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Production and intracellular trafficking of SARS CoV-2 spike protein in insect cells infected with recombinant baculovirus

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ABSTRACT: SARS CoV-2 belongs to the *Coronaviridae* family and is an enveloped virus with a positive polarity single-stranded RNA genome. The virus's spike protein, embedded in the viral membrane, is the most important antigenic protein involved in binding the virus to the host cell receptor. This protein is the basic component of vaccines developed against the virus due to its antigenic character. Therefore, it is crucial to produce this protein heterologously. This study evaluated the potential of ExpiSf9 and Hi5 insect cells infected with recombinant baculoviruses carrying the spike gene to synthesize the spike protein. The synthesis of the spike protein in infected cells was analyzed using SDS-PAGE/silver staining, Western blotting, and immunofluorescence techniques. High levels of spike expression were detected in virus-infected cultures at 72 hours post-infection compared to cellular proteins. The immunostaining results showed that spike proteins were present in the cell cytosol as aggregates, indicating that the proteins were transported via the endoplasmic reticulum-Golgi transport pathway. The Western blot analysis revealed that the spike proteins undergo post-translational modifications, such as glycosylation and proteolytic cleavage, in both insect and mammalian cells. Based on this data, it has been concluded that the baculovirus expression system is a suitable and cost-effective method for producing the spike protein. This protein can be used as an antigenic component in the subunit vaccine against Covid-19.

KEYWORDS: *Coronaviruses*; SARS CoV-2; recombinant spike protein; Covid-19; baculovirus; insect cells

1. INTRODUCTION

Coronaviruses are enveloped, positive-stranded RNA viruses that primarily infect the upper and lower respiratory tract (sinuses, nose, pharynx and lungs) in humans and cause relatively mild clinical symptoms such as pneumonia, bronchitis, pharyngitis, sinusitis and diarrhea in other systems [1]. However, the outbreak of Severe Acute Respiratory Syndrome (SARS) virus (SARS-CoV-1) in China in 2002 [2-4] and the outbreak of Middle East Respiratory Syndrome (MERS) virus (MERS-CoV) in Saudi Arabia in 2012 [5] have shown that coronaviruses can pose a major threat to human health. According to the World Health Organization (WHO), 774 people died in the SARS-CoV-1 epidemic [6] and 866 people died in the MERS-CoV epidemic [7], two local epidemics with high mortality rates that were prevented from becoming a pandemic. The SARS-CoV-2 virus, which first emerged in Wuhan, Hubei Province, China, in late 2019, caused the Covid-19 pandemic [8]. According to the World Health Organization data, the new coronavirus has infected about 774.6 million people worldwide and caused more than 7 million deaths (https://data.who.int/dashboards/covid19/deaths?n=c : 27.02.2024). As a result, all countries unexpectedly faced with this SARS CoV-2 pandemic suffered a major economic and social shock. In the short term, vaccines were considered to be the most effective means of protection against this virus. Inactivated virus vaccines and mRNA vaccines, developed in a very short time under extraordinary conditions, provide significant protection against SARS CoV-2 [9, 10]. In addition, vaccine production and development of new vaccines against the SARS CoV-2 virus is ongoing. One of the types of vaccine used against common infections such as influenza is the vaccine consisting of viral antigens produced by recombinant DNA techniques [11]. Insect cells are used as host organisms for the production of viral antigens, mostly in glycosylated forms, because they are much more cost-effective than mammalian cells [12]. Baculovirus expression systems are most commonly used to produce viral antigens in insect cells [13, 14]. Baculoviruses

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are a family of DNA viruses with closed, circular, double-stranded DNA genomes ranging in size from 80 to 180 kb that infect insects [15]. These viruses are considered safe for humans as they do not infect vertebrates. High levels of recombinant antigen can be obtained by cloning the relevant genes into the genetically engineered virus genomes under the control of promoters that allow constitutive expression, such as the polyhedrin promoter [16].

