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Mosquito Cells Infected with Japanese Encephalitis Virus Release Slowly-Sedimenting Hemagglutinin Particles in Association with Intracellular Formation of Smooth Membrane Structures

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Running head: SMS-SHA association in JEV-infected mosquito cells

Key words: Encephalitis Virus, Japanese: ultrastructure: Virus Particle: Morphogenesis

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Abstract

Arthropod-borne flaviviruses can grow in both arthropod and mammalian cells. Virion morphogenesis, though well studied in mammalian cells, is still unclear in arthropod cells. Here, we compared a mosquito cell line C6/36 and a mammalian cell line Vero in extracellular virus particles and intracellular ultrastructures triggered by infection with Japanese encephalitis virus (JEV). Sedimentation analyses of virion and slowly-sedimenting hemagglutinin (SHA) particles released by infection with the Nakayama strain revealed that C6/36 cells produced higher envelope (E) antigen levels in the SHA than the virion fraction in contrast to Vero cells that showed the opposite pattern. Specific infectivities per ng of E were similar in both cells, whereas specific hemagglutinating activities in the SHA fraction were lower in C6/36 than Vero cells. The precursor membrane protein was less efficiently cleaved to the membrane protein in SHA particles released from C6/36 than Vero cells. Ultrastructural studies showed more remarkable production of smooth membrane structures (SMSs) in C6/36 than in Vero cells. The differences in sedimentation patterns of extracellular virus particles between Nakayama-infected C6/36 and Vero cells were consistently observed in 5 other strains (Beijing P1, Beijing P3, JaTH-160, KE-093 and JaGAr-O1), except for KE-093-infected

C6/36 cells which exhibited the Vero-type sedimentation profile under conditions of open cultivation. By electron microscopy, the production of SMSs from KE-093-infected C6/36 cells under open conditions was markedly less than that under closed conditions where the cells exhibited the C6/36-type sedimentation profile. Thus, intracellular SMS formations were associated with extracellular SHA production in JEV-infected mosquito cells.

Key words: Encephalitis Virus, Japanese: ultrastructure: Virus Particle: Morphogenesis

Introduction

Japanese encephalitis virus (JEV) is a member of the genus Flavivirus family *Flavivirdae*. Flavivirus virions, 40-60 nm in diameter, contain a nucleocapsid about 30 nm in diameter that is surrounded by an envelope. The nucleocapsid is composed of a capsid (C) protein and a single strand of positive-sense RNA, while the envelope is composed of a lipid bilayer derived from the host cell, an envelope (E) protein and a membrane (M) protein. A small amount of the precursor of M (prM) is usually contained in the envelope. In most cases, flavivirus-infected cells release not only virions but also empty particles that do not contain a nucleocapsid. These particles sediment more slowly than do virions in sucrose density gradient centrifugation and are named slowly-sedimenting hemagglutinin (SHA) particles.

Flavivirus virion morphogenesis has been extensively studied (35). Structural proteins C, prM and E, and seven nonstructural (NS) proteins are synthesized in order from the start of a single long open reading frame encoded by the flavivirus genome. Transmembrane domains consisting of hydrophobic amino acids at the carboxy-termini of C and prM play a role as the signal that directs translocation of prM and E to the lumen of the endoplasmic reticulum (ER). Carboxy-termini of C, prM and E are cleaved

on the luminal side of the ER membrane by the host enzyme signalase, and prM and E are fixed on the ER membrane as an N-linked type I membrane glycoprotein with the transmembrane domain as a membrane anchor. Cleavage of the junction of the cytoplasmic and transmembrane domains of C is mediated by the viral protease NS2B/NS3. Budding from the prM/E-accumulated ER membrane into the lumen to envelope a nucleocapsid produces an immature virion, whereas particle formation without nucleocapsids is considered to produce SHA particles (2, 6). These viral particles are transported through a secretory pathway to the trans-Golgi network where prM is cleaved to M by a protease furin (41). Cleavage of prM to M that converts prM/E heterodimers to E homodimers results in the acquisition of infectivity and hemagglutinating (HA) activity. When exposed to low pH, E homodimers are irreversibly converted to a homotrimer form, resulting in the acquisition of the membrane fusion competence (1). Structural analyses of the surface of flavivirus particles by X ray crystallography (5, 39) and cryo-electron microscopy (11, 24, 34, 36, 44, 45) have shown E homodimers on the particles and low pH-induced conversion to E homotrimers; however, it is suggested that a part of E remains to form a heterodimer with prM.

In morphological studies, virions and recombinant subviral particles that are

similar to SHA particles have mainly been observed in the lumen of the ER and the Golgi apparatus in both mammalian and mosquito cells (4, 15, 16, 27, 29, 37). On the other hand, the budding intermediates into the lumen of the ER to form virion or SHA particles have not been frequently observed, probably because the budding process is rapid. The most prominent features observed in flavivirus-infected cells are induction of membrane proliferation particularly in the perinuclear region. Characteristic membrane structures and the dilated ER have been observed in flavivirus-infected mammalian and mosquito cells (10, 14, 26, 30, 43).

JEV is a mosquito-transmitted agent and has the ability to propagate in both mammalian and mosquito cells. However, the course of infection is different between the two cell species, as can be seen in other mosquito-borne flaviviruses (35). Most remarkably, JEV infection induces cytopathic effects and cytolysis in most of mammalian cells, whereas the infection of mosquito cells is usually noncytopathic and may establish persistent infections (19, 38). Since the mechanism of flavivirus virion morphogenesis described above has mainly been established using mammalian cells, it is important to study mosquito cells as another host of JEV infection. The mechanism of virion maturation in mosquito cells may contribute to the development of a strategy for the prevention of JEV transmission, and could include a possible new type of vaccine

targeting this invertebrate vector.

The limited electron microscopic studies conducted to date on virion morphogenesis in JEV-infected mosquito cells have shown virus particles in the ER lumen, Golgi apparatus and coated vesicles; as well, characteristic membrane structures named smooth membrane structures (SMSs) were also observed (14, 16, 26). In this study, as our initial approach to the mechanism of virion maturation in mosquito cells, we performed basic biochemical and biophysical analyses of virus particles released from JEV-infected cells, as well as morphological studies of infected cells utilizing electron microscopy. Our results indicated that JEV-infected mosquito cells released higher levels of E antigen in SHA than virion particles, and that the intracellular formation of SMSs was associated with the release of SHA particles from infected mosquito cells.

