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Efficient production of Japanese encephalitis virus-like particles by recombinant lepidopteran insect cells

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Abstract The production of Japanese encephalitis (JE) virus-like particles (VLPs) in stably transformed lepidopteran insect cells was investigated. The DNA fragment encoding the JE virus (JEV) prM signal peptide, the precursor (prM) of the viral membrane protein (M), and the envelope glycoprotein (E) was cloned into the plasmid vector pIHAbla. The pIHAbla contained the *Bombyx mori* actin promoter downstream of the *B. mori* nucleopolyhedrovirus (BmNPV) IE-1 transactivator and the BmNPV HR3 enhancer for high-level expression, together with a blasticidin resistance gene for use as a selectable marker. DNA encoding a form of prM with a pr/M cleavage site mutation was used to suppress the cell-fusion activity of VLPs. After transfection with the resultant plasmid, *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells were incubated with blasticidin, and cells resistant to the antibiotic were obtained. Western blot analysis and enzyme-linked immunosorbent assay of a culture supernatant showed that transfected High Five cells secreted an E antigen equivalent to the authentic JEV E. Sucrose density-gradient sedimentation analysis of the culture supernatant from recombinant High Five cells indicated that secreted E antigen molecules were produced in a particulate form. VLPs recovered from the supernatant successfully induced neutralizing antibodies in mice, particularly when adsorbed to alum adjuvant. High yields (≈30 μg/ml) of E antigen were achieved in shake-flask cultures. These results indicate that recombinant insect cells may offer a novel approach for efficient VLP production.

Keywords Insect cell culture · Recombinant protein production · Virus-like particles · Japanese encephalitis virus · High Five cells

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Introduction

Structural proteins of many viruses such as envelope and capsid proteins spontaneously assemble into particles that are structurally similar to authentic virus particles or naturally occurring subviral particles. Based on this ability, virus-like particles (VLPs) can be produced following the expression of such viral proteins in heterologous systems using recombinant DNA technology (Grgacic and Anderson 2006; Konishi and Fujii 2002; Konishi et al. 2001; Noad and Roy 2003). Since VLPs do not contain the DNA or

RNA genome of the virus, they are non-infectious and safe. VLPs show a potential for high immunogenicity and antigenicity because of their repetitive, high density display of viral antigens (Grgacic and Anderson 2006; Konishi and Fujii 2002; Konishi et al. 2001; Noad and Roy 2003). Therefore, VLPs offer a promising approach to the development of safe and effective vaccines and diagnostic antigens.

Insect cells are ideal host cells that efficiently and safely produce recombinant proteins with complex folding and post-translational processing and modifications found in higher eukaryotes (Kost et al. 2005; Luckow 1995; Yamaji 2011). The baculovirus–insect cell system has been used extensively for the production of various mammalian virus proteins including a wide range of VLPs (Cox 2012; Kost et al. 2005; Metz and Pijlman 2011; van Oers 2006; Vicente et al. 2011). This system is also employed for the commercial manufacture of a human papillomavirus VLP vaccine, Cervarix, which is indicated for the prevention of cervical cancers (Cox 2012; Schiller et al. 2008; Vicente et al. 2011), demonstrating the potential of insect cells as a practical platform for the production of vaccines and diagnostic antigens. The baculovirus–insect cell system directs transient expression of recombinant proteins upon infection of lepidopteran insect cells with a recombinant nucleopolyhedrovirus (NPV). In this system, continuous protein production is virtually impossible because of the lytic nature of the baculovirus infection process. The lysis of host insect cells and the resultant release of intracellular proteins may lead to protein degradation by proteases and may also complicate downstream processing and purification of products. Moreover, for VLP production using the baculovirus–insect cell system, removal or inactivation of progeny baculoviruses released by budding off from infected insect cells may become a critical problem (Vicente et al. 2011), though baculoviruses are non-pathogenic to vertebrates.