The spike protein of the SARS CoV-2 virus is one of the major targets of the host defence mechanism. For this reason, subunit vaccine development studies have mostly focused on the spike protein, and the strategy of heterologously producing this protein in another host organism of the virus or synthesizing it directly in human cells has been implemented using various vectors developed by DNA technologies [17-21]. Although easy to implement and inexpensive, the glycoprotein structure of the spike protein significantly limits its heterologous production using prokaryotic expression systems. Therefore, baculovirus expression systems, which are much less expensive than mammalian expression systems [22], appear to be an ideal tool for the production of spike proteins.

In this study, the SARS CoV-2 spike protein production and secretion potential of ExpiSf9 and Hi5 insect cells infected with baculoviruses carrying the spike gene were evaluated. Spike protein synthesis in virus-infected cells and protein secretion into the medium over time were analyzed by SDS-PAGE/silver staining and/or Western blotting. The synthesis and subcellular localization of spike proteins in the cells were also examined using immunofluorescence techniques. The results showed that the baculovirus expression system is an effective tool for the production of recombinant spike proteins that can be used as vaccines.

2. RESULTS

2.1. Growth profiles of insect cells

The growth profiles of ExpiSf9 and Hi5 insect cells in serum supplemented and serum-free Sf-900 III SFM medium at 28 °C and 120 rpm shaking conditions were determined by counting live and dead cells at specified intervals. Under these conditions, it was observed that the growth rates of both cell lines slowed significantly with an increase in the number of dead cells after an average incubation period of 120 hours (Figure 1). It has been reported that Hi5 cells proliferate faster than standard Sf9 and divide on average every 24 hours [23]. In this respect, Hi5 cells are favored for recombinant protein production. The growth profile in Figure 1B supports this situation. Sf9 cells, on the other hand, divide every 48-72 hours [24]. In contrast, ExpiSf9 cells were observed to proliferate as rapidly as Hi5 cells (Figure 1A). The addition of serum to Sf-900 III SFM media had a positive effect on cell growth.

2.2. Synthesis of SARS CoV-2 spike protein in cells infected with recombinant baculovirus

The native SARS CoV-2 spike protein is a glycoprotein consisting of 1273 amino acids. This protein is composed of S1 and S2 subunits, which are delimited by a short signal peptide (SP) at the amino-terminus and transmembrane/cytoplasmic (TM/CP) domains at the carboxy-terminus [25, 26] (Figure 2). The recombinant BacV-S virus used in this study encodes a protein that lacks the TM and CP domains consisting of 62 amino acids at the amino terminus of the native spike protein.

The recombinant BcV-dS.Fc virus encodes a hybrid protein consisting of the amino-terminal half of the spike protein (first 685 amino acid residues) fused to the Fc region of a human IgG antibody (Figure 2). The putative molecular weights of these two recombinant proteins were calculated from their amino acid compositions to be 133.2 kD and 116.5 kD, respectively.

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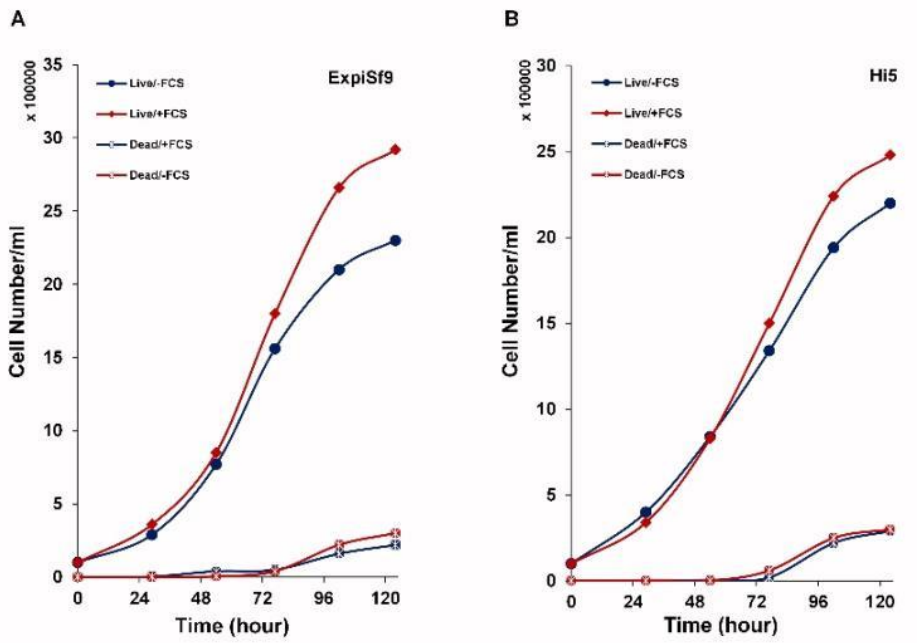

Figure 1. Growth profiles of ExpiSf9 (A) and Hi5 (B) cells in Sf-900 III SFM medium with (+FCS) and without serum. Cells were grown in 100 ml of medium at 28°C and shaking at 120 rpm.

