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Study on Persistent Infection of Japanese Encephalitis Virus Beijing-1 Strain in Serum-free Sf9 Cell Cultures

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Sf9 cells have obvious advantages for the conventional production technology of vaccine. They are useful tools for high concentration and large-scale cultures. Sf9 cells were grown to maximal concentration, 8×10^6 cells/ml in a 500ml spinner flask, with a doubling time at the exponentially growing phase of 24.5 hours, using serum-free media. To explore the ability of Sf9 cells to be infected by the Japanese encephalitis (JE) virus Beijing-1 strain, Sf9 cells were infected with the virus. By 4-5 days post-infection, 10-15% of the Sf9 cells showed cytopathic effect (CPE), from granularity to the formation of syncytia and multinucleated giant cells continuously observed over a period of 35 days. Positive fluorescent reactions were detected in 30-40% of cells infected with the JE virus Beijing-1 strain, and the uninfected Sf9 cells were completely negative. Virus particles, propagated in Sf9 and Vero cells, were concentrated by sedimentation on 40% trehalose cushions by ultracentrifugation, and showed identical patterns of viral morphogenesis. Complete virus particles, 40 to 50 nm in diameter, were observed, and JE virus envelope (E) proteins, at 53 kDa, were found in the western blot analysis to the anti-JE virus E protein monoclonal antibody and reacted as a magenta band in the same position to the glycoprotein staining. To evaluate whether the infectious virus was produced in Sf9 cells inoculated with the JE virus Beijing-1 stain, Sf9 cells were inoculated with the virus, and sample harvested every 5 days. The titers of the JE virus Beijing-1 strain rose from 1.0×10^5 to 1.5×10^6 pfu/ml. The infected Sf9 cells could be subcultured in serum-free medium, with no change in the plaque sizes formed by the JE virus Beijing-1 strain in the plaque assay. It is suggested that the ability of the JE virus Beijing-1 strain to infect Sf9 cells in serum-free media will provide a useful insect cell system, where the JE virus replication, cytopathogenicity and vaccine immunogen can be studied.

Key words: JE virus, Sf9 cell, serum-free, persistent infection

Japanese encephalitis virus (JE virus), a member of the family *Flaviviridae*, is a mosquito-borne virus, which is potentially fatal in humans, and spread throughout eastern Asia, including Korea, India, Japan, China, and southeast Asia. The causative virus is amplified in nature in a cycle involving *Culex* mosquitoes and vertebrate animals. Humans are susceptible at all ages, unless becoming immune by natural infection or vaccination (Da-Wei et al., 1996). The JE virus infection in humans can manifest in a spectrum of diseases, from asymptomatic infection to a mildly febrile symptomatic illness, or to a life-threatening disease that affects the central nervous system (Endy *et al.*, 2002).

Currently, 3 types of JE virus vaccines are in large-scale use, but the mouse brain-derived and inactivated vaccine, based on the Nakayama or Beijing-1 strains, is the only vaccine currently available on the international market. China produces two JE virus vaccines for domestic use, an inactivated JE virus vaccine as well as a live attenuated vaccine, both grown in primary hamster kidney cells. Local reactions, such as tenderness, redness and swelling, occur in 20% of vaccinees. A similar percentage may experience mild systemic symptoms including headache, myalgia, gastro-intestinal symptoms, and fever (WHO, 1994). Side effects of the mouse-brain inactivated JE virus vaccine have been reported to occur with an incidence rate of 0.6% in western immunized adults (WHO, 1994), and on the basis of current World Health Organization vaccine regulatory standards, PHK cells are unacceptable, and thus the vaccine is not suitable for worldwide usage (Wu *et al.*, 2002). Therefore, immunization with this vaccine is not yet recommended for westerners traveling to affected Asian regions (Takahashi *et*

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al., 2000), so large amounts of a cheap and highly immunogenic vaccine is required.

Persistent infections of insect cells with flaviviruses have not been extensively studied. Several arthropod cell lines, including continuous mosquito cell lines, have been shown to support the growth of the JE virus (Igarashi, 1978; Liang *et al.*, 1985) and other arboviruses (Zhang, 1993). Because of its greater optimal characteristics to those of the conventional production technology for vaccines, culturing Japanese encephalitis virus on passage cells for extraction of the vaccine has become a hot topic in this field. As one of the passage cell lines, Sf9 cell has obvious advantages for such application. The Sf9 cell line is a lepidopteran cell line derived from the ovary of the fall armyworm, *Spodoptera frugiperda,* and is widely used to produce recombinant proteins (Luckow, 1991). Sf9 cell cultures are more suitable for high concentration and large scale cultures than other cell lines, and have considerable exploitability. If a new method using Sf9 cells to produce the JE virus vaccine works, it will not only improve the quality of the existing JE virus vaccine, but also lead to a new field for the industry production of tissue culture vaccines all over world. In this report, we investigated the sensitivity of Sf9 cells to the JE virus in a serum-free media and the characterized the JE virus propagated in Sf9 cells, which would provide the principle basis for the large scales production of a new JE virus vaccine.