Stably transformed insect cell lines can be employed as attractive alternative platforms for the continuous production of complex recombinant proteins. Attention has been recently directed towards the production of VLPs by recombinant insect cells. Successful expressions have been demonstrated: Japanese encephalitis (JE) VLPs in a stably transformed *Drosophila* cell line (Zhang et al. 2007); JE VLPs in *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells (Yamaji et al. 2009, 2010); HIV-1 Pr55gag-based VLPs in High Five cells (Tagliamonte et al. 2010); *Rous sarcoma* virus protein-based VLPs in High Five cells (Deo et al. 2011); and, double-layered rotavirus-like particles in *Drosophila melanogaster* S2 cells (Lee et al. 2011). When lepidopteran insect cells, such as Sf9 and High Five cells, are used as host cells for stable expression, the choice of a promoter to drive the heterologous gene expression is crucial as the use of weak promoters results in low recombinant protein yields (Yamaji 2011). Recently, a high-level expression vector containing the *Bombyx mori* cytoplasmic actin promoter, 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 lepidopteran insect cells (Farrell et al. 1998, 1999; Keith et al. 1999). The use of the IE-1 transactivator and the HR3 enhancer allowed an increase of more than 1,000-fold in the stimulation of foreign gene expression through the actin promoter (Lu et al. 1997). In a previous study (Yamaji et al. 2008), we constructed two plasmid vectors, pIHAbla and pIHAneo, which contained the BmNPV IE-1 transactivator, the BmNPV HR3 enhancer, and the *B. mori* actin promoter, together with either a blasticidin or a neomycin resistance gene, respectively, for use as a selectable marker. After cotransfection with these plasmid vectors, into which the heavy- and light-chain genes of an antibody Fab fragment were separately inserted, High Five cells stably secreting a high concentration of the functional Fab fragment were efficiently generated by incubation in the presence of blasticidin and G418.

In the present paper, we describe the production of JE VLPs in stably transformed lepidopteran insect cells. The DNA fragment encoding the JE virus (JEV) prM signal peptide, the precursor (prM) of the viral membrane protein (M), and the envelope glycoprotein (E) was cloned into the above-mentioned plasmid vectors, pIHAbla and pIHAneo. After transfection with the resultant plasmids, High Five cells secreting a high concentration of the E antigen in a particulate form were obtained by incubation with the corresponding antibiotic. High yields of approximately 30 μg/ml of the E antigen were produced in shake-flask cultures of the recombinant High Five cells.

Materials and methods

Insect cell lines and media

A lepidopteran insect cell line, BTI-TN-5B1-4 (High Five; Invitrogen, Carlsbad, CA, USA), derived from the ovarian cells of the cabbage looper, *T. ni*, was used for recombinant protein production. Cell density was determined by microscopically counting the number of cells with a Bürker–Türk hemocytometer, while cell viability was judged by trypan blue dye exclusion (Yamaji et al. 1999). The cells were maintained at 27 °C in T flasks in a non-humidified incubator. The culture medium used was Express Five serum-free medium (Invitrogen), supplemented with 16.5 mM L-glutamine and 10 mg/L gentamicin.

A mosquito cell line, C6/36, was grown in Eagle's minimal essential medium supplemented with 10 % fetal bovine serum (FBS), nonessential amino acids, and 60 mg/L kanamycin at 28 °C in a humidified CO_2 incubator (5 % CO_2).

The maintenance medium used for C6/36 cells following infection with the Nakayama strain of JEV was the growth medium containing 0.1 % bovine serum albumin instead of 10 % FBS (Konishi et al. 2001). The harvested culture supernatant was used as a control in sucrose density-gradient sedimentation analysis.

Plasmid construction

The DNA fragment encoding the JEV (Nakayama strain) signal peptide of prM and the prM and E proteins was PCR amplified from the plasmid pcJEME (Konishi et al. 2001) as a template with primers including *BamH*I or *Sac*II sites. The amplified DNA fragment was inserted into either the plasmid pIHAbla or pIHAneo (Yamaji et al. 2008) using the *BamH*I and *Sac*II sites downstream of the *B. mori* cytoplasmic actin promoter. In a similar manner, the DNA fragment encoding the JEV prM signal peptide, a form of prM containing a mutation of the amino acid sequence at the cleavage site from prM to M, and the E protein was amplified from the plasmid pcJEEP (Konishi et al. 2001) as a template and cloned into either pIHAbla or pIHAneo. The plasmid inserts were confirmed by DNA sequencing.

Transient expression

High Five cells were inoculated into each well of 6-well plates with 2 ml of medium at a cell density of 1×10^5 cells/cm^3 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, Tokyo, Japan). Three days after transfection, the culture supernatant was removed and clarified by centrifugation. The cell-free culture supernatant was stored at −20 °C for subsequent analyses.

