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RESEARCH ARTICLE

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Expression and purification of HCMV glycoprotein B full protein in *Escherichia coli*

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ABSTRACT

Background: Human cytomegalovirus (HCMV) is associated with significant mortality in immunocompromised individuals and neonates and is a major concern of global public health. Some viral glycoproteins such as glycoproteins B have an important role on virus life. This herpesvirus fusion protein is a candidate for the vaccine due to its undeniable role in infection.

Materials and Methods: Here we designed a full-length gB sequence optimization to increase the expression level of this protein. Numerous factors such as the adaptation index of codon, codon context, and adapted GC content based on *E. coli* codon usage. In addition, the ribosome binding site (RBS) of pET-15b was redesigned. Various factors such as IPTG concentration and induction time were optimized. The recombinant gB protein was purified by the Ni-sepharose column.

Result: Among IPTG concentrations, and post inductions, the level of gB was higher in 1mM IPTG, and 8 hours, respectively. In hybrid purification conditions, gB protein was purified completely.

Conclusion: The protein designed in this study be expressed in the prokaryotic system and with an optimal design, it can be purified properly. Our data showed that it can be used as an effective vaccine in vivo immunogenicity evaluation in the future.

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INTRODUCTION

Human cytomegalovirus (HCMV) is considered as a member of the beta-herpesvirinae (HHV) sub family attributing in 40-100% of the population all over the world. Indeed, it can be said that is a ubiquitous human pathogen (1). CMV genome is defined as a double-strand DNA molecule and the length of approximately 230–240 kb encoding more than 200 polypeptides having a significant effect on virus life and interacting with the immune system during latent or acute infections (2). The main cause of neonatal and congenital hearing loss is HCMV which arises from vertical virus transmission as a result of infection or reactivation of the latent virus in pregnant women (3). Evident morbidity and mortality are observed in infected infants with congenital as well as cardiovascular diseases and cancer (4). In addition, HCMV is one of the most common opportunistic pathogens influencing immunosuppressed transplant patients. The infected seronegative cases that received solid organ transplants from seropositive donors have a positive relationship with an incidence of allograft rejection increasingly (5, 6). Although the development of vaccines against HCMV has been listed by the Institute

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of Medicine as a top priority (7, 8) , none has been licensed so far.

Since preconception maternal immunity to HCMV partially protection against vertical transmission of infection, there is a strong interest in producing HCMV vaccines, and assessing different HCMV vaccines in clinical examinations. Interests consisted of vectored and subunit vaccines, as well as live attenuated vaccines, which target the HCMV proteins that appear to be most important in protective immunity (9).

A number of immunogenic CMV proteins have been proposed that could be used in a vaccine. These include glycoprotein B (gB) (10). This protein plays a role in stimulating both cellular and humoral immunity against the virus; it eligible to be used in a CMV vaccine (11). GB is one of the main envelope proteins playing a significant role in viral attaching and transmitting into the cell (2) and is an important component of recombinant vaccines under trial (12). The gB is the focus of most of the recombinant vaccine strategies as it is abundant and highly conserved. The gB protein contains 906 amino acids consisted of an ectodomain, membrane-proximal region (MP), transmembrane region (TM), and cytoplasmic domain (Cy) (4). It possesses 5 discreet antigenic sites targeted by gB-specific antibodies and antibodies against gB can be neutralizing or non-neutralizing. GB is the main goal of neutralizing antibodies and has been identified as the end of the humoral immune system and an applicant of subunit vaccines. Antibodies that detect two different antigenic regions on gB can prevent the virus from binding or fusing to the host cell (13).

Although recent advances have been made in describing the biological, immunological, and antiviral therapeutic targets of the virus considerable work is still needed to design therapeutic and preventive strategies for Cytomegalovirus infections. The studies aimed to produce and purify the major envelope glycoprotein, of HCMV, gB (12). GB is the target for neutralizing and non-neutralizing antibodies, each representing both discontinuous and continuous epitopes (14).

Currently, pET is the most potent system designed to easily clone and express recombinant proteins in *Escherichia coli*. There are over 40 types of pET plasmid. About 80% of proteins whose three-dimensional structure has been registered in the protein database in 2003 are expressed in *Escherichia coli* and 90% of them are made through the pET system (15).

