Production of Japanese encephalitis virus-like particles in insect cells

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Virus-like particles (VLPs) are composed of one or several recombinant viral surface proteins that spontaneously assemble into particulate structures without the incorporation of viral DNA or RNA. The baculovirus-insect cell system has been used extensively for the production of recombinant virus particles including VLPs. While the baculovirus-insect cell system directs the transient expression of recombinant proteins in a batch culture, stably transformed insect cells allow constitutive production. In our recent study, a secretory form of Japanese encephalitis (JE) VLPs was successfully produced by Trichoplusia ni BTI-TN-SB1-4 (High Five) cells engineered to coexpress the JE virus (JEV) preenvelope (pM) and envelope (E) proteins. A higher yield of E protein was attained with recombinant High Five cells than with the baculovirus-insect cell system. This study demonstrated that recombinant insect cells offer a promising approach to the high-level production of VLPs for use as vaccines and diagnostic antigens.

Administration of vaccines has proved to be one of the most effective means of controlling and eliminating life-threatening infectious diseases. Vaccines are a biological preparation used to generate protective immunity to an infectious disease by stimulating the production of antibodies and inducing cell-mediated immunity in the body of a vaccinated individual. Most vaccines consist of inactivated or live-attenuated pathogens that act as antigens, and therefore, they are generally manufactured by propagating infectious pathogens such as viruses and bacteria in large quantities. For instance, influenza vaccines have been traditionally manufactured using embryonated chicken eggs as the substrate for virus propagation. The production process requires very large numbers of eggs from certified farms under strict veterinary control and requires at least a six-month period. Recently, some influenza vaccines have been produced using cultured mammalian cells such as MDCK and Vero cells for virus propagation. On the other hand, until recently the most widely used Japanese encephalitis (JE) vaccine was an inactivated product made from viruses propagated in mouse brain tissue. The vaccine production required a complicated purification process, and there was concern for neurological adverse reactions. More recently, Vero cell-derived inactivated JE vaccines have been licensed in several countries including Japan. The manufacturing of vaccines for viral diseases is switching from slow labor-intensive egg-based or in vivo production to rapid mammalian cell culture-based production.

Cell culture-based vaccine production offers several advantages including short production time and easy scale-up. However, the vaccines are still manufactured from infectious pathogens, a situation that poses potential safety issues. Recombinant protein production systems can provide the next alternatives by synthesizing immunodominant components of a pathogen in vitro. Subunit vaccines composed of a specific protein of the pathogen are safe because they do not contain the genetic material of the pathogen. However, such subunit vaccines often suffer from poor immunogenicity probably due to misfolded conformation of the target protein. Virus-like particles (VLPs) are composed of one or several recombinant viral surface proteins that spontaneously assemble into particulate structures similar to authentic virus particles or naturally occurring subviral particles. VLPs cannot cause infection because they assemble without the incorporation of viral DNA or RNA. VLPs are a highly effective type of subunit vaccine that can elicit strong immune responses because of their repeated dense display of viral antigens in an authentic conformation. Therefore, VLPs offer a promising approach to the development of safe and efficacious vaccines and diagnostic antigens.

Among various recombinant protein production systems, the baculovirus-insect cell system has been one of the most widely used systems for the production of subunit vaccines and VLPs. In this system, following infection with a recombinant nucleopolyhedrovirus (NPV) carrying the foreign gene of interest, lepidopteran insect cells produce extremely large quantities of biologically active proteins with complex folding and post-translational processing and modifications performed in higher eukaryotes during the very late phase of infection. Compared with mammalian cells, insect cells are easy to culture, and the growth rates of both cells are comparable. Insect cells can be maintained at around 27 °C without CO2 supplementation in the
The baculovirus-insect cell system is also employed for the manufacture of a human papillomavirus VLP vaccine, Cervarix, which has been approved for use against cervical cancer.\(^{13,14}\) Quite recently, a seasonal influenza vaccine, Flublok, which contains recombinant hemagglutinin produced using the baculovirus-insect cell system,\(^{15,16}\) was approved in the USA. These examples demonstrate the potential of insect cells as a practical platform for the production of novel recombinant protein vaccines. However, the baculovirus-insect cell system has several inherent limitations. In this system, continuous protein production is virtually impossible because host insect cells are lysed during the baculovirus infection cycle. Release of intracellular proteins from lysed cells may cause protein degradation by proteases and may also complicate the downstream processing and purification of products. Furthermore, in the case of VLP production, removal or inactivation of progeny baculoviruses released from infected insect cells may become a critical problem, though baculoviruses do not infect vertebrates.\(^{17}\)