Stable transformation and cell culture

High Five cells were inoculated into each well of 6-well plates with 2 ml of medium at a cell density of 1×10^5 cells/cm^3 24 h before transfection. The cells were transfected with 1 μg of the plasmids using 6 μl of FuGENE 6. Two days after transfection, the cells were removed from each well and transferred into a 100-mm culture dish with 10 ml of medium. After 24-h incubation, the medium was replaced with fresh medium containing either 20–40 μg/ml blasticidin (Invitrogen) or 200–400 μg/mlG418 (Invitrogen) to select stable expression cells. The selective medium was replaced every 4 days until colonies of blasticidin- or G418-resistant cells were formed. Cells were isolated from a colony into each well of 96-well plates with 100 μl of medium without blasticidin or G418. After 24-h incubation, the medium was replaced with fresh medium containing either 20–40 μg/ml blasticidin or 200–400 μg/mlG418 every 4 days. After the cells had grown to confluence in the presence of blasticidin or G418, cells in the wells of 96-well plates were transferred into each well of 12-well plates with 1 ml of medium containing blasticidin or G418, and the culture supernatants were examined for E antigen levels. Cells yielding a high concentration of the E antigen were expanded in T flasks with medium containing either blasticidin or G418.

Cells in the exponential growth phase were collected and suspended at a density of 2×10^5 cells/cm^3 in fresh medium containing the corresponding antibiotic. In a static culture, 5 ml of cell suspension was transferred into T25 flasks. The cells in the T flasks were statically cultivated at 27 °C in an incubator, and one flask was removed every day to obtain a sample of the cell suspension. In shake-flask cultures, the cells were cultivated using two methods. In the first method, 15 ml of cell suspension was transferred into 100-ml screw-capped Erlenmeyer flasks. The cells in the Erlenmeyer flasks were cultivated at 27 °C on a reciprocal shaker (90 oscillations/min; amplitude 25 mm). In the second method, 30 ml of cell suspension was poured into 200-ml screw-capped Erlenmeyer flasks, and the cells were incubated at 27 °C on a rotary shaker (90 rpm). In both methods, aliquots of the cell suspension were sampled every day. In both static and shake-flask cultures, cell density was measured and culture supernatants were separated via centrifugation and stored at −20 °C for subsequent analyses. For measurement of intracellular E protein, cells were washed with phosphate-buffered saline (PBS), harvested by centrifugation, and lysed in PBS containing 1.0 % Triton X-100. Following centrifugation of the cell lysate, the supernatants were stored at −20 °C for subsequent enzyme-linked immunosorbent assay (ELISA).

Analyses

Culture supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 15 % gel under non-reducing conditions, followed by transfer to a poly(vinylidene difluoride) membrane. Immunoreactive proteins were detected with polyclonal anti-JEV antibodies obtained in the form of hyperimmune mouse ascitic fluid and alkaline phosphatase-conjugated goat anti-mouse IgG, and stained using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium, as described previously (Yamaji et al. 2012).

The concentration of E antigen in the cell-free supernatant was measured by sandwich ELISA, as previously described (Konishi et al. 2001). Briefly, rabbit hyperimmune serum against JEV was allowed to adsorb onto 96-well plates. Each well of the plates was serially incubated with either a supernatant or a standard, mouse monoclonal anti-E

antibody (JE-10B4) (Konishi et al. 2008), horseradish peroxidase-conjugated goat anti-mouse κ chain, and 2,2-azino-di-(3-ethylbenzthiazoline) sulfonic acid and H_2O_2. Antigen concentrations were calculated from the absorbance values obtained with the sample and a reference standard (Yamaji et al. 2012).

For sucrose density-gradient sedimentation analysis, culture supernatants were applied to a 10 to 40 % (*w*/*w*) continuous sucrose density gradient prepared in TN buffer (10 mM Tris–HCl, 100 mM NaCl, pH7.5) in 2.2-ml tubes (Konishi et al. 2001; Yamaji et al. 2012). Following centrifugation at 55,000 rpm for 90 min at 4 °C using an S55S swing rotor in a Himac CS100GX micro-ultracentrifuge (Hitachi Koki, Tokyo, Japan), fractions were collected from the bottom of the tube. The concentration of E antigen in each fraction was determined by ELISA.