Materials and Methods

Cell and viruses

The Beijing-1 strain of the JE virus was obtained from the Central Research Institute, Green Cross Corp. The JE virus stocks were prepared from weaning mouse brains.

The Sf9 cell lines was cloned, by G. E. Smith and C. L. Cherry, in 1983 from the parent line, IPLV-SF21 AE, which was derived from pupa ovarian tissue of the fall armyworm, *Spodoptera frugiperda*, by Vaughn et al. in 1977. The Sf9 cell line, ATCC CRL-1771, was obtained from ATCC at the 16th passage, and adapted to INSECT-XPRESS serum free media (CAMBREX, USA).

Infection of Sf9 cells with Japanese encephalitis virus

Sf9 cells were subcultured prior to infection, and maintained in the logarithmic growth phase with a viability of 98%, using Insect-Xpress™ serum free media (Biowhittaker, USA). The JE viruses were inoculated into the Sf9 cells in a spinner flask (Belco, USA), with a 0.1 multiplicity of infection (m.o.i), and incubated at 27°C. Virus-inoculated cultures were scaled up, and finally transferred to 250 ml/500 ml spinner flasks, at a density of 3×10^5 cell/ml. When virus-inoculated Sf9 cells had reached a density of $3\text{-}4\times10^6$ cell/ml, they were split to a density of 3×10^5 cell/ml, for periods of up to 6 weeks.

Observation of infected cells and collection of samples

After infection the morphological changes of the Sf9 cells were observed daily under microscopy, and the cell numbers counted each week prior to splitting. Cells and supernatants were collected from the infected cultures every 5 days. After centrifugation, at 500×g for 5 min, aliquots of the supernatant were collected, and filtered through a 0.2 μm filter (Gelman, USA). The samples were stored at 70°C for use in plaque assays on Vero cell cultures. The pelleted cells were washed, centrifuged twice and resuspended in phosphate-buffered saline (PBS, pH 7.2). These cells were used in immunofluorescence assays. For other analyses, the virus particles were purified with 40% trehalose (Sigma) step gradient ultracentrifugation. The virus pellets were stored at 70°C until used.

Immunofluorescence assay (IFA)

Sf9 cells infected with the JE virus were washed with PBS (pH 7.2), and dropped onto spot slides (Nunc, Denmark). The slides were kept at room temperature until the drops had dried, then fixed in anhydrous acetone, at 4°C, for 10 minutes, air-dried, and stored at 70°C until used. The slides were placed in a moist chamber to maintain the humidity throughout the procedure. The slides were incubated for 1 hour, at 37°C, with anti-JE virus envelope (E) protein monoclonal antibody (1 : 200 dilution) and washed 3 times with PBS. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (KPL, USA) (20-30 μl, 1 : 50) was added to each spot, and the slides returned to the moist chamber, at 37°C, for 30 min. The slides were washed 3 times with PBS and once with DW, and then air-dried. The slides were mounted with glycine-buffered glycerol (Sigma) under a cover slip, and examined for their fluorescent pattern under fluorescence microscopy. The addition of a counter stain, Evans blue (0.5% w/v in phosphate buffered saline, Sigma) at a dilution of 1 : 100, to the conjugate gave an excellent reddish background, which increased the ease of viewing.