Materials and Methods

Cells. Vero (22) and C6/36 (18) cells were used as mammalian and mosquito cell lines, respectively. Vero cells were cultivated in a growth medium composed of Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated

fetal bovine serum (FBS) at 37 C in a humidified atmosphere of 5% CO_2-95% air. C6/36 cells were grown under the same conditions as in Vero cultures except for the addition of 1 mM nonessential amino acids to the medium and that the cultivation temperature was 28 C.

Viruses. Six strains of JEV were used in this study. The Nakayama and Beijing P3 strains have been described (23). The Beijing P1 strain, which had been passaged eleven times through suckling mouse brains (SMBs) and one time through Vero cells was provided by Dr. Robert E. Shope of Yale Arbovirus Research Unit, Yale University School of Medicine, CT, USA. The JaTH-160 strain, which had been passaged three times through SMBs, and the KE-093 strain, which had been passaged four times through SMBs, were provided by Dr. Ichiro Kurane of National Institute of Infectious Diseases, Japan. The JaGAr-O1 strain, which had been passaged twelve times through SMBs, was provided by Dr. Eiji Kimura of Hyogo Prefectural Institute of Health, Japan. In our laboratory, these strains were passaged one or two times through C6/36 cells and, the infected culture fluid was used in this study.

Virus infection. C6/36 and Vero monolayer cells were infected with each strain of JEV at a multiplicity of infection (m.o.i.) of 5 plaque-forming units (PFU)/cell. After 1-hr adsorption, cells were rinsed three times with phosphate-buffered saline

(PBS), and incubated at temperatures corresponding to each cell line in a maintenance medium that was the growth medium replacing 10% FBS to 0.075% bovine serum albumin. Culture fluids were harvested at 24, 48 and/or 72 hr postinfection (p.i.).

Infected cells were cultivated in culture flasks or dishes (including 6-well microplates). In some experiments, the cap of the culture flask was tightly closed to eliminate gas exchange with the 5% CO_2-95% air environment (closed cultivation), different from the other conditions where the cap was loosened enough to allow gas exchange (open cultivation). Cultivations using culture dishes were regarded as an open condition.

Precipitation with polyethylene glycol (PEG). Culture fluids harvested from infected cells, 40 ml in volume from 4 75-cm^2 culture flasks, were clarified and incubated in 10% PEG and 1.9% NaCl for 2 hr at 4 C. Following centrifugation at 10,000 rpm for 30 min in the R22A rotor of a Himac CR22E (Hitachi Koki Co. Ltd., Ibaraki, Japan), the pellet was suspended in 1 ml TN buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl) and clarified. This suspension was further concentrated by one more PEG precipitation in a similar way, to obtain 200 μl of the concentrate suspended in TN buffer.

Sucrose density gradient centrifugation. Culture fluids or PEG-concentrated

culture fluids were applied to a 10 to 40% (wt/wt) continuous sucrose gradient prepared in TN buffer. Following centrifugation for 90 min at 55,000 rpm at 4 C in the S55S rotor of a Himac CS100GX microultracentrifuge (Hitachi Koki Co. Ltd., Ibaraki, Japan), fractions were collected from the bottom.

ELISA for measuring E antigen levels. E antigen levels were measured by a sandwich ELISA (21). Briefly, 96-well microplates sensitized with rabbit anti-JEV polyclonal antibody were incubated with samples (sucrose gradient fractions) at 1:10 dilution, a monoclonal antibody J3-11B9 (33) recognizing E antigen, alkaline phosphatase-conjugated anti-mouse IgG and then p-nitrophenyl phosphate. The E protein amount was calculated by comparing absorbances at 415 nm with those obtained from a recombinant E protein standard prepared from prM/E-expressing mammalian cells.

Plaque assay and HA test. Infective titers were determined on a monolayer of Vero cells as previously described (22). HA titers were determined by a micro-modification of the method described by Clarke and Casals (9): titers obtained with 25 μl of the sample were expressed in HA unit (HAU). Specific infectivity and HA activities were obtained by calculating infective and HA titers per ng of the E protein, respectively.

Endoglycosidase treatment. The virion and SHA fractions obtained by sucrose density gradient centrifugation from PEG precipitated culture fluids were centrifuged at 55,000 rpm for 90 min at 4 C in the S55S rotor (see above) and the pellets were then dissolved in 20 μl of the "sample" buffer (0.1 M Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 4 mM ethylenediamine-N, N, N', N'-tetraacetic acid, 30% glycerol, 0.2 g/ml bromophenol blue). A half (10 μl) of this sample solution were mixed with 2 μl of the "reaction" buffer (10% SDS, 10% 2-mercaptoethanol) and incubated at 100 C for 5 min. After cooling, one unit of N-glycosidase F (PNGase F; Roche diagnostics, Basel, Switzerland) was added to the sample and incubated at 37 C for 24 hr. For inactivation of PNGase F, the samples were incubated at 100 C for 5 min.

Western blot analysis. The analysis was performed essentially as previously described (21). Briefly, serial 2-fold diluted PNGase F-treated samples were separated by 14% SDS-polyacrylamide gel electrophoresis at 150 V for 75 min and transferred to polyvinylidene difluoride membrane at 200 mA for 120 min. The membrane was incubated with a monoclonal antibody J2-2F1 (33) recognizing prM/M, alkaline phosphatase-conjugated anti-mouse IgG and then nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate. The levels of prM and M were expressed as the reciprocal highest dilution representing a visible band (see Fig. 3). To

compare viral particles obtained from JEV-infected C6/36 and Vero cells, prM:M ratios were calculated from the levels of prM and M.

Electron microscopy. C6/36 and Vero monolayer cells prepared in culture dishes were infected at an m.o.i. of 5, incubated for 48 hr and fixed with 2% paraformaldehyde-0.25% glutaraldehyde in PBS at room temperature for 4 hr. Following rinsing with PBS, cells were further fixed with 2% osmium tetraoxide in PBS at room temperature for 1 hr. Cells were dehydrated with a graded ethanol series and embedded with Epok 812 (Okenshoji Co. Ltd., Tokyo, Japan). Sections were obtained by cutting Epok-embedding samples with a Leica UltracurR (Leica Microsystems GmbH, Vienna, Austria) and stained with uranyl acetate and then with lead citrate,. Sections were observed under a JEM-1220 electron microscope (JEOL Ltd., Tokyo, Japan).

