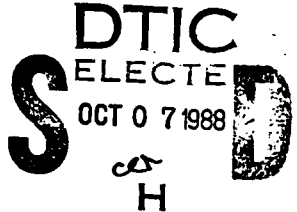


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Maturation process of Japanese encephalitis virus in cultured mosquito cells in vitro and mouse brain cells in vivo

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Summary

The maturation process of Japanese encephalitis (JE) virus in C6/36 cells in vitro and in mouse brain cells in vivo was studied by electron microscopy. In the C6/36 cell infection, 500 to 2250 virions per cell were released into the medium during the period of study; yet, no virus budding process was observed at the host cell membranes. JE virions at various maturation stages appeared within the cisternae of rough endoplasmic reticulum (RER) of infected cells at 24 hours p.i.; and, although C6/36 cells did not show a well-developed Golgi apparatus, the virions appeared to be carried to the cell surface within host-cell secretory vesicles for extracellular release as early as 24 hours p.i. The occurrence of a secretory-type intracellular transport of maturing JE virus particles was well recognizable in brain cells of infected mice, in which JE virus particles were found almost exclusively in the cisternae of RER, in the Golgi apparatus, and in various vesicles, including coated vesicles, in the vicinity of the Golgi apparatus. Our previous study of dengue-2 virus morphogenesis and our present study of JE virus morphogenesis differed substantially at various stages of maturation. Possible mechanisms which explain these differences were discussed.

Introduction

Numerous reports have appeared concerning the developmental pattern of the flaviviruses; yet, so far, no single pattern of morphogenesis has emerged. An area of inconsistency involves the site of nucleocapsid assembly. Several studies have demonstrated flavivirus nucleocapsids in the cytosol (7, 9, 11), while others have demonstrated them in the cisternae of rough endoplasmic reticulum (RER) (4, 8, 10, 12). Recently, we have shown that, during infection of C6/36 mosquito cells with dengue-2 (DEN-2) virus, nucleocapsids assemble themselves in the cytosol and subsequently mature

by budding through host plasma or cytoplasmic membranes, acquiring viral membrane envelopes in the process (7). In contrast, Leary and Blair (8) have reported that, in Vero cells infected with Japanese encephalitis (JE) virus, virions occur within the cisternae of RER, pass through the Golgi apparatus, and appear extracellularly by secretory-type exocytosis. These two reports therefore suggest the presence of at least two different maturation modes among flaviviruses.

So that the morphogenesis of JE virus and DEN-2 virus could be directly compared, the present study examined the infection of C6/36 mosquito cells with JE virus, under conditions similar to those used previously for a DEN-2 virus study (7). In addition, we studied the morphogenesis of JE virus in the brain cells of adult mice intracerebrally inoculated with this virus.

Materials and methods

Virus

JE virus, strain SA₁₁, was obtained from Dr. Yu Yong Xin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). The virus preparations used in this study was passed two times intracerebrally in suckling mice and once in *Aedes albopictus* C6/36 cells. Virus harvest fluids were clarified by centrifugation at $600 \times g$ for 10 minutes, and the virus particles were pelleted at $63,000 \times g$ for 2 hours. The pellet was resuspended to 1/100 original volume in 0.01 M Tris-HCl, pH 7.8, containing 0.15 M NaCl and 0.005 M EDTA. Three ml of the suspension were layered on a 27 ml gradient of 5–25 percent (w/v) sucrose and centrifuged for 2.5 hours at $65,000 \times g$ in a swinging bucket rotor. One ml fractions were collected and screened for hemagglutinating (HA) activity. The fractions with the highest HA activity were pooled, aliquoted, and stored frozen at -70°C until use.

C6/36 cell infection

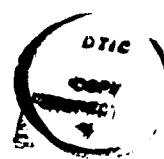
The C6/36 cell line of *Aedes albopictus* were cultured in Beem capsule caps with Eagle's minimum essential medium (Gibco, Cat. no. 410-1500, Grand Island, NY) containing 10 percent fetal bovine serum and supplemented with 100 U of penicillin and 100 μg of streptomycin per ml, according to the method of Asafo-Adjei et al. (1). The C6/36 cells were infected with purified JE virus at a virus-host cell ratio of 80. After adsorption for 1 hour at 35°C , maintenance medium containing 2 percent fetal bovine serum and antibiotics was added to the cells. The cultures were maintained in a humidified atmosphere of 5 percent CO_2 in air at 28°C for 24, 48, and 72 hours. For the virus growth curve study, C6/36 cells were grown in 25 cm^2 tissue culture flasks, and infected with virus at a virus-host cell ratio of 80. Supernatant culture fluids were sampled at 24, 48, and 72 hours post-inoculation (p.i.), and infectious virus titers determined by a standard plaque assay (2).