SDS-PAGE, western blot, and glycoprotein staining

The semi-purified JE virus, cultured in Sf9 cells, was analyzed using 4-20% Tris-glycine pre-cast polyacrylamide gels (Invitrogen, USA), and Tris-glycine running buffer under denaturing conditions, followed by electroblotting onto a nitrocellulose membrane (BioRad, USA), using Novex Xcell II blotting apparatus (Invitrogen, USA). The membranes were blocked with 5% skim milk (Sigma) in phosphate-buffered saline, at 4°C, to prevent non-specific binding. They were then incubated with anti-JE virus envelope (E) protein monoclonal antibody (1 : 200 dilution), overnight, at room temperature, followed by incubation with goat anti-mouse IgG-HRP conjugate (Sigma, USA), for 1 hour, and then visualized with DAB substrate (Sigma, USA). For the glycoprotein staining, a glycoprotein staining kit (Pierce, USA) was used. After electro-

phoresis, the gels were fixed, by complete immersion in 100 ml of 50% methanol for 30 min, and washed twice by gentle agitation in 100 ml of 3% acetic acid for 10 min. The gels were transferred to 25 ml of oxidizing solution, and gently agitated for 15 min. The gels were washed twice by gently agitating with 100 ml of 3% acetic acid for 5 min. The gels were then transferred to 25 ml of GelCode® glycoprotein staining reagent, gently agitated for 15 min, transferred to 25 ml of reducing solution and gently agitated for 5 min. Finally, the gels were extensively washed with 3% acetic acid, and then with DW. The glycoproteins appeared as magenta bands.

Electron microscopy

Formvar-carbon coated, 200 mesh copper grids (TED PELLA, USA), were placed on the surface of the droplet of semi-purified JE virus samples. After 30 sec, most of the solution was removed from the grid with filter paper. The grid was placed on 1% aqueous uranyl acetate solution (Sigma) for 30 sec, and dried. The JE virus was observed under transmission electron microscopy, model JEM1010 (JEOL, Japan) at the NICEM (The National Instrumentation Center of Environmental Management, Seoul National University).

Plaque titration by plaque forming assay

The plaque assay was used to quantify the titer of the JE virus in the Sf9 cell cultures. 5ml of Vero cells (ATCC CRL-1586, USA), at 2.0×10^5 cells/ml, with 5% FBS-MEM media, was seeded to 60mm dishes and incubated at 37°C in a 5% CO_2 incubator. After 24 h incubation, the cells were washed with PBS (pH 7.2) and 0.4 ml/dish of the virus solution, diluted to 10^{-3}~10^{-10}, added. The dishes were incubated for 90 min, at 37°C, with side-to-side rocking, overlaid with the MEM (5% FBS), containing 1% agarose (Sigma, USA). After solidifying the agarose, the dishes were incubated for 72 h, at 37°C, and overlaid with 4 ml of the MEM-1% agarose solution, containing 3% neutral red solution. After solidifying the agarose, the dishes were reversed and incubated at 37°C. After 24 h, the plaques were counted.

Results and Discussion

Infection of Japanese encephalitis virus Beijing-1 strain on Sf9 cells

It is known from the prior part that the Japanese encephalitis virus can propagate on various cell cultures, including cultures of cell lines, in particular Vero cells. However, the culturing methods disclosed satisfactory yields, under large-scale industrial culturing conditions, can not be obtained (Fanget *et al.*, 1996). To explore the ability of Sf9 cells to be infected by the JE virus, Sf9 cells were infected with the JE virus Beijing-1 strain. When the JE virus Beijing-1 strain is used, a broad antigenic spectrum JE virus vaccine can be obtained (Ishikawa *et al.*, 1999). As one of the passage cell lines, Sf9 cells have obvious advantages over conventional production technology for vaccines. They are more suitable for high concentration and large-scale cultures than other cell lines, with considerable exploitability (Summers *et al.*, 1987). The Sf9 cells were normally maintained at cell densities from 3×10^5 to 3×10^6 cells/ml in T- or spinner flasks, and split at 3 or 4 day intervals, using Insect-Xpress serum-free media. The Sf9 cells were grown to maximal concentration, of 8×10^6 cells/ml, in 500 ml spinner flasks, with a doubling time at the exponentially growing phase of 24.5 h. The JE virus was inoculated into Sf9 cells in a spinner flask, with a 0.1 multiplicity of infection (m.o.i), and incubated at 27°C. By 4-5 days post-infection with the JE virus Beijing-1 strain, 10-15% of the Sf9 cells showed cytopathic effects (CPE), from granularity to the formation of syncytia and multinucleated giant cells continuously observed over a period of 35 days (Fig. 1). Arthropod vectors, as a rule, show no pathological effects from infection by most arboviruses, while naturally and experimentally infected vertebrates often do. Similarly, arbovirus infections in cell culture are usually highly cytopathic for permissive vertebrate cell cultures, while permissive arthropod cell cultures may display no detectable CPE, even though they become persistently infected. For the JE virus, no extensive CPE in Diptera and Lepidoptera cells has been reported (Da-Wei *et al.*, 1996). Banerjee *et al.* (1968) reported the JE virus caused CPE in *Aedes albopictus* cells, but after 3-4 passages the cultures appeared morphologically normal. Yunker *et al.* (1968) observed inap-