Results

Comparison of virus particles released from C6/36 and Vero cells infected with the JEV Nakayama strain. To perform a basic characterization of the virus particles released from C6/36 and Vero cells infected with JEV and to assess the effect

of the harvest time point, we first used a prototype Nakayama strain. The Nakayama-infected C6/36 and Vero cells were cultivated in a tissue culture flask under the closed condition and culture fluids obtained at 24, 48 and 72 hr p.i. were analyzed on sucrose density gradients in terms of their E antigen levels, and infective and HA titers (Fig. 1). At any time point, the sedimentation profiles obtained with infected C6/36 cells indicated higher levels of E antigen in the SHA than the virion fraction, whereas infected Vero cells released higher E antigen levels in the virion than the SHA fraction. Based on this profile characteristic of C6/36 cells, subsequent analyses were focused on SHA particles, as well as virions, for directing the events occurring in infected mosquito cells.

The results obtained in Fig. 1 were further analyzed to obtain time courses of the ratio of E antigen levels between the virion and SHA fractions (virion:SHA ratio; Fig. 2a), specific infectivities and HA activities (Fig. 2b, c). The virion:SHA ratios obtained from infected C6/36 cells were <1 and those obtained from infected Vero cells were >1 with differences of >10-fold during the experimental period of 24-72 hr (Fig. 2a). Specific infectivities of the virion fraction obtained with infected C6/36 cells were similar to those obtained with infected Vero cells (Fig. 2b). Specific HA activities of the virion and SHA fractions from infected C6/36 cells were lower than those of the

corresponding fractions from Vero cells (Fig. 2c). In addition, specific HA activities of the virion fractions were higher than those of the SHA fractions in both cells.

Furin-mediated cleavage of prM to M is a critical step for the flavivirus virion to subsequently acquire infectivity and related viral activities. We therefore compared levels of prM and M in each of the virion and SHA fractions. For this purpose, a semiquantitative method based on Western blotting analysis was used. A typical result of this method is shown in Fig. 3, which represents prM and M bands of virion and SHA fractions obtained from JEV-infected C6/36 and Vero cells at 48 hr p.i. Although all 24-hr samples showed undetectable levels and one 72-hr sample showed levels of $\geq$2,048 for both M and prM, the 48-hr samples allowed us to compare 4 data (Fig. 2d) and showed that the prM:M ratios obtained with the SHA fraction were higher than those obtained with the virion fraction in both C6/36 and Vero cells. In addition, the prM:M ratio in the SHA fraction obtained from C6/36 cells was higher than that obtained from Vero cells. Although only the 48-hr samples were used for comparison in Fig. 2d, similar results were obtained in a repeated experiment (data not shown).

These results indicate that virus particles released from JEV-infected C6/36 and Vero cells had different natures and that their surface proteins had different functions.

Ultrastructural changes induced in Nakayama-infected C6/36 and Vero

cells. To compare the ultrastructural changes induced by JEV infection between C6/36 and Vero cells, we used Nakayama-infected C6/36 and Vero cells at 48 hr p.i. for electron microscopic examination. Although culture dishes were used for preparation of cell samples and therefore cells were cultivated under an open condition, the culture fluids obtained from both cell lines showed consistent sedimentation profiles (data not shown) with those of the corresponding harvest time (48 hr) shown in Fig. 1.

At a low magnification, numerous characteristic vesicles of varying sizes were distributed in the whole cytoplasmic area of infected C6/36 cells (Fig. 4a), whereas infected Vero cells did not contain such large-sized vesicles (Fig. 4e). At a high magnification, the dilated ER and SMSs were observed in both infected C6/36 and Vero cells (Fig. 4b, f, respectively), neither of which were found in uninfected cells (data not shown). However, C6/36 cells exhibited a higher degree of membrane proliferation than Vero cells. The production of SMSs was limited mainly to the perinuclear regions in infected Vero cells (Fig. 4g, h), whereas these abnormal membrane structures were observed at similar frequencies both in the perinuclear and peripheral regions of infected C6/36 cells (Fig. 4c, d). Even in the perinuclear regions, SMSs were more remarkably observed in C6/36 than Vero cells (Fig. 4c versus 4g). Virions were observed in the ER in both C6/36 and Vero cells (Fig. 4d, g) and, though in a small

number, in SMSs in C6/36 cells (Fig. 4b, c; arrow heads). No virions were found in SMSs in Vero cells, as far as the samples examined in this study were concerned.

Comparisons of virus particles released from C6/36 and Vero cells infected with other strains of JEV. To examine if the difference between extracellular viral particles released from Nakayama-infected C6/36 and Vero cells shown in Fig. 1 would be a common phenomenon in JEV-infected cells, 5 additional strains of JEV were examined under the same characterization methods. The strains we used were Beijing P1, Beijing P3, JaTH-160, KE-093 and JaGAr-O1: for convenience, infected cells were cultivated in a 6-well culture plate. Since similar results were obtained at any time point after infection (24, 48 and 72 hr) using the Nakayama strain (Fig. 2), culture fluids harvested at 48 hr p.i. were used for sedimentation analyses of the 5 strains (Fig. 5). Infected C6/36 cells released higher levels of E antigens in the SHA than the virion fraction and the virion:SHA ratios were <1 in most strains (Table 1), consistent with the results obtained from Nakayama-infected C6/36 cells. In all cases, infected Vero cells released higher levels of E antigens in the virion than the SHA fraction and the virion:SHA ratios were >1 (Table 1), again consistent with the results obtained from Nakayama-infected Vero cells. However, C6/36 cells infected with the KE-093 strain released higher levels of E antigens in the virion than in the SHA fraction with the

virion:SHA ratio being >1, similar to the pattern obtained with Vero cells infected with Nakayama and the 5 other strains.

Specific infectivities of the virion fraction obtained from infected C6/36 cells ranged from 5.0 to 24.3 x 10^4 in 6 strains including the Nakayama and those obtained from infected Vero cells varied from 2.1 to 15.3 x 10^4 (Table 2), similar to previously obtained specific infectivities from Nakayama-infected C6/36 and Vero cells (Fig. 2b). Comparison of specific HA activities (Table 3) indicated that the activity of the virion and SHA fractions obtained from infected C6/36 cells were lower than the corresponding fractions of Vero cells with some exceptions including the virion fraction obtained with KE-093-infected cells. In all strains and both cell species, specific HA activities were higher in the virion than the SHA fractions. Further, comparison of the prM:M ratios (Table 4) indicated that the ratios obtained with the SHA fraction were higher in C6/36 than Vero cells in any strain and those obtained with the virion fraction were higher in C6/36 than Vero cells in 4 of the 6 strains. In all strains for C6/36 cells and in almost all strains for Vero cells, the prM:M ratios obtained with the SHA fraction were higher than those obtained with the virion fraction: however, there was a low level of prM and an extremely high level of M in the SHA fractions from KE-093-infected C6/36 and Vero cells, respectively.