Mouse infection

Three young adult mice (ICR strain, 4 weeks old) were lightly anesthetized by placing them in a dry-ice chamber and injected intracerebrally with 0.03 ml (10^7 PFU) of JE virus. Three control mice were injected intracerebrally with the same volume of the medium. Since a preliminary study had indicated that a majority of infected mice die on the fifth day p.i., in this study the infected and control mice were sacrificed on the fourth



Fig. 1. JE virus particles (arrowheads) maturing in the dilated tubular and sinusoidal cisternae of RER of a C6/36 cell, 24 hours postinoculation (p. i.). The cisternae also contain multiple smooth membrane structures (SMSs) (*s*) and finely filamentous and granular material. RER membrane is poorly recognizable along rows of ribosomes; the membrane of a vacuole (*V*) is free of budding virions. *C* Dilated cisterna; *N* nucleus. Bar, 100 nm. Insets: Virus particles in the cisternae showing membrane-envelope coverage. Bar, 100 nm



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day p.i. The animals were anesthetized and perfused with 20 ml of phosphate buffered saline (PBS), followed by 10 ml of 4 percent phosphate-buffered paraformaldehyde, by injecting the perfusing fluids from the left ventricle under a mild pressure and draining them from a cut in the right atrium. The perfused mice were placed in a refrigerator at 4°C for 30 minutes. The brain was excised and cut at the coronal plane passing through the pituitary stalk. Small tissue pieces for the electron microscopic study were taken from the cerebral cortex and the basal ganglia at the cut surface and fixed in 1/2 strength Karnovsky fixative for 1 hour at room temperature or at 4°C overnight.

Electron microscopy

The C6/36 cell monolayers grown in Beem capsule caps and infected with JE virus were processed for electron microscopy as described elsewhere (1). The brain tissue pieces fixed in 1/2 strength Karnovsky fixative were washed in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1 percent cacodylate-buffered osmium tetroxide, dehydrated, and embedded in Poly/Bed 812 (Polyscience, Inc., Warrington, PA). Thin sections were cut on a LKB Ultratome, Nova, placed on uncoated grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-500 electron microscope at 50 and 75 kV.

Results

JE virus replication kinetics

In order to establish that C6/36 cells were being optimally infected, with a resultant release of JE virus over the time period studied by electron microscopy, C6/36 cell cultures were inoculated with JE virus at a virus-host cell ratio of 80, and supernatant culture fluids sampled at 24, 48, and 72 hours p.i. Plaque titers of extracellular virus were 500, 1500, and 2250 PFU/cell for the 24, 48, and 72 hour samples. These amounts of extracellular virus indicated that an active replication cycle was established, and the observation times of 24, 48, and 72 hours were optimal for maturation studies.

Electron microscopic observation of JE virus-infected C6/36 cells

Normal C6/36 cells contained abundant ribosomes in their relatively voluminous cytoplasm. The insect cells generally possessed thinner and less-clearly staining cell membranes than mammalian cells, consequently, the structural delineation of C6/36 cells tended to be less sharp in electron micrographs than that of mammalian cells. This was particularly true in the profiles of RER whose membrane was often difficult to recognize. The small Golgi apparatus was occasionally found in the perinuclear region or in the peripheral cytoplasm of C6/36 cells. The cytoplasm also contained varying numbers of vesicles and vacuoles.

In the preparations obtained 24 hours p.i., virus particles measuring 40 to 50 nm in diameter were found in a majority of infected cells within the dilated, tubular or sinusoidal cisternae of RER (Fig. 1). Compared to the DEN-2 virus infection in which multiple viral nucleocapsids occurred in the cytosol (7), the JE virus infection revealed no recognizable nucleocapsids of