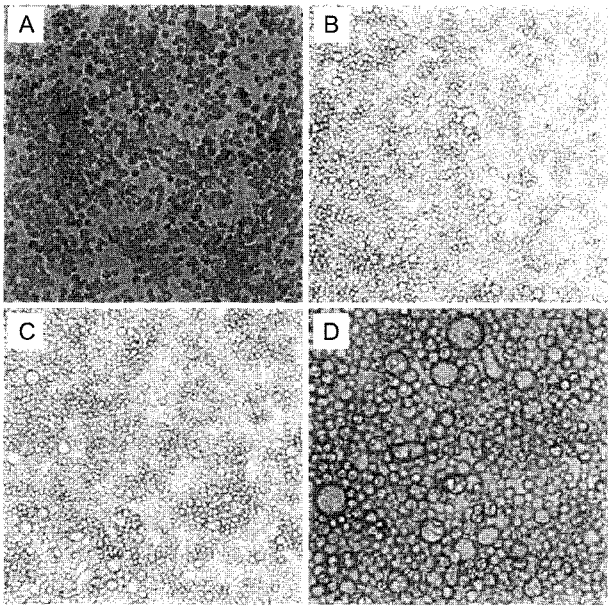

Fig. 1. Comparison of Sf9 cell morphology. (A) normal Sf9 cells (100 X). (B) Sf9 cells infected with JE virus Beijing-1 strain 1 days post-infection (100 X), (C) 3 days post-infection (100 X) and (D) 3 days post-infection (200 X).

parent, or no CPE, and low level of JE virus replication in the *Antheraea eucalypti* cell line. In the present study, the cytopathogenicity of JE virus Beijing-1 strain was characterized by syncytium formation under routine culture conditions.

Detection of virus-specific antigen

To assess whether viral antigens were present over a prolonged period in virus-inoculated Sf9 cells, an immunofluorescence assay was performed using anti-JE virus envelope protein (E) monoclonal antibody (SJ-137), which was prepared in our lab. As shown in Fig. 2, positive fluorescent reactions were detected in 30-40% of cells infected with the JE virus Beijing-1 strain, but the uninfected Sf9 cell control was completely negative. Therefore, the infection with JE virus Beijing-1 strain was accomplished by production of the JE virus specific antigens in infected Sf9 cells, as detected by immunofluorescence, using a monoclonal antibody against the JE virus envelope protein.

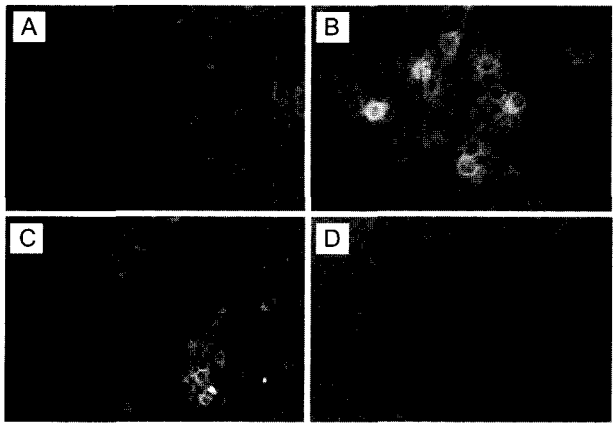

Fig. 2. Indirect immunofluorescence of JE virus Beijing-1 infected Sf9 cells. (A) Sf9 cells infected with JE virus Beijing-1 strain, 1 days postinfection (100 X), (B) 3 days postinfection (100 X), (C) 1 day postinfection (200 X) and (D) normal Sf9 cell (100 X).