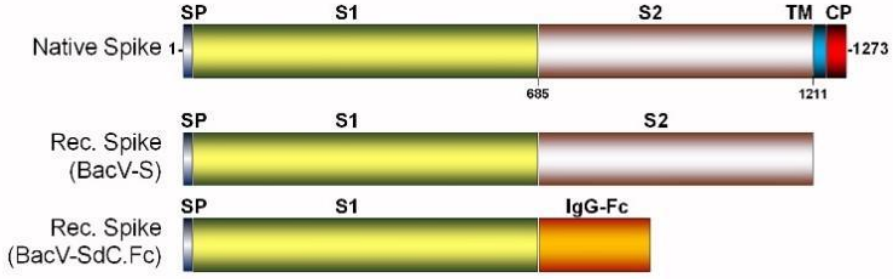

Figure 2. The representative structures of the native SARS-CoV2 spike protein and the recombinant spike proteins encoded by BacV-S and BacV-SdC.Fc viruses. BacV-S virus encodes a spike protein with deleted amino-terminal TM/CP domains. BacV-SdC.Fc virus encodes a hybrid spike protein fused to human IgG Fc.

The number of viable cells over time was determined in ExpiSf9 and Hi5 cell cultures infected with recombinant BacV-S and BacV-dS.Fc viruses at 0.1-0.2 MOI, which have an average of $1x10^6$ viable cells/ml at baseline (Figure 3). A much lower increase in the number of viable cells was observed in both ExpiSf9 and Hi5 cell cultures infected with BacV-S during the first 72 hours compared to uninfected cultures (see Figure 1). In contrast, BacV-dS.Fc-infected cultures did not show a significant increase in viable cells over time, but rather a rapid decrease.

In samples taken at 24-hour intervals from the virus-infected cultures, spike proteins both inside the cells and secreted into the media were analyzed by SDS-PAGE/silver staining and/or SDS-PAGE/Western blotting (Figure 4) . The SDS-PAGE/silver staining results showed that the recombinant spike proteins were synthesized in higher amounts than other cellular proteins within 72 hours of infection (Figure 4A). Western blot analyses showed that the amount of intracellular spike protein in infected cultures increased significantly over time (Figure 4B). Spike proteins secreted by the cells were determined by Western blot analysis of proteins in the culture media. The culture samples were centrifuged at 15,000 rpm and the supernatants were mixed with x4 sample loading dye at a ratio of 3/1 and applied to the gel. The results showed that some spike protein was secreted from the ExpiSf9 and Hi5 cells infected with both BacV-S and BacV-dS.Fc viruses (Figure 4C). Analysis of the protein bands detected in the medium from virus-infected ExpiSf9 cultures after 72 hours showed that the secreted spike proteins were of lower molecular weight than the intracellular spike proteins, and there was an additional protein band of 90-95 kD on the gel labeled with

anti-spike antibody (Figure 4D). These results suggest that the spike proteins are proteolytically cleaved at specific points during the secretion process after translation.

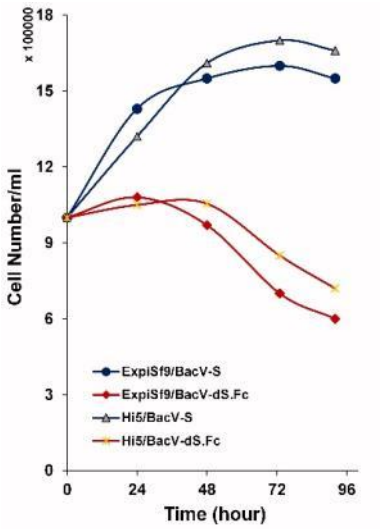

Figure 3. Growth profiles of ExpiSf9 and Hi5 cells in Sf-900 III SFM cultures contaminated with recombinant baculovirus.