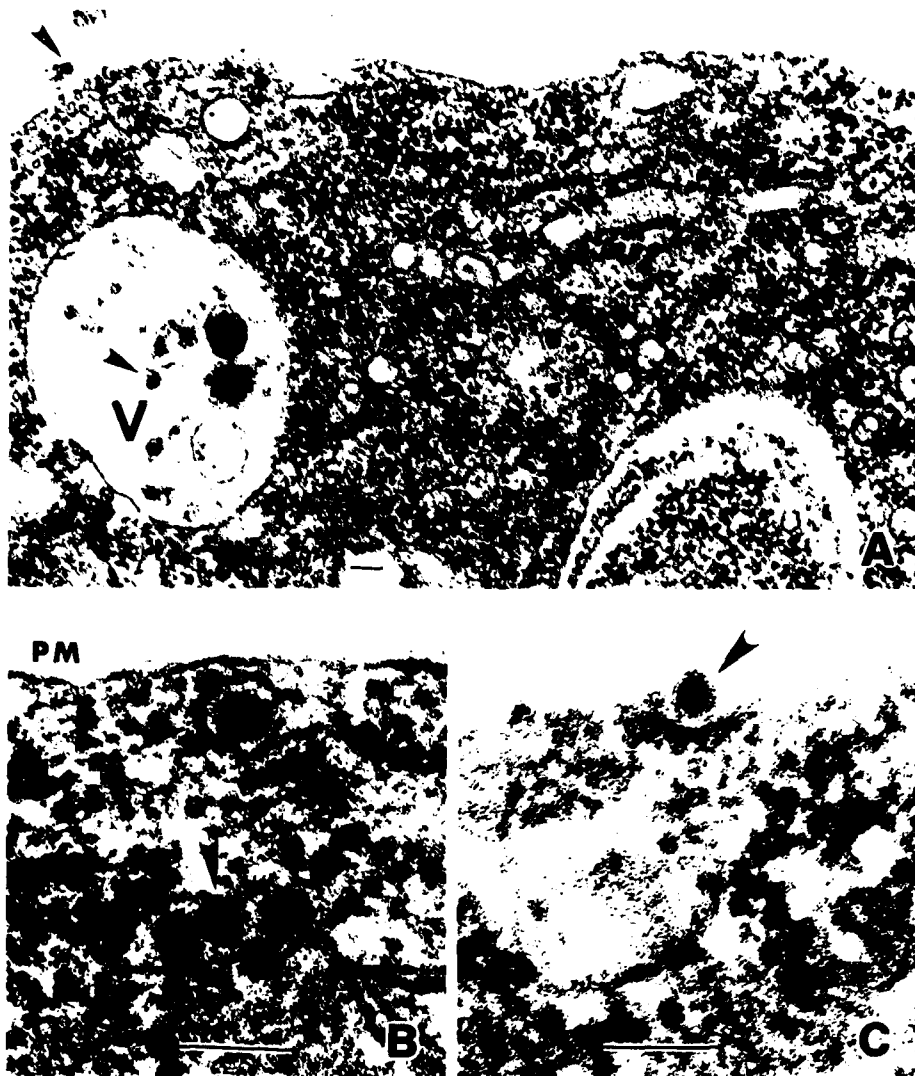


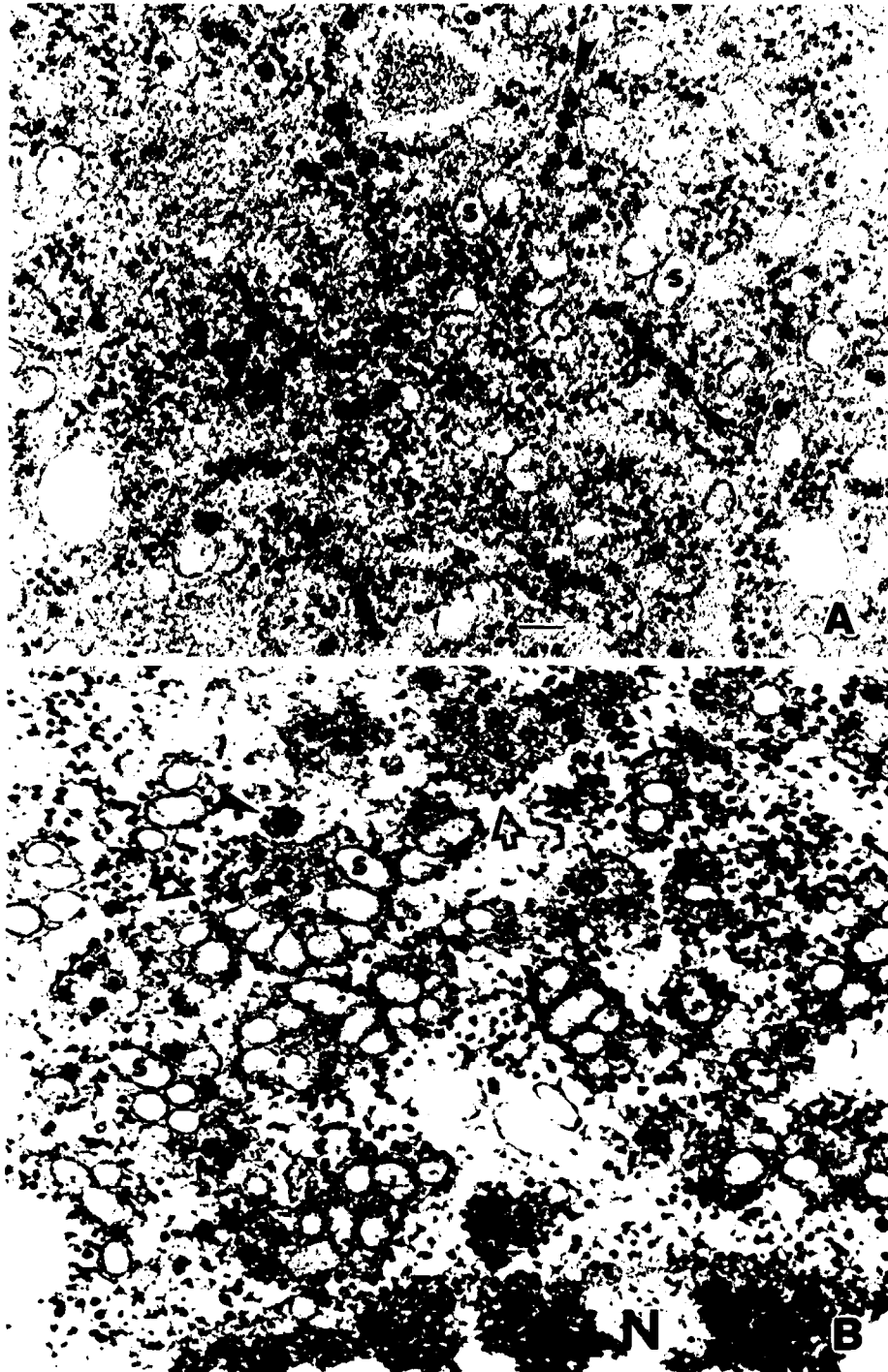
Fig. 2. JE virus particles in the peripheral cytoplasm and on the surface of C6/36 cells, 24 hours p.i. **A** One virion each (large arrowheads) are seen on the cell surface and in the lumen of a vacuole (*V*). One virion (small arrowhead) is recognizable within a vesicle in the peripheral cytoplasm. The rest of virus particles are seen within cisternae in the cytoplasm. Bar, 100 nm. **B** Virus particles (arrowheads) within vesicles beneath the plasma membrane (*PM*). Bar, 100 nm. **C** A virus particle (arrowhead) on the cell surface. Note the clean separation between the viral envelope and the plasma membrane. Bar 100 nm.