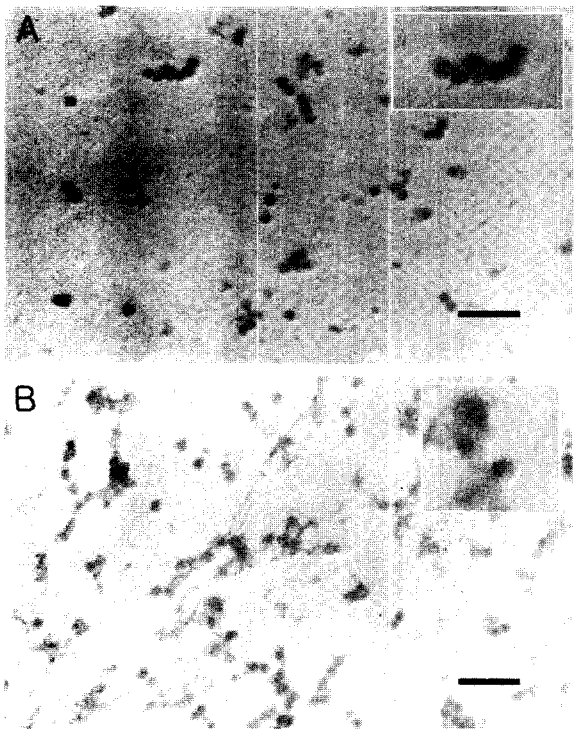

Fig. 3. Detection of JE virus particles by transmission electron microscopy. (A) Purified JE virus Beijing-1 strain cultured in Vero cell and (B) cultured in Sf9 cells. The scale bar is 500 nm.

To further evaluate whether the productive virus infection was occurring, electron microscopy of the JE virus Beijing-1 strain, cultured in Vero and Sf9 cells, was performed, as described in materials and methods, and the viral morphogenesis examined (Fig. 3). There are several reports that the JE virus can grow in certain lines of insect cells (Morimoto *et al.*, 1969; Igarashi *et al.*, 1973). In this work, the JE virus Beijing-1 strain was grown in Sf9 and Vero cells. The virus particles were concentrated by sedimentation on 40% trehalose cushions by ultracentrifugation. Although the Vero cells supported more abundant virus production, the Sf9 and Vero cells showed identical patterns of viral morphogenesis. Complete virus particles, 40 to 50 nm in diameter, were seen. Igarashi *et al.* (1973) reported that they could detect no significant differences between the JE virus preparations grown in *Aedes albopictus* and BHK21 cells, with respect to their apparent sedimentation in a sucrose gradient, buoyant densities, structural polypeptides, optimal pH for hemagglutinin, antigenicity, as revealed by neutralization, or gross morphological structures, as observed by electron microscopy. Therefore, our observation on the viral morphogenesis of the JE virus was comparable to the above report.

For the analysis of Japanese encephalitis virus cultured in Sf9 cell using serum-free media, SDA-PAGE, western blot analysis and a glycoprotein staining assay were performed. As shown in Fig. 4, envelope (E) proteins, at 53 kDa, were found in western blot analysis to the anti-JE virus envelope protein monoclonal antibody (SJ-137). Also, these proteins reacted as a magenta band in the same position to the glycoprotein staining.

Tests for productive infection of Sf9 cells

To evaluate whether an infectious virus was produced in Sf9 cells inoculated with the JE virus Beijing-1 stain, the Sf9 cells were inoculated with the virus, and the samples harvested every 5 days. The samples were inoculated onto Vero cells, and titers determined by a plaque assay. Infectious JE virus was detected in the filtered supernatants from those cultures, indicating that the JE virus established a productive infection in Sf9 cells, with persistent shedding of the virus from the cells. The virus titers were significantly increased when compared with the inoculum (Fig. 5). Titers of the JE virus Beijing-1 strain rose from 1.0×10^5 to 1.5×10^6 pfu/ml. The infected Sf9 cells could be