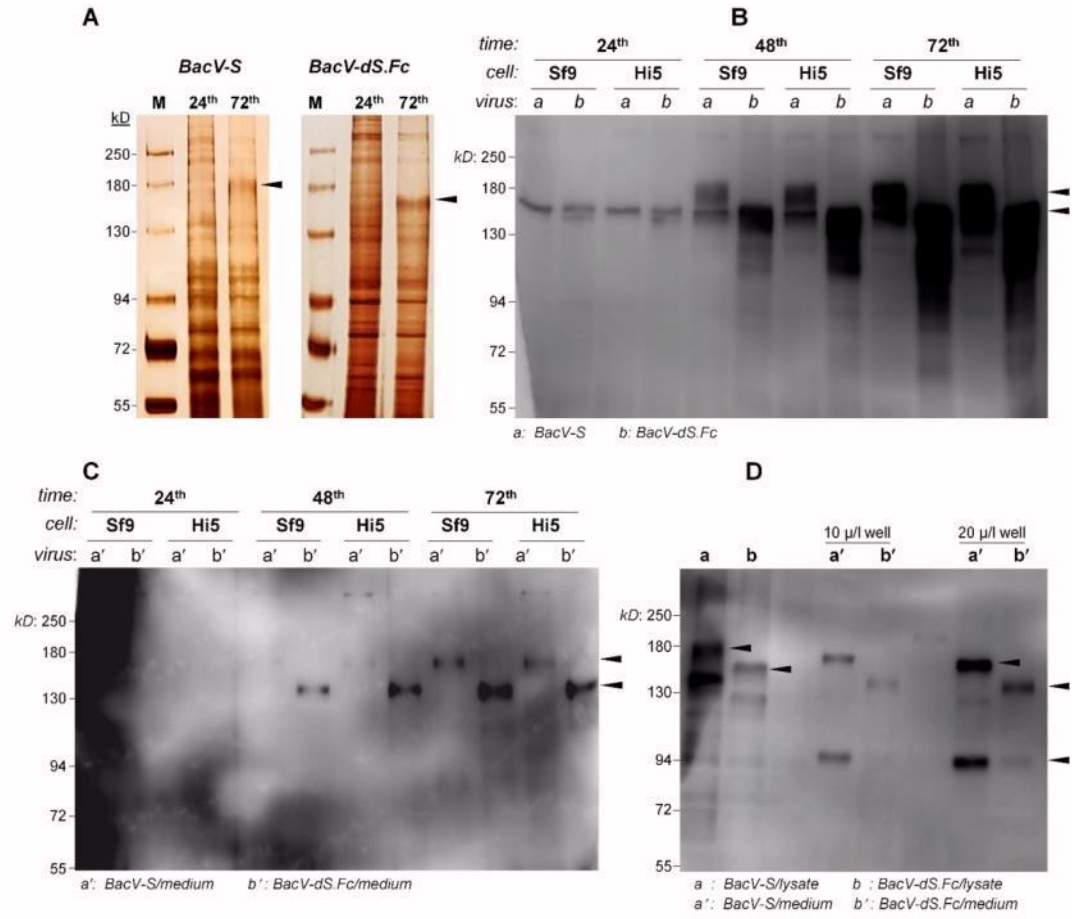

Figure 4. SDS-PAGE/silver staining and Western blot analysis of recombinant spike protein expression in infected ExpiSf9 and Hi5 cells. Samples were taken at time intervals after infection, cells were precipitated by centrifugation, and intracellular spike proteins were detected by SDS-PAGE/silver staining (A) or immunoblotting (B). The secreted spike proteins were detected by analyzing the proteins in the supernatants (media) with immunoblotting (C and D).