a corresponding size in the cytosol; nonetheless, the virus particles within the RER cisternae were covered with membrane envelopes (Fig. 1, insets). The RER cisternae also contained multiple vesicles that were morphologically identical to the "smooth membrane structures (SMSs)" described by

Leary and Blair (8) in JE virus-infected Vero cells (Fig. 1). The SMSs measured 100 nm in diameter on the average and showed an internal structure with a filamentous network; some showed central, electron-dense condensations. In the virus infection the RER cisternae seemed to become dilated through accumulation of mildly electron-dense proteinaceous material, in which rows and groups of maturing virus particles were embedded (Fig. 2 A). Virus particles were also seen in the peripheral cytoplasm, where they were enclosed in vesicles probably of RER origin singly or in groups (Fig. 2 A, B). The plasma and cytoplasmic vacuolar membranes were clean; no or few virus particles were found in the vacuolar lumens (Figs. 1, 2 A, B, C). Virus particles that were found occasionally on the cell surface showed their membrane envelopes cleanly separated from the plasma membrane (Fig. 2 C).

At 48 hours p.i., many infected cells were enlarged mainly as the result of prominent vacuolation of their cytoplasm. The cytoplasm showed varying degrees of disorganization of subcellular structures. Particularly, the membrane of RER became obscure extensively with accumulation of an increasing amount of the intracisternal material (Fig. 3 A). Consequently, the demarcation between RER and the rest of the cytoplasm could only be perceived by the arrangement of rows of ribosomes. Maturing virions were embedded in rows and groups in the abundant intracisternal material. In some areas of the cytoplasm, segments of RER transformed into cystic structures in which virus particles and the SMSs were contained (Fig. 3 B). A row of ribosomes usually lined the cytoplasmic side of the membrane that enclosed a cystic structure, revealing its origin. Virus particles that were individually enclosed in vesicles were also found among the cystic structures (Fig. 3 B). It appeared that from the cisternae and the cystic structures virus particles were carried within vesicles to the cell surface to be released extracellularly. The focal dilatations of RER whose cisternae were filled with the proteinaceous material was prominent in infected C6/36 cells (Fig. 4 A); however, the same kind of change in a lesser degree was observed in control C6/36 cells. On the other hand, the appearance of multiple SMSs together with virus particles within the cisternae was specific to infected cells. In some areas the intracisternal material contained parallel membranous lamellae (Fig. 4 B). This might indicate accumulation of abundant mem-

Fig. 3. JE virus particles in the cytoplasm of C6/36 cells, 48 hours p.i. Bar, 100 nm. **A** Virus particles (arrowheads) embedded in the accumulation of finely filamentous and granular material. Demarcation between RER and the rest of the cytoplasm is mostly obscure. *s* SMS. **B** Virus particles and SMSs enclosed within cystic structures (empty arrows) created by dilatation and segmentation of RER. Note the rows of ribosomes still attached to the cytoplasmic side of the membranes of the cystic structures. Virions individually enclosed in vesicles are also seen (arrowhead). *N* Nucleus



brane constituents in the intracisternal material with the eventual membrane formation. These membranous lamellae formed characteristic stacks of membranous tubules in the following periods as shown below.

At 72 hours p.i., many infected cells showed marked vacuolation. The cytoplasm contained relatively few remnants of virus-containing cisternae of RER, apparently indicating that a large portion of the dilated RER merged with the rest of the cytoplasm. Numerous SMSs persisted in the cytoplasm, but their exact location, either in the cytosol or in the cisternae, could not be determined because of extensive obliteration of cytoplasmic membrane (Fig. 5). The cytoplasm also contained stacks of membranous tubular structures, which were apparently derived from the membranous components that appeared in the intracisternal material in the preceding period: cross sections of the structure revealed conglomerations of membranous tubules measuring approximately 35 nm in diameter. In the cytoplasm, the membranous tubular structures interspersed with groups of ribosomes and the SMSs without clear demarcation (Fig. 5). Segments of fairly normal-looking RER, possibly representing regenerated ones, were seen, sometimes in close apposition to stacks of the membranous tubular structures (Fig. 5). The plasma and vacuolar membranes of infected cells remained free of virus particles.