These results indicated that quantitative and qualitative differences in virus particles released from C6/36 and Vero cells infected with the Nakayama strain were a common feature of JEV infections, with the exception of the KE-093 strain.

Comparison of virus particles released from KE-093-infected C6/36 cells under the open and closed cultivating conditions. To further examine the exceptional nature of the virus particles released from KE-093-infected C6/36 cells, we attempted to compare sedimentation profiles of culture fluids obtained from cells cultivated in culture flasks under the open and closed conditions (Fig. 6). Under the closed cultivating condition, KE-093-infected C6/36 cells released higher levels of E antigens in the SHA than the virion fraction, showing the original C6/36-type sedimentation profile shown by the other strains of JEV. On the other hand, the open cultivation of KE-093-infected C6/36 cells released higher levels of E antigens in the virion than the SHA fraction, showing the Vero-type sedimentation profile. The virion:SHA ratio obtained with the open and closed cultivating conditions were 2.73 and 0.34, respectively; comparable to the results obtained with Vero and C6/36 cells infected with the other strains.

Specific infectivities of the virion fraction obtained under the open (48.73) and closed (67.23) cultivating conditions were equivalent. Although specific HA activities

of the virion fraction obtained from the open (3.00) and closed (2.69) cultivating conditions were higher than those of the SHA fraction (<0.1 under both the open and closed cultivating conditions), the values obtained under both cultivating conditions were similar. In addition, the prM:M ratios obtained with virion (0.06) and SHA (1.00) under the closed cultivating condition did not differ from previously obtained values (0.031 for virion, 1.00 for SHA, Table 4). These results indicated that although particle formation was affected by the cultivating condition in KE-093-infected C6/36 cells as shown by sedimentation profiles, the effect of the cultivating condition was not large enough to detect the difference in the nature of virus particles.

Ultrastructural changes of KE-093-infected C6/36 cells under the open and closed cultivating conditions. In addition to the above morphological approach using different cell species (C6/36 and Vero cells), a study of comparisons in a single cell species (C6/36) showing differences in extracellular virus particles under different cultivating conditions is another model appropriate for studying associations of extracellular virus particles with intracellular morphological changes. We therefore compared the ultrastructural features of KE-093-infected C6/36 cells under the open and closed cultivating conditions (Fig. 7). Although SMSs were observed throughout the cytoplasmic area under both conditions, remarkably reduced SMS production was noted

under the open cultivating condition. The formation of the dilated ER was also reduced in KE-093-infected C6/36 cells under the open cultivating condition. Virions were observed in the ER and much less in SMSs under both open and closed cultivating conditions.

Discussion

This study demonstrated that virus particles released from JEV-infected cells have different natures, respectively, in two host cell species, C6/36 and Vero. C6/36 cells released higher levels of E antigens in the SHA than the virion fraction, whereas Vero cells released higher levels of E antigens in the virion than the SHA fraction. The difference between these mosquito and mammalian cells was also observed in ultrastructural approaches, which indicated that SMSs were more markedly observed in C6/36 cells than in Vero cells. Thus, the present comparative studies using C6/36 and Vero cells showed preferred release of SHA particles from JEV-susceptible cells in which increased intracellular SMS formation occurred. Further, the association of SMS formations with SHA releases was strongly supported by comparisons between open and closed cultivating conditions using a single cell species (C6/36) infected with the

KE-093 strain: the use of the single cell species could rule out any possible effect derived from the difference of host cells.

Virus-induced membrane structures have generally been observed in flavivirus-infected cells. Smooth membrane vesicles similar to SMSs were shown in dengue virus-infected mammalian and mosquito cells (4, 29). In Kunjin virus-infected mammalian cells, vesicle packets (VPs) containing SMS-like vesicles, convoluted membranes localized adjacent to VPs and paracrystalline arrays, which were similar to the structures observed when microtubules were disrupted , have been reported (28, 32, 43). In hepatitis C virus-infected cells, contiguous vesicles and membranous webs are observed (10). Moreover, such membrane proliferations have been found in cells contained in the midgut epithelium, midgut muscles, and salivary grands in West Nile virus-infected mosquitoes *in vivo* (12). Thus, flavivirus infections can induce a variety of intracellular membrane structures in mammalian and mosquito cells.

The roles of these induced membrane structures are still unclear. However, the association of virus-induced membranes with viral RNA and nonstructural proteins has been reported. dsRNA, NS1 and NS3 were localized in association with the induced membrane structures of Kunjin virus-infected cells (43). In dengue virus-infected cells, the induced membrane structures were related with the localization of genome RNA

(13), dsRNA and NS1 (30) and NS3 (8). In JEV-infected cells, NS3 was found localized in association with virus-induced microtubules (7). These results suggest that the induced membrane structures are related to particle formation and the subsequent release of virion and SHA particles in flavivirus-infected cells.

In the present study, JE virions were observed in SMSs in infected C6/36 cells, although the number was much smaller than that in the ER that is considered as a major factory of viral particles. The presence of virions in SMSs suggests that this type of membrane structure is another factory related to both virion and SHA formations. Since it is unlikely that viral particles produced in SMSs are transported through a normal intracellular secretory pathway, these particles are considered different in nature from those released by an anterograde transport from the ER through the Golgi apparatus to the periphery, particularly in terms of furin-mediated prM cleavage. Consistent with this hypothesis, extremely higher prM:M ratios were shown in SHA particles released from C6/36 than Vero cells. Further, specific HA activities in SHA samples obtained from C6/36 cells were lower than those from Vero cells in most of the JEV strains. It is probable that preferred release of SHA to virion particles in C6/36 cells with increased SMS formation may be attributed to an inefficient envelopment of the nucleocapsid in an abnormal factory (SMS), resulting in the marked differences in the prM:M ratio

between SHA particles released from C6/36 and Vero cells. The potential production of viral particles in induced membrane structures and their subsequent secretion in C6/36 cells may be supported by reports that an anterograde protein transport inhibitor, brefeldin A that inhibits transport from the ER, inhibited the release of West Nile virions from Vero but not C6/36 cells (37). Further, the two sizes of subviral particles reported in tick borne encephalitis virus (TBEV)-infected mammalian cells (3) may relate to the existence of at least two factories and their own secretory pathways.