2.3. Subcellular localization of SARS CoV-2 spike protein encoded by recombinant baculovirus

The time course of expression and subcellular localization of spike proteins encoded by recombinant BacV-S and BacV-dS.Fc viruses in ExpiSf9 cells were examined by immunostaining (Figure 5). It was observed that spike proteins were detectable in infected cells at 16 hours of infection and the proportion of infected cells increased over time. After 64 hours of infection, almost all cells were infected and expressed high levels of spike proteins. The recombinant spike proteins accumulated as aggregates in the cytosol of ExpiSf9 cells. No nuclear localization of spike protein was observed even at the late infection stage. This localization pattern suggests that spike protein, a membrane-bound glycoprotein, accumulates in intracellular organelles (endoplasmic reticulum and Golgi) and in the plasma membrane of insect cells.

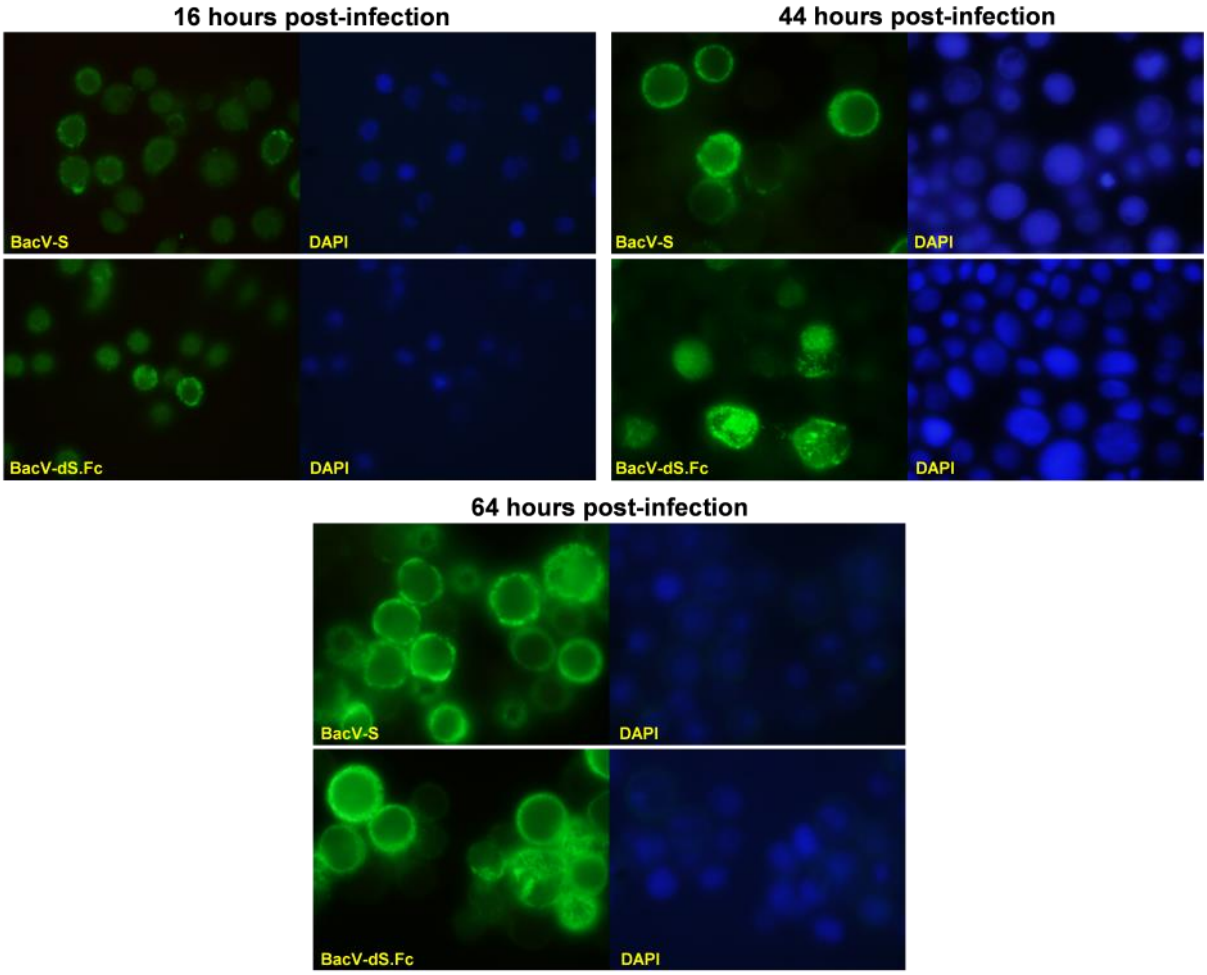

Figure 5. Immunodetection of spike protein expression in ExpiSf9 cells infected with recombinant baculovirus. The ExpiSf9 cells grown on coverslips were infected with an average of 1 MOI of baculovirus, and after fixation with 3% paraformaldehyde at the indicated times, immunostaining was performed. Proteins were labelled with rabbit polyclonal anti-spike primary antibody and anti-rabbit Alexa 488 conjugated secondary antibody. The nucleus was visualized with DAPI.

3. DISCUSSION

In this study, the spike protein synthesis potential of ExpiSf9 and Hi5 cells infected with two different recombinant baculoviruses carrying the spike gene was evaluated. It was observed that both cell lines showed similar growth profiles in Sf-900 III SFM medium, and the addition of FCS to the medium had a positive effect on cell growth (Figure 1). On the other hand, the growth rate of the insect cells decreased dramatically after infection with the viruses (Figure 3). A more significant decrease in the number of viable cells was observed in cultures infected with BacV-dS.Fc viruses. This is thought to be related to the titer of the virus rather than the type of virus. It has been reported that within the first 18 hours of virus infection,

the synthesis of all cellular proteins is inhibited, in parallel with the blocking of the cell cycle in the G2/M phase [27-29]. Therefore, in order to obtain large amounts of recombinant protein, the number of cells in the initial culture and the cell viability rate are as important as the viral titer used for infection. Protein analysis by SDS-PAGE/silver staining showed that spike protein levels were much higher than other cellular proteins in cells infected with both virus types (Figure 4A).