Electron microscopic observation of JE virus-infected mouse brain cells

Gross examination of excised whole brains from the infected mice indicated some degree of swelling. Light microscopic examination of toluidine blue-stained, semi-thin sections of the brain samples were rather unremarkable; neither perivascular cuffing nor localized lesions were observed. Electron microscopic examination of the cerebral cortex and the basal ganglia revealed JE virus-infected nerve cells and neuroglial cells. The most frequently and prominently infected cells were nerve cells. RER of the Nissl body in the cytoplasm of infected nerve cells were variably disoriented, and multiple virus particles together with the SMSs were found within dilated RER cisternae (Fig. 6, 7). Ribosomes that lined the cytoplasmic side of virus-containing RER appeared to be relatively undisturbed until the

Fig. 4. Accumulation of intracisternal material in infected C6/36 cells, 48 hours p.i. Bar, 100 nm. **A** A cystic dilatation (*CD*) of a segment of the cisterna, filled with the finely filamentous and granular material. An arrow points to the junction between the cystic dilatation and the cisterna. Arrowheads point to SMSs (*s*) that appear to be forming by invagination of the RER membrane. No virus particle is seen in the picture. **M** mitochondrion. **B** Numerous SMSs and membrane lamellae (*ML*) that are seen presumably in the intracisternal material, although RER is mostly undefinable. Arrowheads point to virus particles embedded in the material

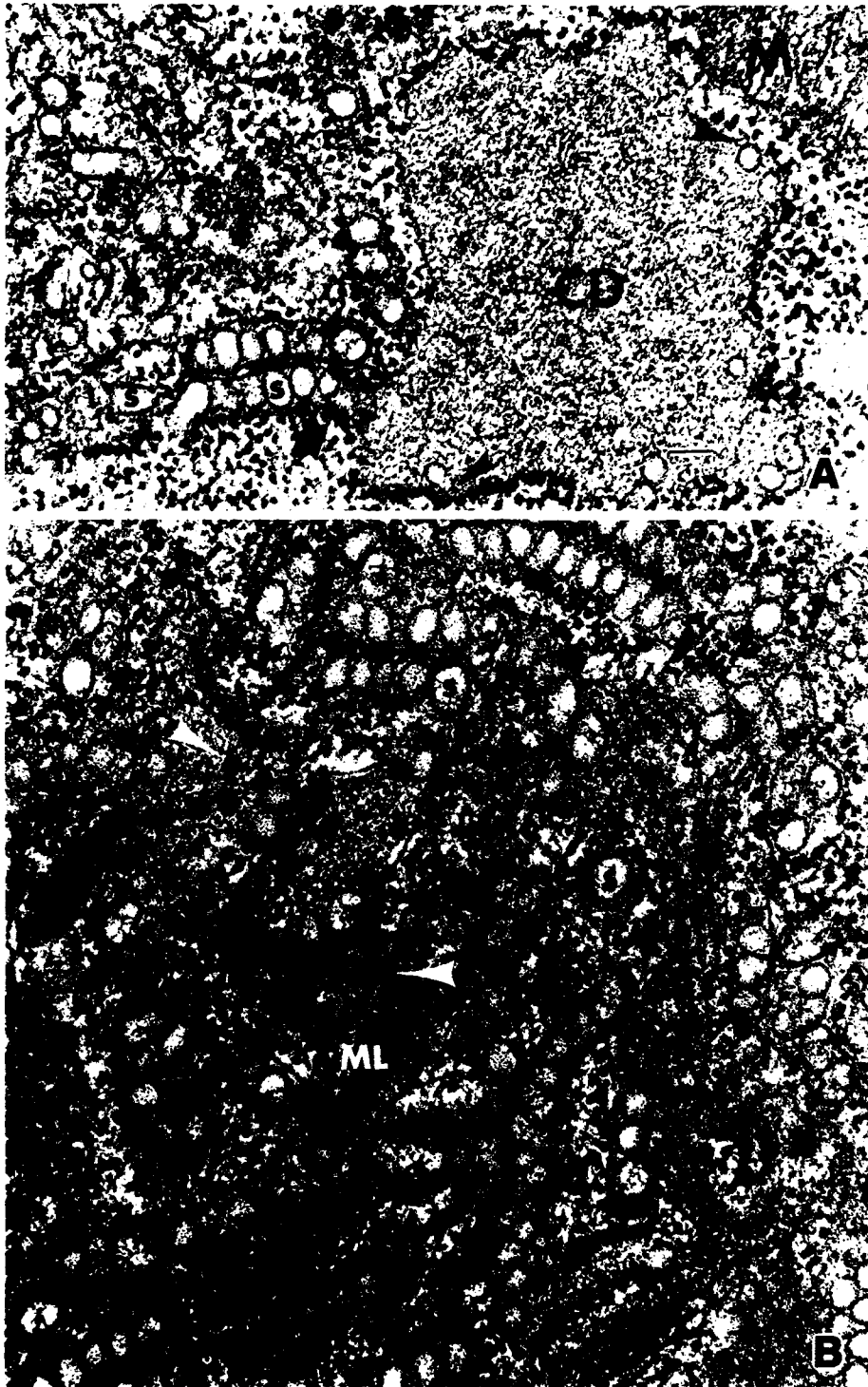




Fig. 5. Membrane proliferation in an infected C6/36 cell, 72 hours p.i. The cytoplasm contains stacks of membranous tubular structures (*MTS*) whose tubular nature is revealed in cross sections (empty arrows). Profiles of normal-looking RER segments (arrows) are seen. Only a few virus particles (arrowhead) are found in the cytoplasm. *L* Lipid; *N* nucleus. Bar, 100 nm