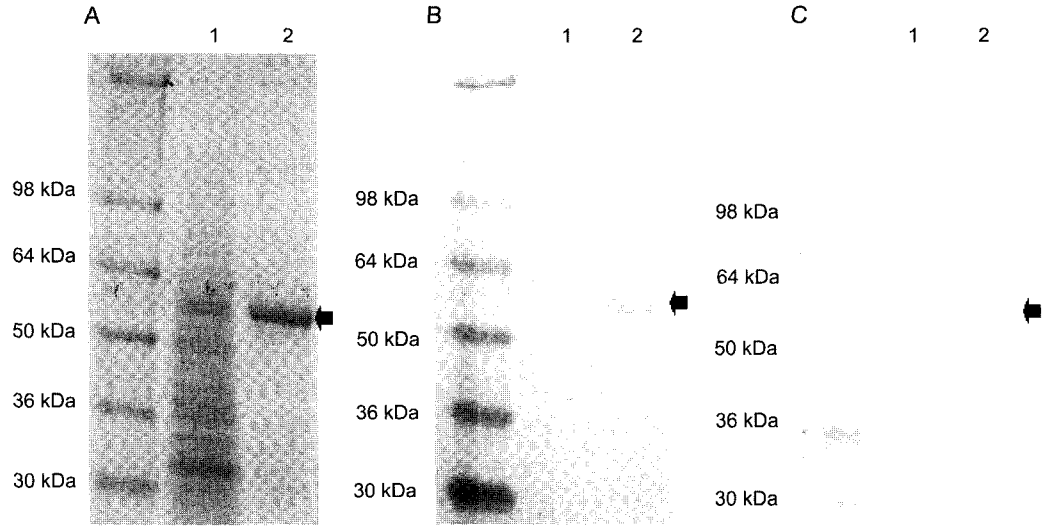

Fig. 4. SDS-PAGE: (A) western blot, (B) and glycoprotein analysis and (C) JE virus propagated in Sf9 cells, and purified with 40% trehalose step gradient ultracentrifugation. The arrows show the JE virus envelope protein. Lane 1, normal Sf9 cell lysate and lane 2, semi-purified Japanese encephalitis virus, cultured in Sf9 cells.

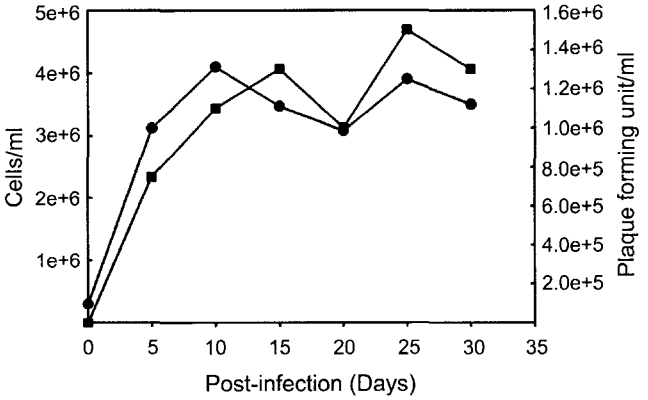

Fig. 5. Growth and replication curves of Sf9 cells infected with JE virus Beijing-1 strain, in spinner flask culture. The cultures were split, and adjusted 3×10^5 cells/ml every 5 days. Symbols: (●), Sf9 cells; (■), Plaque forming unit of JE virus.

sub-cultured in serum-free medium, with the maximum titers showing little difference among the generations. Wang *et al.* (1996) reported that Sf9 cells were very sensitive to the JE virus, which can propagate in both serum and serum-free media. From the above results, the same tendency was observed in the Insect-Xpress™ serum-free medium, with the result showing the JE virus established as a persistent infection of the Sf9 cells in serum-free medium. During a cultivation period of 35 days for the JE virus-inoculated Sf9 cells, multinucleated giant cells were continuously evident, consistently displayed positive immunofluorescence in 30-40% of cells, and the titers of the cell-free viruses remained constant. The susceptibility of a cell line to the JE virus infection may be determined by a number of factors, including the availability of specific receptors, the compatibility of biochemical process within the cell, with requirements for viral replication, and the growth conditions of the cells in the serum free media. Factors that may contribute to the ability of cells to support propagation of the JE virus include; temperature, pH, multiplicity of infection, virus strain, viral passage history, split ratios and composition of the medium. All these factors can potentially influence the growth of the JE virus on Sf9 cells, resulting in differences in the levels of viral replication, the extent of CPE or the establishment of a persistent infection.

Temperature is a critical element in the development of viral infections. For each virus, there is an optimum temperature for its successful replication, above or below which multiplication will be reduced or inhibited. The optimum temperature for the growth of Sf9 cells is 27°C, for other arthropod cell lines it is 29 and 35°C (Igarashi *et al.*, 1973). Igarashi *et al.* (1973) have shown the development of spontaneous temperature sensitive mutants of the JE virus in chick embryo fibroblast cells cultured at 35°C, and the influence of temperature on the production and quality of togavirus plaques in vertebrate cell lines (Brown, 1963; Johnson, 1969). Arboviruses may not generally produce CPE in arthropod cells, due to the low temperature of cultivation. Low temperatures may favor persistent infection, which may explain why temperature shock of St Louis encephalitis virus (SLE)-infected cloned *A. albopiclus* mosquito cells induced plaque formation (Randolph *et al.*, 1990). However, the present data, showing that the JE virus Beijing-1 strain produced syncytia in sf9 cells cultured at 27°C, suggest that in Sf9 cells, syncytia can form at low temperatures. For the investigation of the effects of temperature on the virus productivity, attempts were made at propagation of the JE virus on Sf9 cells cultured at temperatures ranging from 23 to 37°C. However, no difference in the productivity could be found