The fact that the spike protein is glycosylated and a relatively large protein severely limits its production in a suitable form and at desired levels in other expression systems. For this reason, it is mostly synthesized in smaller portions covering specific functional domains of the spike proteins [30-31]. In this respect, baculovirus expression systems appear to have a significant advantage in the production of glycosylated and relatively large recombinant viral antigens. It was observed that the synthesis of recombinant spike proteins in infected cell cultures was not significant during the first 24 h, whereas the amount of protein increased significantly at 48 and 72 h (Figure 4B). IF results also correlate with SDS-PAGE/Western blot results, and high spike protein expression is observed in almost all cells at 64 hours of infection (Figure 5). It is clear that starting infection with the virus at a high MOI increases the rate and amount of recombinant proteins.

The spike protein exists in a trimeric form embedded in the viral membrane with a short transmembrane domain at the carboxy terminus [32]. This glycosylated membrane protein is post-translationally modified by the endoplasmic reticulum (ER) and Golgi complex [33]. IF results show that recombinant spike proteins are found in aggregates in the cytosol of infected cells and involved in the ER-Golgi complex vesicle pathway (Figure 5).

In immunoblot analysis of proteins in the medium of virus-infected cell cultures, two proteins with different molecular weights were labelled with polyclonal anti-spike antibodies. The larger protein band has a lower molecular weight than the recombinant spike proteins detected in infected cell lysates. The smaller band corresponds to a protein with an average weight of 90-95 kD (Figures 4C and 4D). This suggests that the proteins detected in the medium are not the proteins released by the lysis of infected cells, but rather the proteins secreted from the cells after specific proteolytic cleavage. In human cells, spike protein is cleaved into S1 and S2 functional subunits by a "furin-like protease" during processes in the ER-Golgi pathway [34, 35]. Kiefer et al. [36] have shown that the spike protein expressed in the green alga *Chlamydomonas reinhardtii* is also proteolytically cleaved and the fragments with average sizes of 117 and 90 kD are secreted into the medium. These data indicate that the small protein bands found in culture media infected with both BacV-S and BacV-dS.Fc viruses are the S1 subunit. On the other hand, the observation of larger protein bands on the polyacrylamide gel, compared with the putative molecular weights of the recombinant spike proteins, calculated to be 133.2 kD and 116.5 kD, indicates effective glycosylation of the recombinant proteins in insect cells.