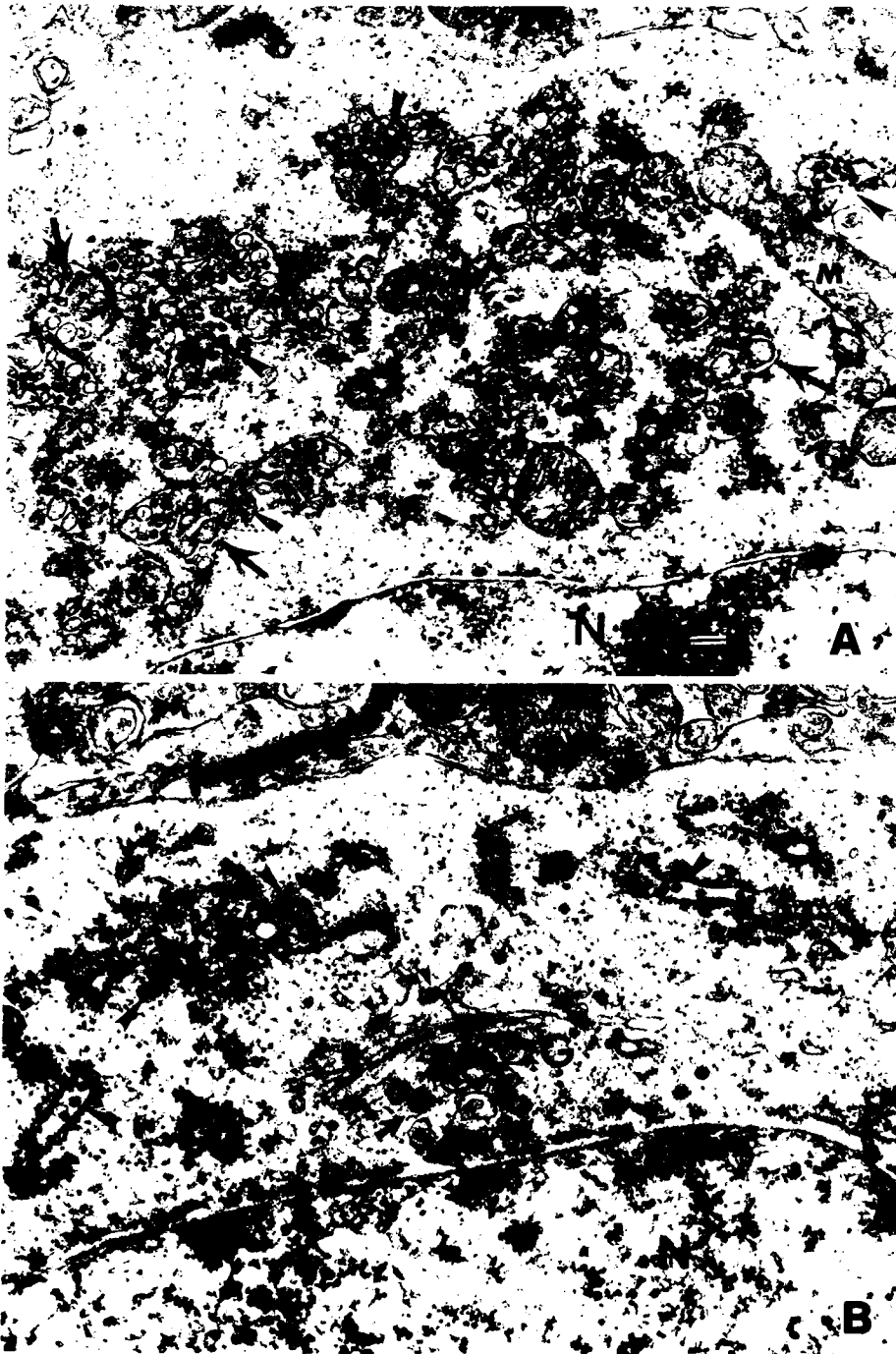


Fig. 6. Infected nerve cells of the mouse brain, 4 days p.i. Bar, 200 nm. **A** Virus particles (arrowheads) and SMSs (arrows) are seen exclusively in the dilated cisternae of RER of the Nissl body. *M* Mitochondrion; *N* nucleus. **B** Virus particles are seen in the RER cisternae (large arrowheads), in the Golgi saccules (small arrowheads), and within vesicles (arrow). *G* Golgi apparatus; *N* nucleus

structure of RER eventually broke down. Virus particles were also found within the saccules of the Golgi apparatus and within vesicles, including coated vesicles (Fig. 6 B, 7). In some infected cells the Golgi apparatus showed what appeared to be degenerative changes with partial dissolution of its membrane and vacuolation of its saccules (Fig. 7). In the vicinity of the

