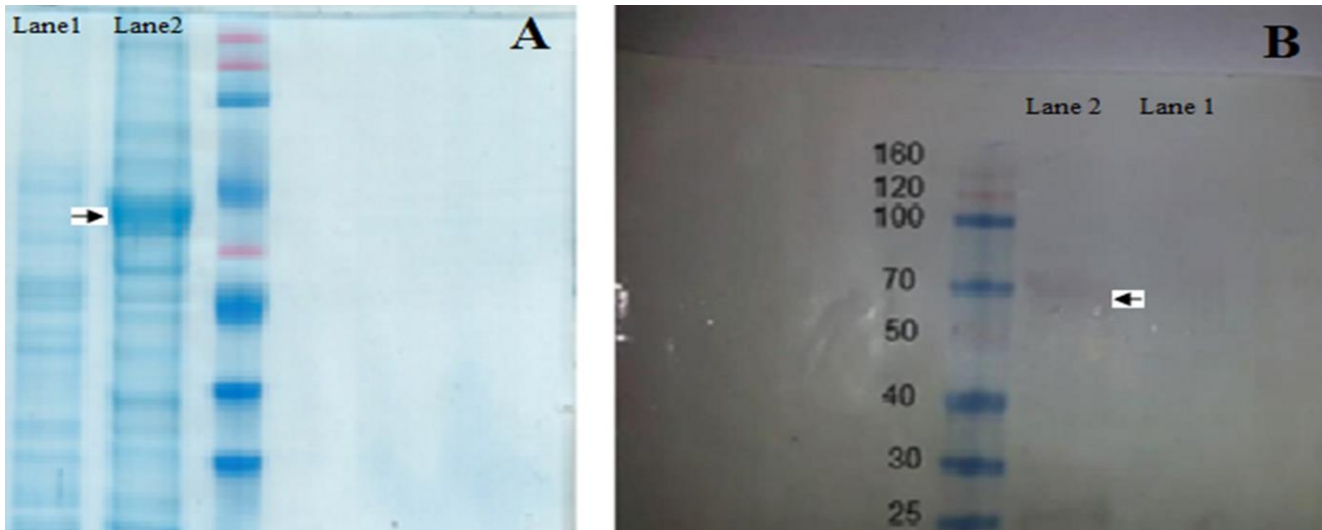
### Verification and Purification of truncated HCMV gB by Western Blotting

Following infection of Sf9 cells by the recombinant baculovirus (MOI = 1), the cell lysate was subjected to SDS-PAGE (Fig. 3A) and the separated protein fractions were analyzed by Western blotting. A specific band of ~70 kDa was detected (Fig.3B). The recombinant protein was purified at native condition to use for immunization (data not shown).

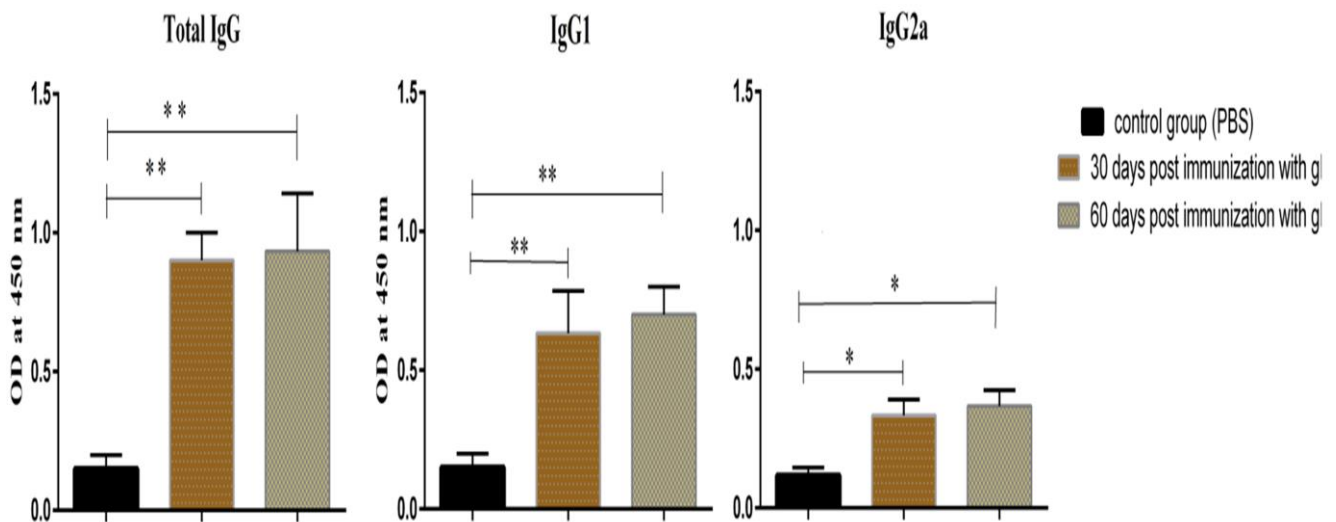
### Vaccination with truncated HCMV gB Induces high levels of IgG antibody in mice