Mouse experiment

VLPs were purified as previously described (Konishi et al. 2001; Kuwahara and Konishi 2010). Briefly, culture supernatants were clarified by centrifugation and precipitated with 10 % polyethylene glycol (molecular mass, approximately 6,000 Da). Following centrifugation, the pellets were suspended in TN buffer and applied to a sucrose density gradient as described above. The collected fractions were examined for E antigen levels by ELISA. The fractions containing the highest levels of E antigen were used as purified VLPs.

Groups of six 4-week-old male C3H/He mice (Japan SLC, Shizuoka, Japan) were immunized twice, at an interval of 2 weeks between immunizations, with purified VLPs either alone or formulated in alum adjuvant at a ratio of 1 μl of 1.3 % aluminum hydroxide suspension (Serva, Heidelberg, Germany) per 1 μg of E antigen, by intramuscular injections. Mice were bled retro-orbitally, and pooled sera were examined for neutralizing antibody using plaque reduction assays performed with JEV strain Nakayama in the presence of rabbit complement (Imoto and Konishi 2007). The neutralizing antibody titers were expressed as the maximum serum dilution yielding a 70 % reduction in plaque numbers (Kuwahara and Konishi 2010). All of the animal experiments were conducted according to the Kobe University Animal Experimentation Regulation.

Results

Transient expression of JEV prM and E genes

In the course of flavivirus virion maturation, the prM protein is cleaved to M protein by a cellular protease in infected cells. The cleavage from prM to M causes rearrangement of E proteins on flavivirus particles, leading to the formation of mature virions that can show cell-fusion activity (Konishi and Fujii 2002; Konishi et al. 2001; Li et al. 2008; Mukhopadhyay et al. 2005; Stiasny and Heinz 2006; Yu et al. 2008). As reported previously, when CHO-K1 cells were transfected with the authentic form of the JEV prM gene and the E gene, stable expression cell lines were not obtained due to the cell-fusing ability of VLPs containing E and M proteins (Konishi et al. 2001). By contrast, a stable CHO cell line secreting JE VLPs was successfully established by transfecting the E gene and a DNA fragment encoding a mutated form of prM containing a modification of the amino acid sequence at the pr/M cleavage site. Biochemical alteration of the prM protein is critical for the successful generation of JE VLP-producing mammalian cell lines (Konishi et al. 2001). Nevertheless, the yields of the E antigen produced by recombinant CHO cells were low and would not meet the requirements for practical applications.

In the present study, the effect of the authentic and mutated form of the JEV prM gene was investigated in transient expression in lepidopteran insect cells. High Five cells were employed because they are an excellent host for the production of recombinant secreted proteins (Farrell et al. 1999; Keith et al. 1999; Yamaji et al. 2008). Figure 1a shows the results of western blot analysis of the culture supernatants from High Five cells transfected with the JEV authentic (JEME) or mutated (JEEP) prM gene and the E gene cloned into pIHAneo (neo) and pIHAbla (bla). In each culture supernatant, a specific protein band was detected at an electrophoretic mobility of approximately 50 kDa, which coincided with the molecular weight of the JEV E protein. Moreover, the prM protein was detected in the culture supernatants (Fig. 1a). The culture supernatants of transfected High Five cells were also analyzed by ELISA. As shown in Fig. 1b, the JEV E antigen was identified in each culture supernatant. These results suggest that High Five cells transfected with the JEV prM and E genes were able to secrete the E and prM proteins into the culture medium. Figure 1b also shows that the yield of E antigen obtained with the mutated prM gene was significantly higher than that with the authentic prM gene regardless of whether either pIHA-neo or pIHAblao was used. As shown in Fig. 1a, a higher-level expression of the prM protein was observed with the mutated prM gene than with the authentic prM gene, indicating a decreased pr/M cleavage with the former gene. These results indicate that the expression of the authentic prM gene may be unfavorable even in lepidopteran insect cells, while the cytotoxicity of JEV proteins against insect cells may be lower than that against mammalian cells. Therefore, the use of the mutated prM gene would be effective in generating highly productive recombinant insect cells.