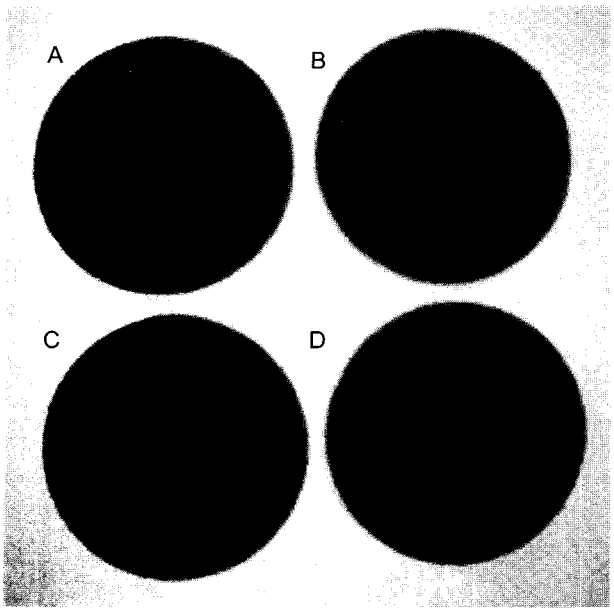

Fig. 6. Plaque formation on Vero cells by the JE virus Beijing-1 strain in the supernatant harvested from persistently infected cultures of Sf9 cells. The supernatants were harvested 5 days post-infection (A), 15 days post-infection (B), 25 days post-infection (C) and 35 days post-infection (D).

(data not shown).

The optimum pH, in addition to the temperature, may be another critical parameter for JE virus replication (Gangodkar *et al.,* 1997). The pH of the media ranges from pH 6.0-6.5, under routine serum-free Sf9 culture conditions, and the optimum pH range for the JE virus stability was between pH 7.0-9.0. Relatively low titers of the JE virus were produced in serum-free Sf9 cell cultures, compared to those, such as C6/36 (8.0-9.5 pfu/ml), BHK-21 (7.0-8.5 pfu/ml) and Vero cells (7.0-8.5 pfu/ml) (Liang *et al.,* 1985). To investigate the effects of pH on the productivity, release and stability of the JE virus in Sf9 cell cultures, serum-free media with pH levels ranging from 5.5 to 8.0 were studied. For the cell growth, normal serum-free media (pH 6.0-6.2) were used, and pH adjusted serum-free media were used for the infection and replication phases of the JE virus. As with the temperature result, difference could be found (data not shown). For the evaluation of the replication level, additional studies of these issues will be required, including the media compositions.

Studies have shown that progeny flaviviruses, derived from arthropod cell cultures, may differ phenotypically from the parent virus used to infect the cultures (Sinarachatanant *et al.,* 1973; Ng *et al.,* 1980). Also, temperature sensitive and small plaque mutants have been detected, after long-term passage, in several mosquito cell lines infected with the St Louis encephalitis virus (Zhang *et al.,* 1993). In order to understand if the plaque formation capacity of the JE virus produced in serum-free Sf9 cell cultures could be changed, plaque assays were performed using the JE virus, sampled at 5, 15, 25 and 35 days post-infection, in persistently infected serum-free Sf9 cell cultures. The plaque forming capability by the JE virus, from persistently infected Sf9 cells, was monitored according to the passage number and time (Fig. 6). The results showed no change in the plaque sizes formed by the JE virus Beijing-1 strain.

The sensitivity of the Sf9 cells to the Japanese encephalitis virus in serum free media, the infection type of the JE virus in Sf9 cells and the passage stability of the JE virus have been studied. The ability of the JE virus Beijing-1 strain to infect Sf9 cells in serum-free media will provide a useful insect cell system, where the concurrent JE virus replication, cytopathogenicity and factors involved in viral persistence, or isolation of field strains, can be studied. This may also prove useful for the large-scale production of a virus, as Sf9 cells can be readily cultured in bioreactors. Additional studies on the possibility of using a serum-free Sf9 cell culture system on a large-scale, as a JE virus vaccine candidate, will be performed in our laboratory, including productivity and potency issues.

References

Banerjee, K., and K.R.P. Singh. 1968. Establishment of carrier cultures of *Aedes albopictus* cell line infected with arboviruses. *Indian Med. Res.* 56, 812-814.

Brown, A. 1963. Differences in maximum and minimum temperature among selected group A arboviruses. *Virology.* 21, 362-372.