In previous studies, many peptides have been employed as a vaccine against CMV. The most plentiful covering protein (gB) is a major target of the cellular and humoral immunity. It seems that it is the best option to prepare a suitable vaccine for CMV, synchronously by taking advantage of epitopes dealing with the immune dominant protein derived from these proteins. In this study, a full-length gB gene of HCMV have been designed (for the prototype AD169 strain) in the pET15 vector using *E. coli* BL21. Furthermore, we present a different hybrid purification condition to purify full-length gB. Further studies have shown that gB could be an attractive targeting for antibody-based immunotherapy. This has prompted significant interest in developing a subunit vaccine for HCMV and several viral envelope glycoproteins, such as gB, are considered excellent candidates for this purpose.

MATERIALS AND METHODS

Design of codon-optimized gB gene:

The full-length sequence of gB from HCMV (GenBank accession no. **FJ527563.1**) was designed to increase the expression levels of this protein. In this study, researcher employed *E.coli* codon usage database (http://www.kazusa.or.jp/codon/) for evaluating and assessing the index of codon adaptation (CAI), frequency of optimal codon (FOP), codon context and GC content related to the coding sequence. Also, the sequence of ribosome binding site (RBS) of pET-15b was removed by *XbaI* and *XhoI* and it was redesigned by the RBS calculator to avoid entering of the RBS into the secondary structure with its downstream and upstream sequences. In addition, the secondary structure of gB mRNA was optimized in the whole length of coding sequence and for assessing the hairpins' stability, researcher used RNA fold web server, Vienna RNA (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The final nucleic acid sequence was integrated by Biomatik Co. (Canada) and cloned into the two restricted enzyme sites (*XbaI* and *XhoI*) of pET-15b. To confirm the cloning of the correct sequence, the resulting sequence was compared with the NCBI database using BLAST.

Protein expression

Enzyme digestion and PCR with specific primers for gB gene emphasized Purified plasmids. The confirmation of the constructs was done both methods and the constructs were used for subsequent valuation of protein expression. The process of culturing transformed bacterial cells overnight in LB broth (supplemented with 100 µg/mL ampicillin) is at 37°C in a shaking incubator (180 rpm) for 16 hrs. Dilution of cultures was 1:100 into fresh LB (containing 100 µg/mL ampicillin and 1% glucose) and cultures grew to reach an OD600 of 0.6-

0.7. When the OD of the culture at 600 nm reached 0.6, isopropyl Beta-D-thiogalactoside (IPTG) was added at different densities (0.2, 0.5, 1, 1/5, 2, and 3 mM). Additionally, samples were collected at different post-induction times (0, 1, 2, 3, 4, 5, 6, 7, 8, and 14 hours) to optimize the induction conditions.

Purification of recombinant gB protein

In the process of purification recombinant glycoprotein B under native condition, lysis buffer (50 mM NaH_2PO_4, 10mM Imidazole, and 300mM NaCl, pH 8.0) was counted up into the cell pellet and sonicated for 10 times (30s on, 30s off). After the solvent was centrifuged, the supernatant was loaded onto 500 µl pre-packed Ni–NTA affinity chromatography column (Qiagen-CN: 31014). The washing process of column was done with five bed volumes of the wash buffers (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM Imidazole, pH 8.0) two times. The elution of gB protein was conducted with two bed volumes of the elution buffer (50 mM NaH_2PO_4, 250 mM imidazole, 300 mM NaCl, pH 8.0) three times. Three different concentrations of imidazole (750-500-250 mM) were used for the optimization of purification in three different pH (7-7.5-8). To increase the purification band, 2 ml of Triton x100 was added to the first solution and the salt concentration was changed from 300 Mm to 1 M in all solutions. But the outcome was not favorable and many additional bands were withdrawn.

Denaturing conditions

As condition was denatured, lysis buffer (8 M Urea, 10mM Tris, 100mM NaH_2PO_4, pH 8.0) was included into the cell pellet and sonicated for 10 times. When the solvent was centrifuged, the supernatant was loaded on to 500 µl pre-packed Ni–NTA affinity chromatography columns. The five bed volumes of the wash buffers were used for washing the colmns(100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, pH 6.3) three times. Two bed volumes of two elution buffers eluted gB protein (100 mM NaH2PO4, 10 mM Tris 8 M Urea, pH 4.5 and 3.5) which had different pH 4.5 and 3.5. But the outcome was not favorable and many additional bands were withdrawn.