JEV prM had the highest cleavability to pr and M among 4 representative flaviviruses, as demonstrated in a study using a full-length cDNA clone of dengue type 2 virus in which the 13 sequential amino acids at the pr-M junction was replaced by the corresponding region of JEV, TBEV and yellow fever virus (20). This study also reported that virions containing a JE sequence at the pr-M junction showed the most delayed export outside infected cells (20): one reason for the export retardation would be the premature expression of fusion-competent virions that may fuse intracellular membranes. In the present study, induction of membrane structures was observed upon infection with the Nakayama strain in C6/36 cells, but much less in Vero cells. Since Vero cells are known to be resistant to JEV-induced polykaryocyte formation (17) in contrast to C6/36 cells that are highly sensitive (42), it is probable that the fusion of

intracellular membranes mediated by fusion-competent virions induced membrane structures like the SMSs in JEV-infected C6/36 cells.

The present system utilizing mosquito and mammalian cells demonstrates a unique approach to the morphogenesis of flavivirus virions. Although we analyzed only virus particles released from infected cells, analyses of intracellular virus particles should provide information about production level of virion and SHA particles and their export efficiency, which may cause differences in extracellular particles. In addition, further studies using gene engineering technologies in the present comparative system will contribute to elucidating functions of each of the viral proteins related to flavivirus virion morphogenesis.

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KE-093 strains, Dr. Eiji Kimura of Hyogo Prefectural Institute of Health, Japan for providing the JEV JaGAr-O1 strain and Dr. Akinori Miki of Kobe University School of Medicine, Japan for technical supervision on electron microscopy.

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1 Table 1. Virion:SHA ratios obtained from infected C6/36 and Vero cells using six strains

2 of JEV.

JEV strain	Virion:SHA ratio[a)]	
	C6/36	Vero
Nakayama[b)]	0.18	1.98
Beijing P1	0.51	2.66
Beijing P3	0.13	8.00
JATH-160	0.25	7.01
KE-093	6.50	14.79
JaGAr-O1	0.60	3.76

3 [a)]Calculated based on E antigen levels contained in each of the virion and SHA fractions

4 obtained from culture fluids of JEV-infected cells by sucrose density gradient

5 centrifugation.

6 [b)]Data of the Nakayama strain are in Fig. 2.

Table 2. Specific infectivities of the virion fraction obtained from C6/36 and Vero cells infected with one each of the six strains of JEV.

JEV strain	Specific infectivity (x 10^4) [a)]	
	C6/36	Vero
Nakayama[b)]	13.6	7.9
Beijing P1	7.2	8.8
Beijing P3	5.0	6.2
JaTH-160	10.7	15.3
KE-093	24.3	4.4
JaGAr-O1	16.3	2.1

[a)]Defined as infective titers per ng of E.

[b)]Data of the Nakayama strain are in Fig. 2.

1 Table 3. Specific HA activities of the virion and SHA fractions obtained with C6/36 and

2 Vero cells infected with one each of the six strains of JEV.

	Specific HA activity (x 10) [a]			
	C6/36		Vero	
JEV strain	Virion	SHA	Virion	SHA
Nakayama[b]	1.09	<0.024	5.06	0.16
Beijing P1	0.82	<0.052	2.83	0.23
Beijing P3	0.50	0.033	1.59	0.40
JaTH-160	0.09	0.029	0.07	<0.01
KE-093	1.94	<0.039	0.62	0.29
JaGAr-O1	0.40	0.001	0.90	0.05

3 [a]Defined as the HA titer per ng of E.

4 [b]Data of the Nakayama strain are in Fig. 2.

Table 4. prM:M ratios in the virion and SHA fractions obtained with C6/36 and Vero cells infected with one each of the six strains of JEV.

		Virion			SHA		
		prM	M	prM:M	prM	M	prM:M
Cell	JEV strain	level [a)]	level [a)]	ratio	level [a)]	level [a)]	ratio
C6/36	Nakayama[b)]	4	128	0.031	128	64	2.00
	Beijing P1	<1	16	<0.063	512	216	2.00
	Beijing P3	1	16	0.063	64	128	0.50
	JaTH-160	1	64	0.016	128	256	0.50
	KE-093	4	128	0.031	8	8	1.00
	JaGAr-O1	2	128	0.016	16	32	0.50
Vero	Nakayama[b)]	32	256	0.125	8	16	0.50
	Beijing P1	<1	128	<0.008	<1	16	<0.06
	Beijing P3	16	≥1024	<0.016	4	64	0.06
	JaTH-160	<8	512	<0.016	4	64	0.06
	KE-093	32	≥2048	<0.016	8	1024	0.06
	JaGAr-O1	8	128	0.063	8	32	0.25

[a)]Determined by semiquantitation on Western blots (see Materials and Methods).

[b)]Data of the Nakayama strain are in Fig. 2.

Figure legend

Fig. 1. Sedimentation profiles of viral particles released from Nakayama-infected C6/36 and Vero cells. Culture fluids harvested at 24, 48 and 72 hr p.i. were fractionated following sucrose density gradient centrifugation. Each fraction was examined for the E antigen level (closed circle with solid line), infective titer (dotted line) and HA titer (bloken line).

Fig. 2. Analysis of the virion and SHA fractions released from Nakayama-infected C6/36 and Vero cells. (a) The ratio of the E protein amount contained in the virion to SHA fractions obtained from infected C6/36 (open circle) and Vero (closed circle) cells. (b) The specific infectivity of the virion fraction obtained from C6/36 (open circle) and Vero (closed circle) cells. Since the E antigen contained in the virion fraction obtained from C6/36 cells at 24 hr p.i. was undetectable, the specific infectivity was not calculated. (c) The specific HA activity of the virion (triangle) and SHA (square) fractions obtained from C6/36 (open symbol) and Vero (closed symbol) cells. The E antigen contained in the virion fraction obtained from C6/36 cells at 24 hr p.i. was undetectable, and so the specific HA activity was not calculated. (d) The ratio of the

prM to M antigen level of the virion (triangle) and SHA (square) fractions obtained from C6/36 (open symbol) and Vero (closed symbol) cells.. The prM:M ratio was not available in all samples at 24 hr p.i. in which prM and M levels were undetectable and the SHA sample obtained from C6/36 cells at 72 hr p.i. in which both of the prM and M levels were $\geq$2048.

Fig. 3. Semiquantification of prM and M levels based on Western blotting analysis. Four examples of the results are shown, which were obtained with virion and SHA fractions from Nakayama-infected C6/36 and Vero cells at 48 hr p.i. The levels of prM and M were determined by the highest dilution showing a visible band. Thus, the prM and M levels were 4 and 128 in the virion sample from C6/36 cells (C6/36 virion), 128 and 64 in the SHA sample from C6/36 cells (C6/36 SHA), 32 and 256 in the virion sample from Vero cells (Vero virion), and 8 and 16 in the SHA sample from Vero cells (Vero SHA), respectively.