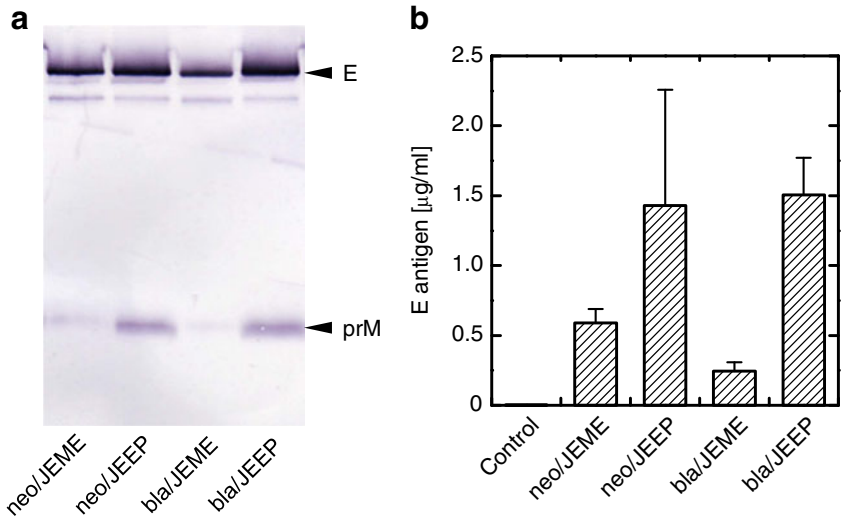

Fig. 1 Analyses of culture supernatant from *T. ni* BTI-TN-5B1-4 (High Five) cells in transient expression of Japanese encephalitis virus (JEV) prM and E genes. **a** Western blot analysis; **b** enzyme-linked immunosorbent assay (ELISA). After transfection with pIHAneo (neo) or pIHAbla (bla), which contained the JEV prM signal sequence, the authentic form of the prM gene, and the E gene (JEME) or the JEV prM signal sequence, a DNA encoding a form of prM containing a mutation of the amino acid sequence at the cleavage site from prM to M, and the E gene (JEEP), cells were incubated for 3 days in static culture. Control, untransfected cells. Bars represent the means ± S.D. obtained from three different determinations

Characterization of E antigens produced by stably transformed cells

Next, the JEV prM genes and the plasmid vectors were compared for the generation of highly productive lepidopteran insect cells stably secreting VLPs. High Five cells were transfected with either the JEV authentic or the mutated prM gene and the E gene using either pIHAneo or pIHAbla. After incubation for about 2 weeks in the presence of respective antibiotic, the formation of colonies was observed. Cells isolated from each colony were expanded in medium containing either G418 or blasticidin. After cells were screened using ELISA of the culture supernatant, the most productive clone was obtained from cells transfected with the JEV mutated prM gene and the E gene using pIHAbla.

The culture supernatant of the established recombinant High Five cells was fractionated by 10–40 % sucrose density-gradient centrifugation, and the concentration of E antigen in each fraction was determined by ELISA (Fig. 2b). The E antigens present in each fraction were compared with those in fractions similarly prepared from the culture supernatant of JEV-infected C6/36 mosquito cells (Fig. 2a). Flavivirus-infected cells release non-infectious subviral particles containing only the envelope proteins, known as slowly sedimenting hemagglutinin (SHA) particles, as well as infectious viral particles (Stiasny and Heinz 2006). As previously demonstrated (Konishi et al. 2001), two peaks of E antigen were identified in the culture supernatant of JEV-infected C6/36 mosquito cells: one for the infectious JEV

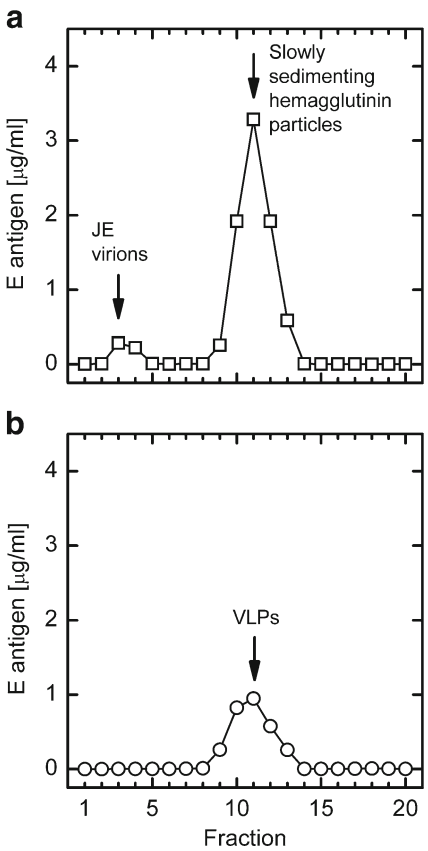

Fig. 2 Sucrose density-gradient sedimentation analysis of culture supernatant from JEV-infected C6/36 mosquito cells (**a**) and recombinant High Five cells transfected with bla/JEEP (**b**). Fractions were harvested in numerical order from the bottom of the gradient, and the E antigen concentration in each fraction was determined by ELISA