Hybrid condition

Lysis buffer in which cell pellet was re-suspended possesses guanidinium hydrochloride (100 mM NaH_2PO_4, 10 mM Tris, 6 M GuHCL, pH 8.0). After centrifuging the solvent, the supernatant could pass through the Ni-NTA agarose column under denaturing conditions. Five bed volumes of the native wash buffers were employed for washing the column three times. Elution process of gB protein was performed with two bed volumes of two native elution buffers containing different imidazole concentrations (250 mM and 500 mM) and in the last phase, th elution of proteins was just once with the assistance of denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, pH 4.5). The systematic modification process of parameters in this fundamental protocol optimized to purify gB by different incubation times (2 min for wash buffer and 5 min for elution buffer) and pH 7 for wash buffer. The gB protein purification was measured by SDS-PAGE.

SDS-PAGE, Western blot

Samples were combined with buffer sample (0.5 M Tris-HCl [pH 6.9], 0.25% bromophenol blue, 10% glycerol, 5% SDS, 5% b-mercaptoethanol). They were boiled at 95°C for 5min, resolved by 10% SDS-polyacrylamide gel, and were identified by taking advantage of Coomassie blue stain (Bio-Rad). For Western blotting, the Bis-Tris gel was moved into a nitrocellulose membrane. Then, the nitrocellulose was blocked at 4°C for 24 h with 5% non-fat milk in PBS–Tween 20 and was incubated with a 1:5000 dilution of goat polyclonal anti-gb-HRP (Abcam, USA) at room temperature for 60 min. The 3, 3′-Diaminobenzidine (DAB, Sigma Aldrich, and USA) was used for visualizing antigen-antibody reaction.

RESULTS

Codon optimization

In this study, the GC content before and after the optimization process showed an increased from 52.08% in the normal state to 55.23% after optimization. Also, the amount of CAI indicates how much of a codon in a gene resembles that of codons in highly-expressed genes from 0.50 before optimization to 0.96. FOP after optimization, the highest amount of codons was changed to a value of 80 to 100. After optimization, alignment between the DNA sequence and the wild-type gene was detected. The alignment of NCBI site showed both sequences encoding a protein with the identical amino acid sequences.

Expression

A high level of expression was observed on SDS-PAGE induced with 1 mM of IPTG in comparison to 0.2, 0.5, 1, 1/5, 2, and 3 mM (Figure 1A). At the time gradient, IPTG was induced at a concentration of 1 mM and then precipitated at 1, 2, 3, 4, 5, 6, 7, and 14 hrs (Figure 1B). Since the half-life of gB protein in bacteria was more than 10 h, the protein expression increased from 1 to 14 h. Therefore, optimal conditions applied for gB expression in the present study were as follows. The overnight culture from a plate that has

been freshly inoculated would be extended in a 50 ml flask until an OD600 = 0.6 at 37°C, and then the induction of extended bacteria would be done by 1 mM IPTG and cultured constantly at 37 °C for 8 h.