Fig. 4. Electron micrographs of Nakayama-infected C6/36 (a-d) and Vero (e-h) cells at 48 hr p.i. (a, e) Observations at a low magnification. Due to a larger size of Vero than C6/36 cells, a portion of a Vero cell was shown. (b, f) a cytoplasmic area containing

SMSs and the dilated ER. (c, g) Observations of the perinuclear region. (d, h) Observations of the peripheral region. Magnification, x 4,000; scale bar = 2 μm (a, e): magnification, x 50,000; scale bar = 100 nm (b-d, f-h). Arrows indicate SMSs, and arrow heads indicate virions in the ER (d, g) and in SMSs (b, c). Abbreviation: N, nucleus; PM, plasma membrane.

Fig. 5. Sedimentation profiles of viral particles released from infected C6/36 and Vero cells using six strains of JEV. Culture fluids harvested at 48 hr p.i. from C6/36 and Vero cells infected with the JEV Nakayama, Beijing P1, Beijing P3, JaTH-160, KE-093 and JaGAr-O1 strains were fractionated following sucrose density gradient centrifugation. Each fraction was examined for the E antigen level (closed circle with solid line), infective titer (dotted line) and HA titer (bloken line). Data of the Nakayama strain are in Fig. 2.

Fig. 6. Sedimentation profiles of viral particles released from KE-093-infected C6/36 cells under both the open and closed cultivating conditions. Culture fluids harvested at 48 hr p.i. were fractionated following sucrose density gradient centrifugation. Each fraction was examined for the E antigen level (closed circle with solid line), infective

titer (dotted line) and HA titer (bloken line).

Fig. 7. Electron micrographs of KE-093-infected C6/36 cells of the perinuclear (a) and peripheral (b) regions under the closed cultivating condition, and the perinuclear (c) and peripheral (d) regions under the open cultivating conditions at 48 hr p.i. Arrows indicate SMSs. Open and closed arrow heads indicate virions in the ER and SMSs, respectively. Magnification, x 50,000: scale bar = 100 nm. Abbreviation: N, nucleus; PM, plasma membrane.

Abbreviations

C, capsid; E, envelope; ER, endoplasmic reticulum; FBS, fetal bovine serum; HA, hemagglutination; HAU, hemaggltinating unit; JEV, Japanese encephalitis virus; M, membrane; MEM, minimal essential medium; m.o.i., multiplicity of infection; N, nucleus; NS, nonstructural; PBS, phosphate-buffered saline; PFU, plaque-forming unit; p.i., postinfection; PM, plasma membrane; prM, precursor of M; SHA, slowly-sedimenting hemagglutinin; SMB, suckling mouse brain; SMS, smooth membrane structure; TBEV, tick-borne encephalitis virus; VP, vesicle packet.

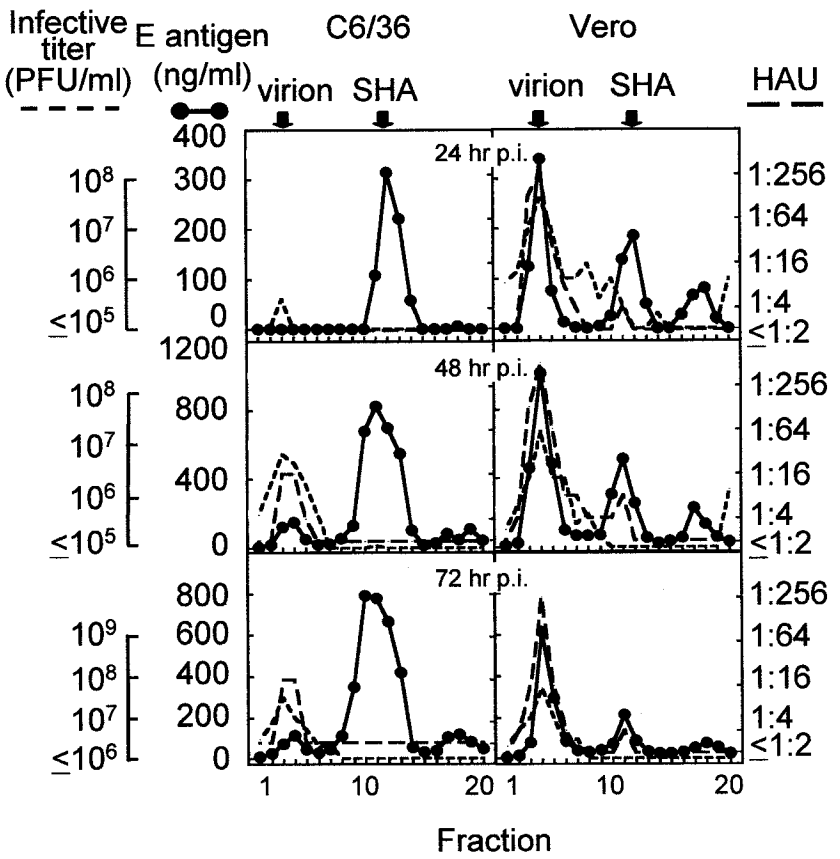
Infective titer (PFU/ml)
E antigen (ng/ml)
C6/36
Vero
virion
SHA
virion
SHA
HAU
24 hr p.i.
48 hr p.i.
72 hr p.i.
10^8
10^7
10^6
$\leq 10^5$
10^9
$\leq 10^6$
400
300
200
100
0
1200
800
600
1:256
1:64
1:16
1:4
$\leq$1:2
1
10
20
Fraction

Fig. 1

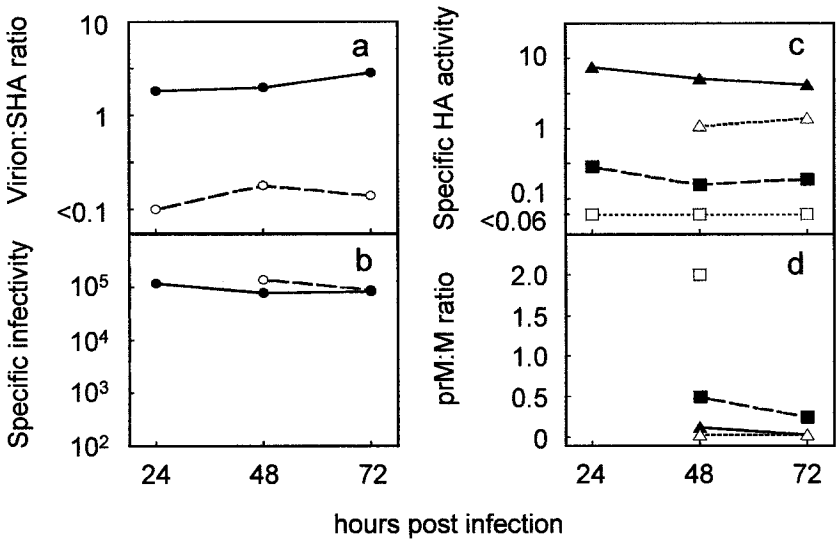
Virion:SHA ratio
10
1
<0.1
a
Specific infectivity
10^5
10^4
10^3
10^2
b
24
48
72
hours post infection
Specific HA activity
10
1
0.1
<0.06
c
prM:M ratio
2.0
1.5
1.0
0.5
0
d
24
48
72