4. CONCLUSION

The SARS CoV-2 spike protein is the main component of vaccines against Covid-19, as it is the main target of host defense. It is of great importance to produce this glycosylated and relatively large protein in a suitable form and in sufficient quantities. In this study, it was shown that the spike protein was efficiently synthesized in insect cells infected with recombinant baculoviruses as full size (only 62 residues deleted at the C-terminus) and as a fusion with human IgG Fc protein. Data were obtained showing that the recombinant spike proteins undergo post-translational modifications such as glycosylation and proteolytic cleavage as in mammalian cells. It was concluded that the baculovirus expression system is a suitable low cost system for the production of spike protein as the main component of the subunit vaccine effective against Covid-19.

5. MATERIALS AND METHODS

5.1. Cell lines and cell culture media

SARS CoV-2 spike proteins were produced in *Spodoptera frugiperda* originated-Sf9 (ExpiSf9 Cells: Invitrogen #A38841) and Trichoplusia ni originated-High Five (Hi5) (Gibco #B85502) cells by infection with recombinant baculovirus. Cells were grown and maintained in 100 ml of ready-to-use serum-free Sf-900 III SFM (1265803-Gibco) and/or Express Five SFM (10486025-Gibco) medium at 28°C, shaking at 90-120 rpm. When the cell titer reached an average of 2-3x10^6/ml, passaging was performed by diluting the culture at 1/9 in fresh medium. Heparin (H3393-50KU / Sigma) was added as required at a concentration of 10 U/ml

to prevent aggregation of the cells. For long-term cell storage, suspension cultures were centrifuged at 500g for 5 minutes in the logarithmic growth phase with high viability, and the cells were resuspended in the medium (9 volumes of fresh / 1 volume of used Sf-900 III SFM) containing 10% DMSO at an average of $2x10^6$/ml and stored in liquid nitrogen vapour.

5.2. Recombinant baculoviruses and their production

Two different types of recombinant baculovirus encoding the spike protein, developed as part of a project to develop a recombinant vaccine against SARS CoV-2, were used. The viruses were developed using the Bac-to-Bac Baculovirus Expression System (Invitrogen) by cloning the spike genes under the control of the strong polyhedrin promoter. One of the viruses (BacV-S) encodes a spike protein lacking the TM and CD domains at the carboxy terminus, while the other (BacV-dS.Fc) encodes the amino-terminal half of the spike protein in fusion with the human IgG Fc region.

Viruses were grown in 80-90% confluent ExpiSf9 cell cultures in 150 cm^2 culture flasks (TPP#90150) containing Express Five SFM medium. The culture medium was removed and the cells were inoculated with the viruses at an average of 0.1-0.2 MOI in 10 ml of medium at 28 ^oC for 1 hour. The inoculum was then removed and the cells were incubated in 50 ml Express Five SFM medium (9 volumes fresh / 1 volume used) for four days at low shaking speed (50 rpm). At the end of incubation, the media were collected, centrifuged at 5000g for 10 minutes and the supernatants were filtered through a 0.45 µm filter and stored at -80 ^oC.

5.3. Determination of ExpiSf9 and Hi5 cell growth profiles in liquid culture

ExpiSf9 and Hi5 cell cultures in the exponential growth phase were centrifuged at 500g for 5 minutes to precipitate the cells. The precipitates were resuspended in fresh Sf-900 III SFM medium and the number of viable cells was determined using a hemocytometer after treatment with 0.4% trypan blue (in PBS). Cells were diluted in 100 ml Sf-900 III SFM medium (9/1; fresh / used medium) in 1 liter flasks to a titer of $1x10^5$ cells/ml. Cultures were incubated at 28 °C, shaking at 120 rpm. Samples of 0.5 ml taken from the cultures at 24-hour intervals were mixed with an equal volume of 0.4% trypan blue and the number of live and dead cells was determined by counting on a hemocytometer.