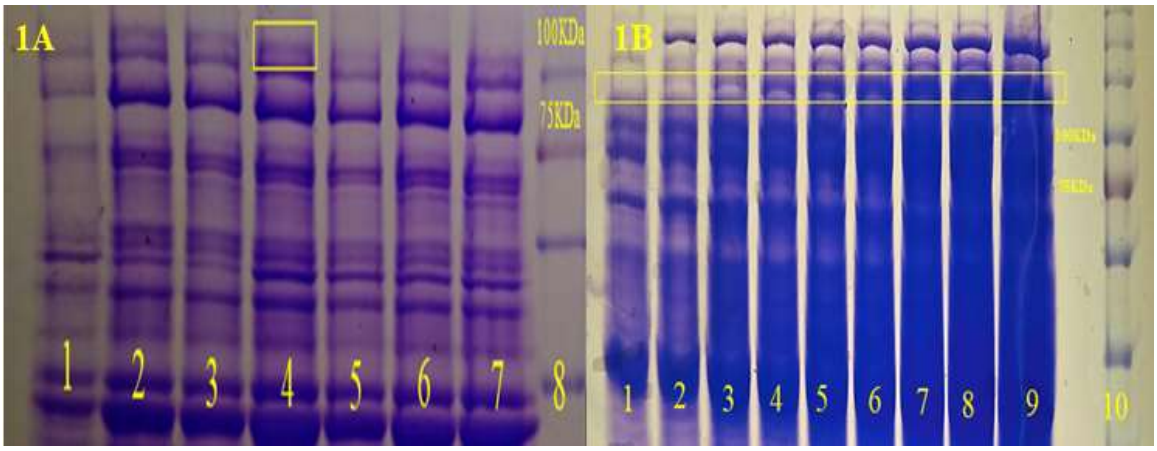

Figure 1A Coomassie blue-stained SDS-PAGE of the pellet fractions of cells developed at various concentrations of IPTG. Uninduced total cell lysate of *E. coli* BL21 (DE3)-PET15b-gB, (lane 1), 0.2 mM IPTG (lane 2), 0.5 mM IPTG (lane 3), 1 mM IPTG (lane 4), 1.5 mM IPTG (lane 5), 2 mM IPTG (lane 6), 3 mM IPTG (lane 7), Protein size marker (lane 8). Figure 1B Coomassie blue-stained SDS-PAGE of gB expression level analysis at different points of post-induction; lane 1, uninduced total cell lysate, lanes 2-9, whole lysate of *E. coli* BL21(DE3)-PET15b-gB post-induction at 1, 2, 3, 4, 5, 6, 7, 8, and 14 h, respectively. Lane 10, protein molecular weight marker (kDa).

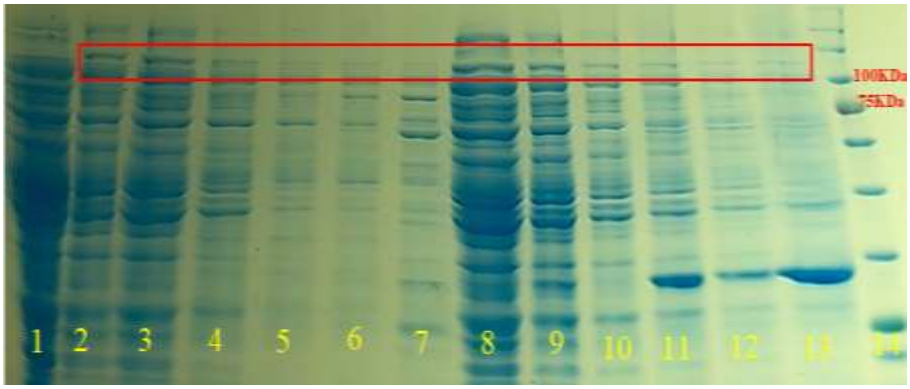

Figure 2 gB was expressed and purified in Native and Denature conditions from *E. coli* BL21 (DE3) cells. Samples were separated on 10% SDS-PAGE: lane 1, uninduced total cell lysate, purified gB protein from *E. coli* BL21 (DE3) cells at different steps of native purification including, lysis (lane 1), washing (lanes 3-4), and elution (lanes 5-7); purified gB protein from *E. coli* BL21 (DE3) cells at different steps of denature purification including lysis (lane 8), washing (lanes 9-11), and elution (lanes 12-13); and protein molecular weight marker (kDa) (Lane 14).

Expression

High level of expression was observed on SDS-PAGE induced with 1 mM of IPTG in comparison to 0.2, 0.5, 1, 1/5, 2, 3 mM (Figure 1A). At the time gradient, IPTG was induced at a concentration of 1 mM and then precipitated at 1, 2, 3, 4, 5, 6, 7, and 14 hrs (Figure 1B). Since the half-life of gB protein in bacteria was more than 10 h, protein expression increased from 1 to 14 h. Hence we applied the optimal conditions for gB expression in our study were as follows. The overnight culture from a freshly inoculated plate would be expanded in a 50 ml flask until an OD600 = 0.6 at 37 °C, and then the expanded bacteria would be induced by 1 mM IPTG and continually cultured at 37 °C for 8 h.

Purification

Purification under both completely denaturing and native conditions (Figure 2) did not lead to products with satisfactory purification and so different hybrid purification protocols mixing denaturing conditions for both connecting to the column and cell lysis with native buffers have been assessed for eluting and washing the proteins. Optimization for purification

conditions was done and the by SDS-PAGE was used for evaluating the purification of protein purity (Figure 3). Also, immunoassay of purified gB by coat anti-gB antibody depicted an intense brown color reaction with the protein size corresponding to ~103 kDa for gB protein (Figure 4).