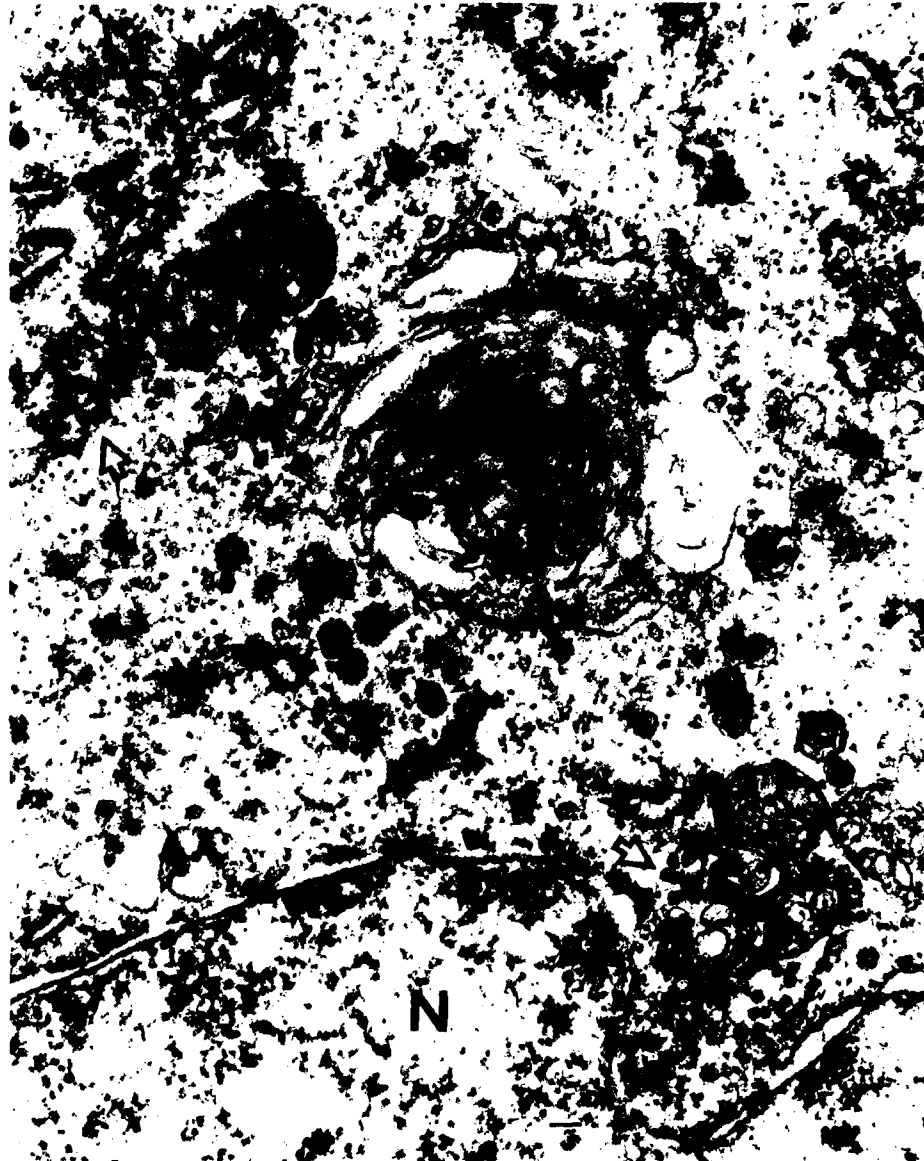


Fig. 7. A perinuclear area of an infected nerve cell showing distribution of virus particles in dilated cisternae (empty arrows), in the Golgi apparatus (*G*), and within vesicles including coated vesicles (arrowheads), 4 days p.i. *M* Mitochondrion; *N* nucleus. Bar, 100 nm

Golgi apparatus, many cytoplasmic vesicles, both coated and uncoated, were found. Particularly, virus-containing coated vesicles occurred in close topographic relationship to the Golgi apparatus. Oligodendroglial cells also contained virus particles in the cisternae of their well-developed RER, in the Golgi apparatus, and within vesicles including coated vesicles in the Golgi area. Virus particles were also found in astrocytes, exhibiting essentially the same distribution pattern as those found in infected nerve cells and oligodendroglial cells. We failed to find virus particles in microglial cells, vascular endothelial cells, and perivascular cells (data not shown).