Fig. 2

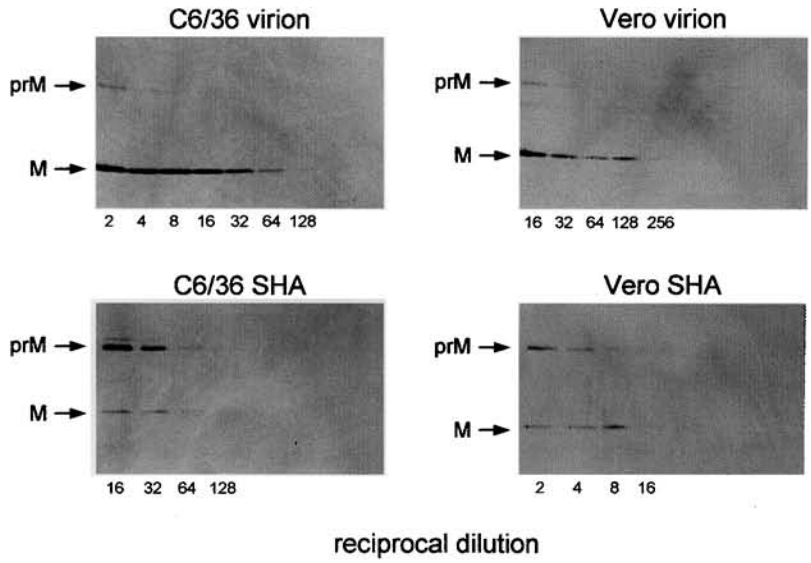
C6/36 virion
prM
M
2 4 8 16 32 64 128
Vero virion
prM
M
16 32 64 128 256
C6/36 SHA
prM
M
16 32 64 128
Vero SHA
prM
M
2 4 8 16
reciprocal dilution