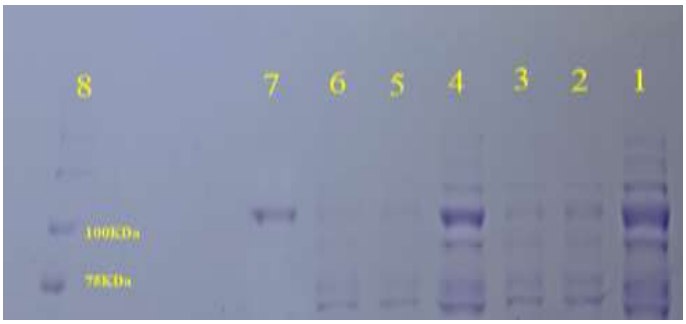

Figure 3 gB was expressed and purified from *E. coli* BL21 (DE3) cells in the hybrid condition. Samples were separated on 10% SDS-PAGE: purified gB protein from *E. coli* BL21 (DE3) cells at different steps including, lysis (lane 1), washing in native condition (lane 2-4), elution imidazole 250 mM (lane 5), elution imidazole 500 mM (lane 6), elution denature buffer pH 4.5 (lane 7), and protein molecular weight marker (kDa) (Lane 8).

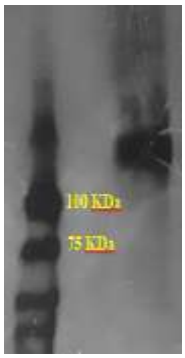

Figure 4 Western blot: Proteins on SDS-PAGE gel were transferred onto nitrocellulose membrane and gB proteins were detected using the anti-HCMV-gB antibody. Protein size of ~103 kDa corresponds to gB protein.

DISCUSSION

Antiviral antibodies can be a great substitute strategy for confining the clinical complications of viral diseases in patients (10). The improvement of effective, inexpensive, and free risk vaccines is one of the significant challenges in medical science. To design an effective peptide vaccine, it is important to identify proper viral antigens for the induction of protective immune responses (16). The present study, described a highly efficient way of producing large quantities of gB protein using the *E.coli* expression system. One approach that has been proven to be useful in herpesviruses and other viral systems is to express and purify recombinant, and soluble versions of envelope glycoproteins and evaluate their functional features. Although such lines of experimentations are limited in the sense that cooperative interactions between multiple envelope proteins are not addressed, they are often very revealing as to functional roles and sufficient quantities of these proteins in the native state can lead to practical studies (17). gB is a major objective for neutralizing antibodies. In addition, it is an attractive molecule for the intrusion of strategies including passive immunotherapy or vaccination (14). So far, various studies have been conducted on the antigenic components of gB, most of which have focused on humoral immunity. For example, Vahabpour selected ectodomain of HCMV gB (18) and Tabaei evaluated three immuno dominant sequences of gB (16). Since gB has different domains that stimulate neutral and non-neutralizing antibodies, as well as both humoral and cellular immunity, it can be a target for a vaccine, which is why we chose the full length of this protein. In the Gill study, full length gB was selected that was extracted from the clinical sample (19) but in order to prevent problems such as low solubility or low expression in the prokaryotic expression system, we designed a full-length gB fragment using genetic engineering.

The protein purification is disputing due to similarities between the features of the targeted

protein and other remaining proteins in a complex protein mixture. The present study examined the cloning and expression of the target gene fragments chose regarding bioinformatics analyses. These had been cloned by employing genomic sequence data in *E. coli* BL21 using a vector that counted up a 6xHis tag to the cloned protein for purifying on a nickel-conjugated agarose resin (20).

In this study, the benefit of codon optimization was assessed for developing the expression of gB in *E.coli*. This study designed the full-length gB gene by selecting the most favorable codons avoiding the formation of stable secondary structures in the corresponding mRNA sequence. mRNA stability is generally demonstrated by free energy changes. It also breaks the loop-stem structures that can dramatically reduce translation efficiency with the algorithm as far as possible, resulting in better ribosomal binding and mRNA stability. FOP is the distribution percentage of codons in computed codon quality groups. The value of 100 is defined for a codon with the highest usage frequency for a specific amino acid in the expression organism. Recombinant proteins are highly expressed by optimizing codon. Since preferred codons are various in a variety of organisms, a direct relationship was observed between the level of expression and the host codons. Codon optimization defined with respect to different meanings as adjusting the GC content, removing the motif, which is not conducive to useful expression and add the assistant one, simplifying mRNA's secondary structure after gene transcription, avoiding rare codons with low usage, and other methods to re-design genes (13).