Stably transformed insect cells allow the constitutive production of recombinant proteins and can be employed as attractive alternative platforms to the baculovirus-insect cell system.\(^{18-20}\) They are particularly useful for the production of secreted complex proteins, because the protein synthesis and processing machinery of the host insect cell is not compromised by baculoviral infection. Recently, we investigated the production of JE VLPs in stably transformed lepidopteran insect cells.\(^{21}\) The virion of flaviviruses, such as JE, dengue, West Nile, and yellow fever viruses, consists of a nucleocapsid structure surrounded by a lipid bilayer inserted with an envelope (E) glycoprotein and a membrane (M) protein.\(^{22-24}\) The E protein is the major surface protein with a role in cellular receptor binding and membrane fusion and induces neutralizing antibodies that protect hosts against disease. The M protein is synthesized as the precursor membrane (prM) protein in infected cells, which is then cleaved to M by a cellular protease, furin, during virion maturation. This cleavage event results in changes in the oligomerization of these surface proteins from a prM/E heterodimer to an E homodimer, and, thereby, the formation of mature virions that can display cell-fusion activity. In our recent study,\(^{13}\) Triatoma brasiliensis mitochondria (Tb-M) were found to stabilize virion maturation, thereby enhancing the expression of the JEV E protein. This study shows the potential of insect cells for the production of recombinant proteins and can be employed as attractive alternative platforms to the baculovirus-insect cell system.\(^{18-20}\) Second, we used a powerful expression vector to generate recombinant High Five cells. In stable transformation, the choice of a promoter to drive the heterologous gene expression is critical, because the use of weak promoters results in low recombinant protein yields. The activity of promoters used for insect cell expression systems can be enhanced by certain cis- or trans-acting elements derived from baculoviruses.\(^{25}\) Recently, an expression vector containing the BmNPV IE-1 transactivator and the HR3 enhancer, from which foreign gene expression is stimulated with the B. mori NPV (BmNPV) IE-1 transactivator and the BmNPV HR3 enhancer, has been developed for high-level expression of recombinant proteins in lepidopteran insect cells.\(^{26}\) Use of the IE-1 transactivator and the HR3 enhancer with the actin promoter has resulted in a more than 1000-fold increase in the stimulation of foreign gene expression through the promoter.\(^{27,28}\) We used plasmid vectors that contained the B. mori actin promoter downstream from the BmNPV IE-1 transactivator and the BmNPV HR3 enhancer for high-level expression, together with either a bacteriophage or a yeast protein affinity tag for use as a selectable marker (Fig. 1).\(^{29}\) High Five cells stably secreting a high concentration of E protein were efficiently developed by incubation in the presence of the respective antibiotic. Third, DNA encoding a form of prM with a pr/M cleavage site mutation was used to suppress the toxic cell-fusion activity of VLPs (Fig. 1). During flavivirus virion maturation, the cleavage from prM to M causes the rearrangement of E proteins on virus particles, leading to the formation of mature virions that can show cell-fusion activity. As reported previously for mammalian cells,\(^{30}\) the use of the gene encoding a mutated (furin proteinase resistant) form of prM was effective in generating highly productive recombinant insect cells. Finally, suspension culture was useful in improving productivity. The viable cell density and the E protein yield in a shake-flask culture were increased considerably compared with a static culture, probably due to a better oxygen supply in the shake-flask culture than in the static culture. Suspension culture has practical advantages for large-scale production of recombinant proteins. In a simple shake-flask culture, however, the environmental conditions are not optimal. We are currently investigating the JEV VLP production by recombinant High Five cells in a bioreactor system. Figure 2 shows the preliminary results obtained in a 2.5-L, stirred-tank bioreactor with 1 L of Express Five serum-free medium (Invitrogen). Agitation in the bioreactor was provided by an impeller rotating at 90 rpm, and aeration was performed by bubbling a mixed gas of O\(_2\) and N\(_2\) from a nozzle.\(^{31}\) The dissolved oxygen (DO) concentration
recombinant High Five cells could be attained in a bioreactor system by optimizing the culture conditions. Additionally, we investigated the effects of signal peptides on the secretory production of JEV E proteins in transient expression experiments using High Five cells. The JEV prM signal sequence used in our recent study was a transmembrane signal located adjacent to prM (Fig. 1), which directed the translocation of prM into the lumen of the endoplasmic reticulum (ER) from its site of synthesis on the surface of the ER. Use of insect- or baculovirus-derived signal peptide sequences may affect the E protein production in High Five cells. A plasmid vector pIHAbla (Fig. 1) containing the mutated prM and E genes downstream from one of the three signal peptide sequences, the Drosophila BiP signal sequence, 20 the honeybee melittin signal sequence, 31 or the Autographa californica NPV (AcNPV) gp64 signal sequence, 32 instead of the JEV authentic prM signal sequence, was constructed, and High Five cells were transfected with the resultant plasmids. High Five cells were inoculated into each well of 6-well plates with 2 ml of in the culture medium was controlled at 20% oxygen saturation by automatically changing the composition of the supplied gas based on the reading of a DO sensor. The temperature of the culture medium was maintained at 27 °C, but the pH was not controlled. In a batch culture using a stirred-tank bioreactor, the density of viable cells reached $6.4 \times 10^6$ cells/cm$^3$ on day 5 (Fig. 2), which was as high as that found in shake-flask cultures. Recombinant High Five cells also showed an E protein yield that was nearly comparable to the yield obtained in shake-flask cultures. More efficient production of JEV proteins by
medium at a cell density of 1 x 10^6 cells/cm² 24 h before transfection. The cells were transfected with 1 μg of the constructed plasmids using 6 μl of FuGENE 6 transfection reagent (Roche Diagnostics). Three days after transfection, the culture supernatants were removed and clarified by centrifugation. In ELISA, the culture supernatants, significantly low yields of E protein were obtained when the signal sequences of honeybee melittin and baculovirus gp64 were used (Fig. 3A). Interestingly, however, by comparison with the authentic JEV prM signal sequence, the Drosophila BiP signal sequence gave a yield of E protein that was comparable or rather high. In western blot analysis of the culture supernatants, specific protein bands were detected at an equivalent position of approximately 50 kDa regardless of the signal peptide (Fig. 3B). These results suggest that in High Five cells the Drosophila BiP signal sequence may function as efficiently as the JEV prM signal sequence and could be used for the generation of highly productive cells secreting VLPs.

In conclusion, recombinant insect cells may provide a breakthrough in the development and production of VLPs for use as the next generation vaccines and diagnostic antigens. The strategy used for producing JE VLPs in our recent study would be applicable to other flaviviruses including dengue[12] and West Nile viruses. Further developments of an optimal bioreactor system for the long-term, high-density culture of recombinant insect cells would allow the large-scale production of VLPs and improve their productivity.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

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