particles and the other for subviral SHA particles without a nucleocapsid. In the culture supernatant of recombinant High Five cells, E antigens were detected only in fractions corresponding to the SHA particles (Fig. 2b). These results suggest that E antigens secreted by the recombinant High Five cells were produced in a particulate form similar to that of subviral particles without a nucleocapsid.

Immunogenicity of VLPs produced by recombinant insect cells

Previous reports showed that recombinant JEV E protein produced in the baculovirus–insect cell system successfully induced neutralizing antibodies in mice (Matsuura et al. 1989; McCown et al. 1990; Wu et al. 2003; Yang et al. 2005). VLPs secreted by Sf9 cells in transient expression of the JE prM and E genes also showed the ability to elicit neutralizing antibodies in mice (Kuwahara and Konishi 2010). In the present study, to evaluate the potential of VLPs produced by recombinant High Five cells for use as vaccine antigens, their ability to induce neutralizing antibodies in mice was examined. Mice were immunized twice, at an interval of 2 weeks between immunizations, with VLPs containing 1–10 μg of E antigen. Four weeks after the first immunization with purified VLPs alone, mice developed neutralizing antibody titers of 1:40 to 1:160, as determined from pooled sera (Fig. 3a). When mice were immunized with VLPs formulated in alum adjuvant, sera obtained from these mice showed high neutralizing antibody titers of 1:160 or >1:1280 (Fig. 3b). Although further investigations to examine the immunogenicity of the VLPs including a challenge experiment are needed, the results obtained in the present study show that the VLPs produced by recombinant High Five cells can induce neutralizing antibodies in mice.

Production of VLPs by recombinant High Five cells

Finally, the productivity of E antigen was investigated in static and shake-flask cultures of recombinant High Five cells. As shown in Fig. 4, in a static culture, the viable cell density reached 3.1×10^6 cells/cm^3 on day 5 and 9 μg/ml of E antigen was secreted by day 5. In a shake-flask culture with 15 ml of cell suspension on a reciprocal shaker, however, the maximum cell density achieved was 5.3×10^6 cells/cm^3 on day 5, and the yield of E antigen reached around 30 μg/ml, which was three times as high as in the static culture. The increased cell density and E antigen yield were probably due to better oxygen supply in a shake-flask culture than in a static culture. Increases in cell density and product yield in a shake-flask culture compared with a static culture have also been reported for recombinant High Five cells (Sonoda et al. 2012; Yamaji et al. 2008). In a shake-flask culture with 30 ml of cell suspension on a rotary shaker, the secretion efficiency by recombinant High Five cells was evaluated. As shown in Fig. 5, approximately 30 μg/ml of E antigen yield in the culture supernatant was obtained again in the shake-flask culture. Figure 5 also shows that E antigen synthesized in recombinant High Five cells were efficiently secreted into the culture medium.

Discussion

The virion of flaviviruses including JEV has a nucleocapsid structure surrounded by a lipid bilayer containing E and M proteins (Konishi and Fujii 2002; Konishi et al. 2001; Li et al. 2008; Mukhopadhyay et al. 2005; Stiasny and Heinz 2006; Yu et al. 2008). The E protein is the major surface protein with a cellular receptor-binding site and a membrane