Fig. 3

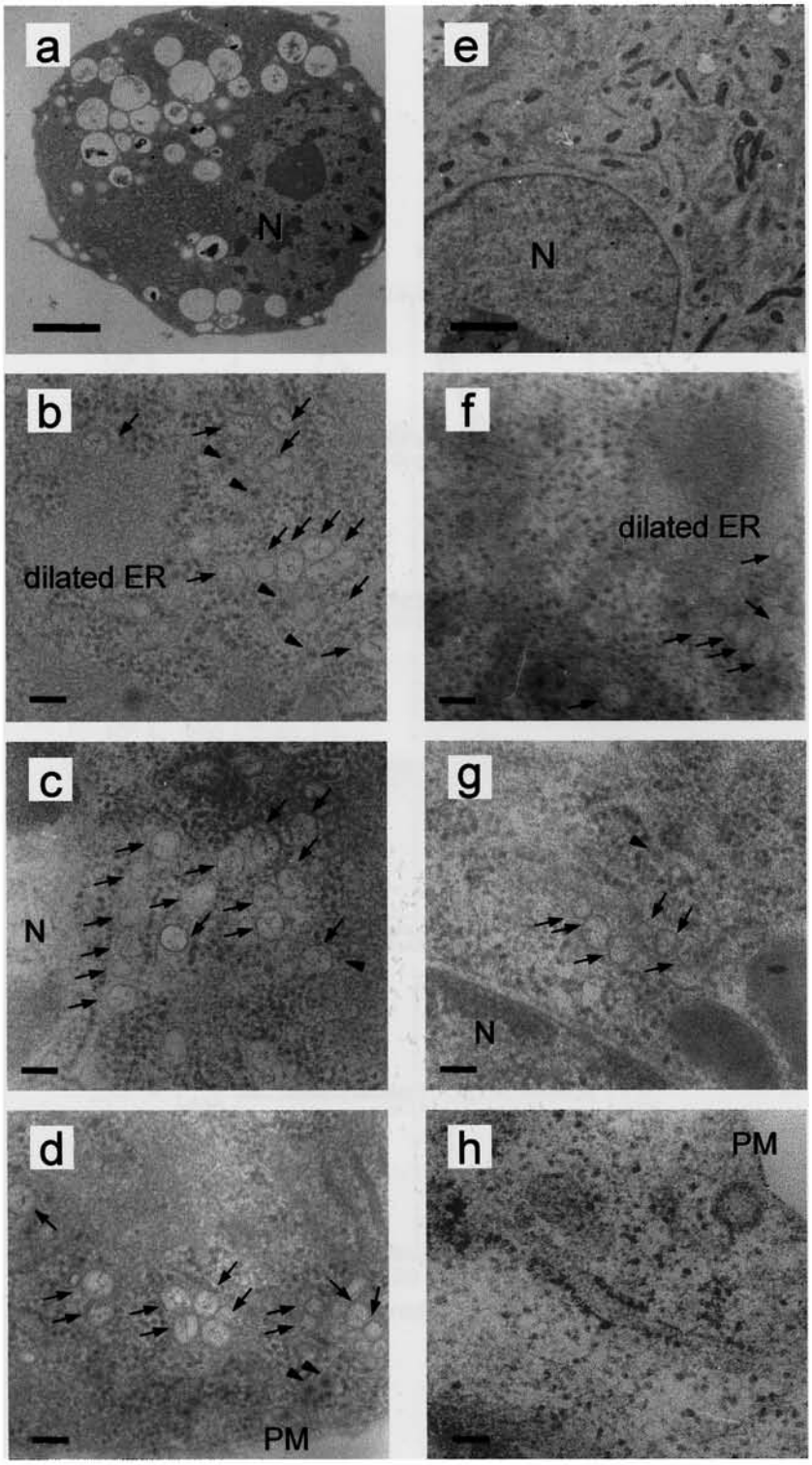

Fig. 4

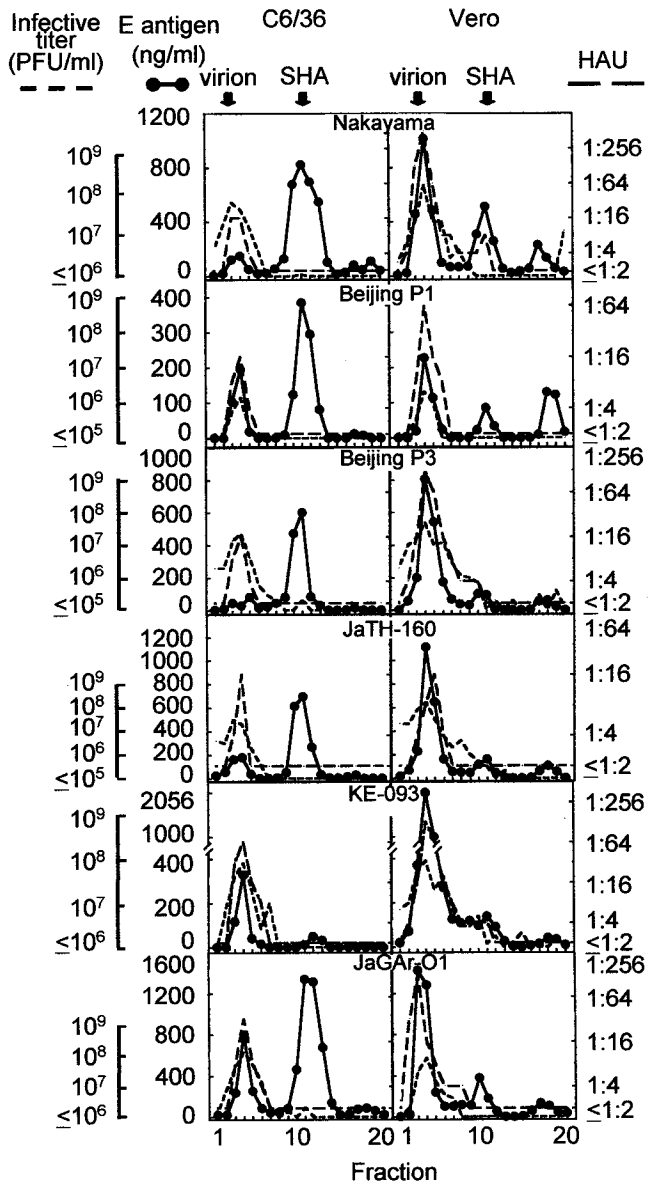
Infective titer (PFU/ml)
E antigen (ng/ml)
C6/36
Vero
virion
SHA
virion
SHA
HAU
Nakayama
Beijing P1
Beijing P3
JaTH-160
KE-093
JaGAr01
Fraction

Fig. 5

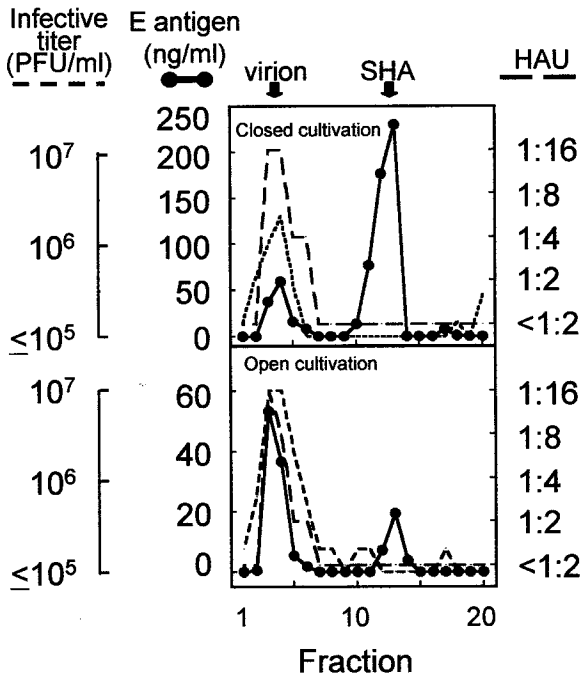
Infective titer (PFU/ml)
E antigen (ng/ml)
virion
SHA
HAU
Closed cultivation
Open cultivation
10^7
10^6
≤10^5
250
200
150
100
50
0
60
40
20
1:16
1:8
1:4
1:2
<1:2
1
10
20
Fraction

Fig. 6

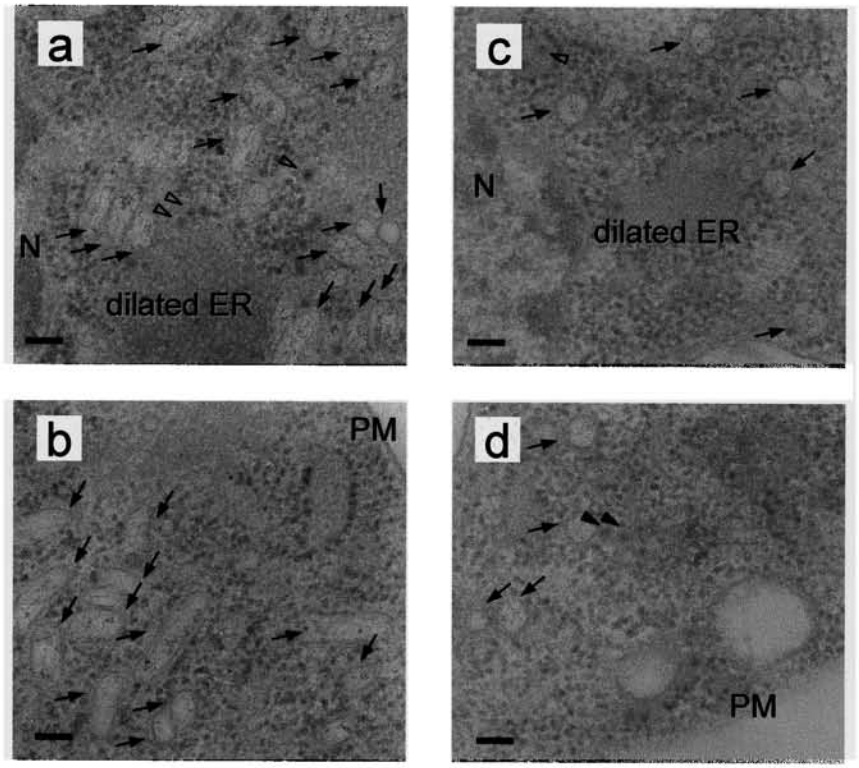

Fig. 7