In our study, the native method was initially used depending on the type of protein. Imidazole concentrations from 250 to 750 were tested for protein purification, but the protein alone was not purified. Also, a hybrid purification strategy was turned to account. In this strategy, aforementioned cells were lysed and the proteins bound to the column under denaturing conditions. These cells were washed under native conditions and elusion was done under native and denaturing conditions with various buffer pH, urea, and imidazole concentrations.

Some optimization processes related to purification conditions have been done consisting of imidazole concentrations and pH of the elution and wash buffers. In Liu's study, the purification findings under completely denatured or native conditions were not recognized acceptable. Therefore, different hybrid purification conditions were optimized for protein (13).

Optimal purification depends on various factors including the amount of necessary 6xHis-tagged protein and expression level. The quantity of culture is based on the level at which the protein is expressed, which must empirically be specified for each expression experiment. If the protein is not significantly expressed, bacteria from a large culture volume should be lysed in a certain volume of lysis buffer in consistence with a high "concentration factor". In the native condition, the prediction of the amount of protein soluble in lysate is difficult. In general, 50-100 times the concentration is suggested for purifying an evident amount of the protein labeled with 6xHis. Protein expression under native conditions is recommended for optimal purification of 50-100 ml, which is greater than 1% of the protein expression in 50 ml culture medium if more than 50 µg of protein is labeled with 6xHis. It occurs if the expression is less than 1% compared to 100 ml culture and less than 100 µg of 6xHis protein is obtained after purification. In the present study, the desired points of steps were observed, and there was no difference in the amount of protein between the volumes 50 ml and 100 ml.

The interaction occurring between the 6xHis tag of the recombinant protein and Ni-NTA is not based on the third structure so that the proteins can be purified under both denaturing and native conditions. For designing the best purification strategy, determination of whether the protein is located in the cytoplasmic inclusion body or soluble in the cytoplasm is essential (21, 22). Providing a universal protocol for purifying 6xHis-tagged proteins under native conditions is hard as there is significant variation in the proteins' structure interfering with binding. Strong denaturants including 8M urea or 6M GuHCl completely solubilize 6xHis-tagged proteins and inclusion bodies. In our study, the addition of GuHCl was effective in the first step and resulted in a better binding of the protein to the resin due to the exposure of the 6xHis-tagg. Different examinations on general protocol for the purifying 6xHis-tagged proteins have revealed the importance of purification in the function of protein (22, 23). In our study, many tests were performed to optimally purify the proteins. Finally, a hybrid protocol containing natural and denaturant solutions resulted in gB purification. In this study, an effort was made to design a full fragment of the gB cytomegalovirus that is capable of stimulating both cellular and humoral immune systems and it is valuable.

We did not evaluate the levels of immunogenicity, leaving many unanswered questions regarding gB immunogenicity determinants. The synthesis of these recombinant proteins is an important issue that

needs to be addressed by researchers. Studies show that we need to reconsider our knowledge about supported mechanisms of gB vaccine's response and assumptions about vaccine-induced correlates of protection against acquisition of CMV infection and the relationship between vaccine usage and patient populations and polymorphisms in gB epitopes is a matter a fact (24, 25). According to the study by Baraniak et al, in gB-vaccinated CMV SOT patients, the AD-2 domain is significant in mediating protection against CMV disease in seropositives because antibody's response to this epitope was remarkably lower in seropositive subjects who improved CMV viremia following SOT, in compare to seropositives remaining free of viremia (26).

Isolating a stable prefusion and quality in terms of purification form of CMV gB can be an advantage for future vaccine design. These examinations and other experimental approaches in finding the immunological foundation of protection conferred by gB/MF59, as the most successful CMV vaccine tested so far, will help inform and direct the development of improved “next-generation” gB vaccines for this crucial public health problem.

CONCLUSION

The protein designed in this study can be expressed in the prokaryotic system and with the optimal design; it is possible to be purified properly. Our data suggest that it can be used as an effective vaccine in the in vivo immunogenicity evaluation in the future. It is hoped that, given the challenges involved, the need to introduce optimal combinations of CMV vaccine, the need to introduce protective criteria for obtaining a vaccine for congenital CMV infection, and needs to address the challenge of successful vaccine design against reinfection. It was later used as an effective vaccine in the in vivo immunogenicity evaluation.

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