Da Wei Fu, and P.F. Zhang. 1996. Establishment and characterization of Japanese B encephalitis virus persistent infection in the Sf9 insect cell line. *Biologicals.* 24, 225-233.

Endy, T.P. and A. Nisalak. 2002. Japanese encephalitis virus: Ecology and epidemiology. *Curr. Top. Microbial. Immunol.* 267, 11-48.

Fanget Bernard, Francon Alain, and Heimendinger Pierre. 1996. Method for industrially producing a Japanese encephalitis vaccine, and resulting vaccine. *WO 97/04803.*

Gangodkar, S.V., S.P. Paranjape, V.D. Kadam, and R.P. Deolankar. 1997. Optimal pH conditions for the growth, release and stability of Japanese encephalitis virus in PS cell line. *Curr. Sci.* 73, 12, 1108-1109.

Igarashi, A., F. Sasao, S. Wungkobkiat, and K. Fukai. 1973. Growth of Japanese encephalitis virus in established lines of mosquito cells. *Biken J.* 16, 17-23

Igarashi, A. 1978. Isolation of a Singhs Aedes albopictus cell clone sensitive to dengue and chikungunya viruses. *J. Gen. Virol.* 40, 531-544.

Ishikawa Toyokazu, Yoshii Hironori, Imagawa Tadashi, and Ishibashi Masahide. 1999. Enhanced immunogen for inactivated vaccine for infection with Japanese encephalitis viruses and process for producing the same. *EP 1 057 889 A1. 99923858.7.*

Johnson, J.W. 1969. Growth of Venezuelan and eastern equine encephalomyelitis viruses in tissue cultures of minced *Aedes aegypti* larvae. *Am. J. Trop. Med. Hyg.* 18, 103-114.

Liang, X.J. and Y.M. Wang. 1985. Study of infection of C6/36 cells with Japanese encephalitis virus. *Chin. J. Microbiol. Immunol.* 5, 85-87.

Luckow, V.A. 1991. Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In recombinant DNA technology and Application, 97-152, Elsevier press, New York.

Morimoto, T., T. Sugimor, Y. Miura, H. Sazawa, and M. Watanabe. 1969. Growth characteristics and persistent infection of Japanese encephalitis virus in bovine kidney cell culture at different temperatures. *Nat. Inst. Anim. Hlth. Quart.* 9, 65-73.

Ng, M.L. and E.G. Westaway. 1980. Establishment of persistent infections by flaviviruses in *Aedes albopictus* cell. In Invertebrate systems. In Vitro. 389-402. New York & Oxford: Elsevier/ North-Holland Biomedical Press.

Randolph, V.B. and V. Stollar. 1990. Low pH-induced cell fusion in flavivirus type 2 in Aedes albopictus cell culture. *J. Gen. Virol.* 71, 1845-1850.

Sinarachatanant, P. and L.C. Olson. 1973. Replication of dengue virus type 2 in *Aedes albopictus* cell culture. *J. Gen. Virol.* 12, 1845-1850.

Summers, M.D. and G.E. Smith. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station and Texas A&M University College Station.

Takahashi, H., V. Pool, T.F. Tsail, and R.T. Chen. 2000. Adverse events after Japanese encephalitis vaccination: review of post-marketing surveillance data from Japan and the United States. The VAERS Working Group. *Vaccine*, 18, 2963-2969.

Vaughn, J.L., R.J. Goodwin, G.J. Tompkins, and P. McCawley. 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda. In vitro.* 13, 213-217.

Wang, X., Qi Yihua, and Ouyang Fan. 1996. Study on the sensitivity of sf9 cells to the Japanese B encephalitis virus. 4th Asia-Pacific Biochemical Engineering Conference, 181-183.

WHO. 1994. Japanese encephalitis. *Wkly. epidemiol. rec.* 69, 113-120.

Wu, S.C. and G.Y. Huang. 2002. Stationary and microcarrier cell culture processes for propagating Japanese encephalitis virus. *Biotechnol. Progr.* 18, 124-128.

Yunker, C.E. and J. Cory. 1968. Infection of grace's Antheraea cell with arboviruses. *Am. J. Trop. Med. Hyg.* 17, 889-893.

Zhang Peng-fei., K. Michael, M. Jacqueline, and J.M. Carol. 1993. St Louis encephalitis virus establishes a productive, cytopathic and persistent infection of sf9 cells. *J. Gen. Virol.* 74, 1703-1708.