5.4. Infection of ExpiSf9 and Hi5 cells with the viruses and analysis of spike protein expression in infected cell cultures

As described above (5.1), ExpiSf9 and Hi5 liquid cell cultures in the exponential growth phase were centrifuged at 500g for 5 minutes and the cells counted using a hemocytometer. 10 ml of the cell suspension at a titer of $1x10^7$ cells/ml was combined with 5 ml of BacV-S or BacV-dS.Fc recombinant virus suspension at an average titer of $2x10^6$ pfu/ml (0.1 MOI) and mixed for 60 minutes at low speed in a vertical tube mixer at room temperature. The virus-infected cell suspensions were then diluted to 100 ml with fresh Sf-900 III SFM medium in 1 liter Erlenmeyer flasks and incubated at 28 °C, shaking at 120 rpm. To determine the expression of spike proteins and their secretion into the medium, 250 µl samples taken from infected cultures at specified intervals were centrifuged at 5000g for 5 minutes and the cells and supernatants (media) were separated. Cell precipitates were lysed in 200 µl x2 SDS-PAGE sample loading buffer by passing through a 27G needle. The supernatants were mixed with 3/1 x4 SDS-PAGE sample loading buffer. Both cell lysates and media were analyzed for SARS CoV-2 spike protein by SDS-PAGE silver staining and/or Western blotting.

Cell lysates and medium samples prepared in SDS-PAGE loading buffer were denatured at 95 ^oC for 5 minutes and then loaded onto 6% polyacrylamide gels in 10x10x1 mm cassettes. Proteins were separated for 90 minutes at 50 V for 15 minutes followed by 100 V until the dye reached the bottom of the gel cassette. The proteins were visualized by silver staining or analyzed by Western blotting. The proteins were transferred to a 0.45 µm PVDF membrane at a constant voltage of 45 V for 45 min followed by 90 min at 90 V. The membranes were blocked in 5% skimmed-milk solution prepared in TBS (50 mM Tris pH.8, 138 mM NaCl, 2.7 mM KCl) for 60 min at room temperature with gentle shaking. The membranes were then exposed overnight to rabbit anti-spike antibody (Invitrogen ## PA5-116916) diluted 1/500 in blocking solution. The membranes were washed once with TBS-T (TBS + 0.1% Triton X-100), twice with TBS for 20 min and treated with anti-rabbit IgG-HRP antibody (Thermo# 31423) diluted 1/7500 in TBS for 20 min. The membranes were

washed again with TBS-T and TBS in the same manner. Spike proteins were visualized using a gel imaging system (DNR Bio-Imaging system) with chemiluminescent substrate (GE Healthcare # RPN2235).

5.5. Immunofluorescence Assay

The time course expression and intracellular localization of SARS CoV-2 spike proteins in the recombinant baculovirus-infected cells were detected by immunofluorescence (IF) assay. ExpiSf9 cells were seeded on coverslips in 12-well plates ($2x10^5$ cells/well) and incubated at 28 ºC for 6 hours to allow the cells to adhere to the coverslips. The medium was then removed and the cells were infected with 300 µl of virus suspension at an average MOI of 1 for 60 minutes, with gentle mixing at regular intervals. The virus inoculum was discarded and 1 ml of Sf-900 III SFM medium (9/1; fresh / used medium) was added to the cells and incubated at 28°C. At 16, 44 and 64 hours after infection, spike protein synthesis and localization in the infected cells were analyzed by IF. The cells were washed once with PBS and then fixed in 3% paraformaldehyde solution for 20 minutes at room temperature and then washed twice with PBS for 10 minutes. To prevent non-specific antibody binding, the cells were incubated in blocking solution (1% skim milk in PBS) for 30 minutes. After blocking, the cells were treated with rabbit polyclonal anti-spike antibody diluted in blocking solution (1/500) for 60 minutes. The cells were washed three times with PBS for 5 minutes, kept in blocking solution for a second time for 20 minutes and then treated with Alexa-488 conjugated anti-rabbit IgG diluted at 1/300 for 60 minutes. To visualize cell nuclei, DAPI (4′, 6-diamidino-2-phenylindole) was added to the secondary antibody solutions at a concentration of 0.5 µg/ml. After washing, the cells were mounted on coverslips in PBS, placed upside down on 3-5 µL antifade mounting medium (1 mg/mL p-phenylenediamine in 90% glycerol and 10% 20 mM Tris, pH 8.8) and sealed with nail polish. Spike proteins were analyzed using a conventional fluorescence microscope equipped with a digital camera (Olympus, DP72).

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