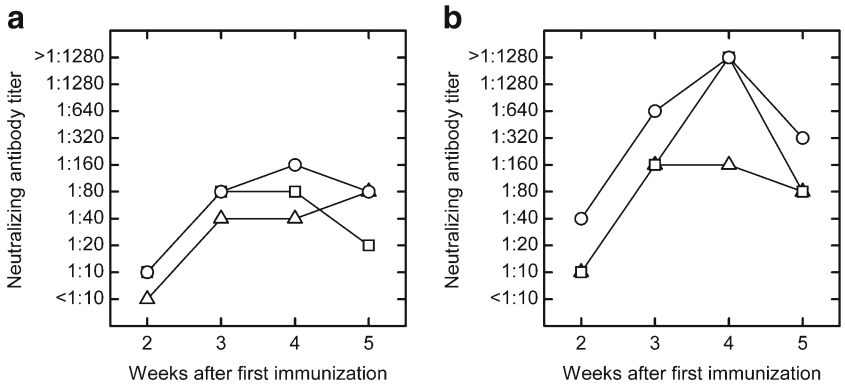

Fig. 3 Immunogenicity of VLPs produced by recombinant High Five cells. Groups of six mice (4-week-old male C3H/He) were immunized twice, at an interval of 2 weeks between immunizations, with purified VLPs either alone (**a**) or formulated in alum adjuvant at a ratio of 1 μl of 1.3 % aluminum hydroxide suspension per 1 μg of E antigen (**b**). Neutralizing antibody titers were determined by plaque reduction assays and were expressed as the maximum serum dilution yielding a 70 % reduction in plaque numbers. Dose of E antigen: (*white triangle*), 1 μg; (*white square*), 3 μg; (*white cicle*), 10 μg

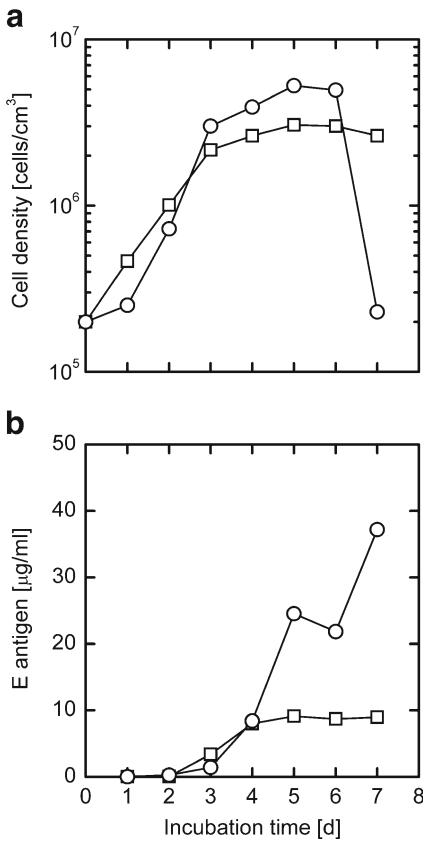

Fig. 4 Growth of recombinant High Five cells and production of E antigen in static (*white square*) and shake-flask (*white circle*) cultures. **a** Density of viable cells; **b** concentration of E antigen in the culture medium. In the shake-flask culture, 15 ml of cell suspension was incubated in 100-ml Erlenmeyer flasks on a reciprocal shaker

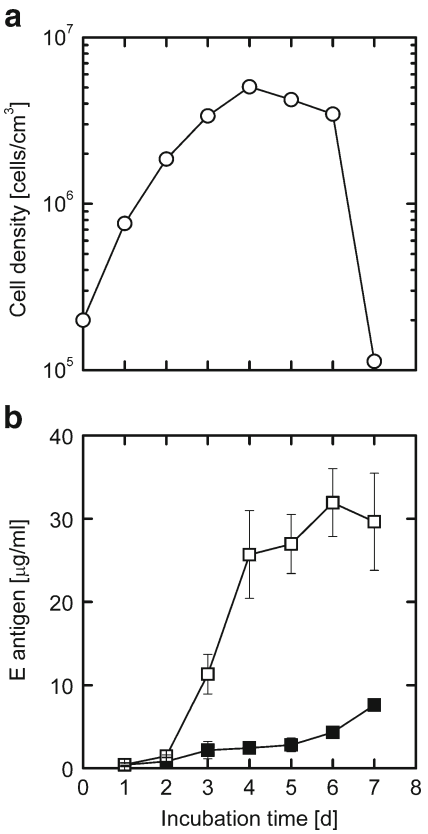

Fig. 5 Secretory production of E antigen by recombinant cells in shake-flask culture. **a** Density of viable cells; **b** secreted (extracellular) E antigen concentration (*white square*) and intracellular E antigen concentration (*black square*). Thirty milliliters of cell suspension was incubated in 200-ml Erlenmeyer flasks on a rotary shaker. Each plot represents the mean ± S.D. obtained from three different determinations (**b**)