The HCMVgB-specific humoral responses of mice were analyzed by ELISA, at 4 and 8 weeks after the last immunization. The total IgG, IgG1 and IgG2a antibody responses to HCMV gB epitope are depicted in Fig.4. Total anti-CMV gB IgG was significantly higher than the control group ( $P < 0.001$ ). The measurements of IgG subclasses were also conducted to evaluate the T-helper (Th) response involved. As shown in Fig. 4, a significantly higher titer of HCMVgB-specific IgG, IgG1, and IgG2a were observed in the vaccinated mice ( $P < 0.001$ ) whereas the IgG2a/IgG1 ratios for the vaccinated group at days 30 and 60 post immunization were 0.8 and 0.6, respectively. This data indicated the humoral immune response against HCMVgB with a moderate dominance of Th2 cells. Additionally, the antibody titer (IgG) were stable for at least 8 weeks after the last immunization for all test groups.





**Fig. 3.** A) Lysate of Sf9 cells infected with the recombinant baculovirus, subjected to SDS-PAGE. B) Western blot analysis using HRP conjugated anti-HCMV gB. The expression of recombinant HCMVgB protein (~70 kDa) was detected in insect cells which were infected by recombinant baculovirus (Lane 2) as compared to un-infected cells (Lane1). The arrows show the location of the recombinant protein.



**Fig. 4.** Analyses of total IgG response and the subclasses, elicited by HCMV gB. P-values were calculated by t-test to evaluate the statistically significant differences (\*  $P < 0.05$ ; \*\* $P < 0.001$ ).

## DISCUSSION

The development of a subunit vaccine is still one of the promising approaches to prevent HCMV infection. A great number of such subunit vaccines incorporate HCMV gB protein in their design. CMV gB facilitates entry into the majority of all cell types and is a prominent target of CMV-specific antibody responses. In particular, the application of HCMV gB subunit vaccine, adjuvanted with MF59 (a squalene-based oil-in-water emulsion) is one of the most promising approaches to elicit high and efficient neutralizing antibodies against HCMV infections. The first model among these vaccine candidates, consisted of a modified gB protein with a deletion in its transmembrane domain, formulated with MF59 adjuvant [14]. This subunit vaccine incorporated the N-terminal 676 and C-terminal 131 amino acids of the wild-type gB protein [15]. Another study revealed that gB/MF59 vaccine boosted the neutralizing antibody titers in HCMV-infected seropositive women with 14–40 years of age [16]. Additionally, the

gB/MF59 vaccine has been assessed for boosting immune response in individuals who had previously received ALVAC-gB vaccine and for simultaneous administration of the ALVAC-gB vaccine; however, no benefit of these regimens has been documented [17].

In this study, we presented an example of HCMV subunit vaccine targeted for eliciting the humoral immune response. The originality of our study is the use of BEVS to express HCMV glycoprotein B. Our Western blot data demonstrated a specific band with size of ~70 kDa for ectodomain of HCMV gB protein in infected Sf9 cells, as compared to normal insect cells. Our immunological experiments showed that the mice immunized with the recombinant gB protein elicited significantly higher total IgG antibodies, compared to unimmunized mice. In addition, a mixed IgG1/IgG2a response was demonstrated with rather high intensity toward IgG1. Evaluation of ELISA results also revealed that vaccination with HCMV gB applied a major impact on the stimulation of Th1/Th2 immunity with higher level of IgG1 subclass which is commonly considered as the dominance of Th2 subpopulation

response. This phenomenon might be the result of not using an adjuvant through the administration. Taken together, these data indicate that antibodies induced by this HCMV gB subunit vaccine strategy are capable of producing a robust humoral response even without using the conventional adjuvants. The application of such HCMV gB subunit vaccine might significantly improve the survival of immunocompromised people and could be more effective at decreasing the viral load.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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