fusion peptide, and it induces neutralizing antibodies to protect hosts against disease. The M protein is synthesized as prM in infected cells, which is then cleaved to M by a cellular protease during virion maturation. This cleavage event results in changes in the oligomerization of prM/M and E, and, thereby, the formation of mature virions that can show cell-fusion activity (Konishi and Fujii 2002; Konishi et al. 2001; Li et al. 2008; Mukhopadhyay et al. 2005; Stiasny and Heinz 2006; Yu et al. 2008). Coexpression of the prM and E proteins is known to lead to the formation and secretion of recombinant capsidless VLPs in mammalian cells. However, biochemical alteration of prM in the pr/M cleavage site to suppress the cell-fusion activity is crucial for the successful generation of mammalian cell lines producing JE and dengue type 2 VLPs (Konishi and Fujii 2002; Konishi et al. 2001). In the present study, the authentic and mutated form of the JEV prM gene was transiently expressed with the E gene in lepidopteran insect cells. The results obtained show that the mutated prM gene at a pr/M cleavage site improved the expression of the E protein in High Five cells (Fig. 1). The use of the mutated prM gene was effective in generating highly productive recombinant insect cells, as the most productive stably transformed clone was obtained from cells transfected with the mutated prM gene in the present study. Recombinant High Five cells efficiently secreted a particulate form of the E antigen molecules equivalent to the authentic JEV E (Figs. 2 and 5). Moreover, the VLPs produced by the recombinant cells successfully induced neutralizing antibodies in mice, particularly when adsorbed to alum adjuvant (Fig. 3). These results indicate the potential of the VLPs produced by recombinant High Five cells for use as vaccine and diagnostic antigens.

In shake-flask cultures of the recombinant High Five cells, high yields of approximately 30 μg/ml of E antigen were attained (Figs. 4 and 5). Such a high productivity would have been achieved by the synergistic effect of the use of highly productive host insect cells, the powerful expression vector, and the mutated prM gene. As reported previously, the yield of JEV E antigen from recombinant CHO cells was approximately 0.1 μg/ml (Konishi et al. 2001). Therefore, more than 100 times higher productivity of E antigen was achieved with recombinant High Five

cells. While a stable mammalian cell line highly producing JE VLPs (2.5 μg per 10^4 cells) was established by transfecting rabbit kidney-derived RK13 cells, which were resistant to JEV infection and the cytopathic effects of JEV, with the prM and E genes, the yields of E antigen appeared to remain around 15 μg/ml (Kojima et al. 2003; Mutoh et al. 2004). In a previous study, yields of JEV E antigen of 2–3 μg/ml were obtained by Sf9 cells infected with a recombinant baculovirus that contained the JEV prM and E genes (Yamaji et al. 2012). Although a simple comparison of the results from recombinant insect cells with those from baculovirus-infected insect cells is difficult, a considerably higher yield of E antigen was obtained by recombinant High Five cells. A significantly high productivity of JEV E antigen, approximately 60 μg released from 10^6 expressing cells, was achieved with Sf9 cells transiently transfected with the JEV prM and E genes (Kuwahara and Konishi 2010). However, recombinant protein production continues only for a limited time in the transient expression system. In contrast to the baculovirus–insect cell system and the transient expression system, stably transformed insect cells allow constitutive production of recombinant proteins (Yamaji 2011; Yamaji et al. 2008).

Insect cells provide safety advantages for the production of biopharmaceuticals because they do not support the growth of mammalian viruses or mycoplasmas. Baculovirus-infected High Five cells, or the subclone derived from them, are employed for the industrial-scale production of a human papillomavirus vaccine, Cervarix (Schiller et al. 2008), and, therefore, recombinant High Five cells are a promising candidate for vaccine production. The results obtained in the present study indicate that recombinant insect cells may offer a novel approach for efficient production of VLPs for use as vaccines and diagnostic antigens. More efficient production of JE VLPs by recombinant High Five cells could be attained in a bioreactor system and an immobilized cell culture (Yamaji et al. 2006). Meanwhile, as shown in Figs. 4 and 5, viable cell density markedly decreased on day 7 in shake-flask cultures. Such a sudden drop in viable cell density was also observed in a shake-flask culture of untransfected High Five cells (data not shown), indicating that the rapid decrease in cell viability was probably caused by apoptotic death but not by the cytotoxicity of the JEV proteins. Further advances including medium optimization and fed-batch culture strategies could extend the culture longevity and improve the productivity of VLPs by recombinant insect cells.

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