Discussion

In this study, the morphogenesis of JE virus in C6/36 cells was investigated, so that the findings could be compared with those for DEN-2 virus morphogenesis in the same cells reported previously (7). In C6/36 cells infected with JE virus at a virus-host cell ratio roughly equivalent to that used in the DEN-2 virus study, virus particles that were covered by membrane envelopes were found in the RER cisternae as early as 24 hours p.i., without nucleocapsids appearing in the cytosolic side of RER. The present findings, therefore, differ significantly from those of DEN-2 virus morphogenesis in the same cells over the same time interval, for, in the latter, distinct nucleocapsids appeared in large numbers in the cytosol and matured at the host plasma or cytoplasmic membrane, starting at 24 hours p.i. (7). Therefore, whereas the maturation of DEN-2 virus from infected C6/36 cells involved attachment to and budding at the host cell membrane of virus particles, in this study neither nucleocapsids nor maturing virions appeared at the host cell membrane. Instead, JE virions were found exclusively within the cisternae of RER and cytoplasmic vesicles. Although, like many undifferentiated cells, C6/36 cells exhibited poorly differentiated RER and the Golgi apparatus, and their secretory activity is difficult to follow, the distribution pattern of virus particles in the cytoplasm of infected cells suggests virus transport through a host cell secretory channel in that virus particles assemble in the RER cisternae and are carried within cytoplasmic vesicles to the cell surface for release. The SMSs are probably identical to "round membranous vesicles" and "cytopathic vesicles" reported by various investigators studying replicating flaviviruses (10, 12). The membranous lamellae and membranous tubular structures also occurred in the cytoplasm, probably originating from the intracisternal material. Abundant proliferation of cytoplasmic membranes has been described by Murphy et al. (10) in brain nerve cells of suckling mice infected with St. Louis encephalitis virus, with the appearance of the round membranous vesicles and convoluted membranous masses within the RER cisternae and in the cytoplasm respectively. Leary and Blair (8) thought

that the SMSs were precursors of virions; however, we could not establish a direct developmental relationship between the SMSs and maturing virions.

The intracellular transport of JE virus particles through the host secretory channel was more clearly demonstrated in infected nerve cells of the mouse brain. In these cells, which possessed clearly definable RER and a well-developed Golgi apparatus, the cisternae of RER, the Golgi apparatus, and various vesicles including coated vesicles in the Golgi area all contained virus particles. The distribution pattern of JE virus in infected nerve cells, therefore, clearly indicated that virus particles were assembled in the cisternae of RER, transported to the Golgi apparatus, and carried within secretory vesicles to the cell surface for extracellular release. The involvement of clathrin and coated vesicles at the trans face of the Golgi apparatus in the secretory process of mammalian cells has been well documented (3, 5, 6, 13). JE virus apparently utilized the host secretory channel for its maturation within and for its exit from infected nerve cells. Filshie and Rehacek (4) reported distribution of Murray Valley encephalitis and JE viruses exclusively within the cisternae of endoplasmic reticulum of infected cultured mosquito cells. The characteristic distribution pattern of flaviviruses in RER, the Golgi apparatus, and cytoplasmic vesicles of the host cell was reported by Murphy et al. (10) in mouse brain nerve cells infected with St. Louis encephalitis virus, and Leary and Blair (8) in Vero cells infected with JE virus. Leary and Blair (8) postulated that JE virus particles in their infectious system were released from infected cells by a secretory-type exocytosis. The present observations of JE virus-infected C6/36 cells and mouse brain nerve cells, therefore, corroborate the findings of the above investigators.

On the basis of our previous study on DEN-2 virus (7) and of our present study on JE virus, it is interesting to propose two types of maturation for flaviviruses as a working hypothesis for future investigations, as illustrated in Fig. 8 with the well-developed cytoplasmic secretory system of mammalian cells in mind. In the model of DEN-2 virus maturation, termed cis-type maturation, the viral structural proteins that are synthesized on host ribosomes are presumed to be released into the cytosol; the assembled nucleocapsids in the cytosol then move to the host cell membrane for budding. On the other hand, in the model of JE virus maturation, termed trans-type maturation, the viral structural proteins that are synthesized on host ribosomes are presumed to appear within the cisternae of RER for viral assembly, and the assembled virions then pass through the host secretory channel including the Golgi apparatus, and are eventually carried within secretory vesicles to the cell surface for secretory-type exocytosis. The proposed models raise interesting questions that need to be answered. For example, the mechanism by which the structural proteins of a given virus are placed in a particular cytoplasmic compartment needs to be clarified; in

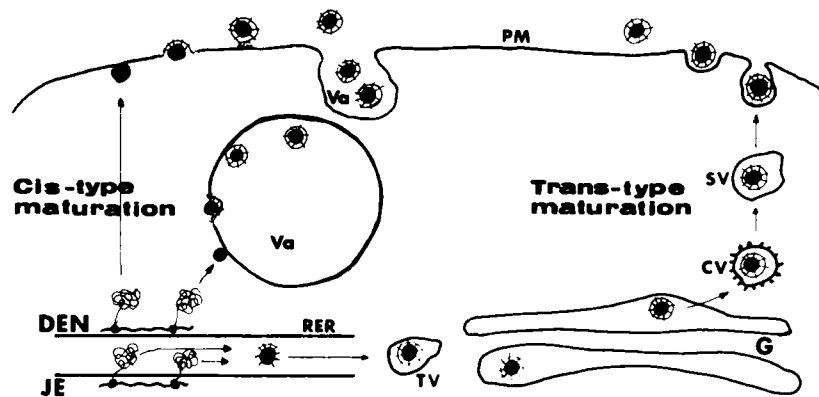


Fig. 8. A schematic illustration of the cis-type and trans-type maturation exhibited by DEN-2 virus and JE virus, respectively. *CV* Coated vesicle; *DEN* DEN-2 virus genome on host ribosomes, synthesizing the viral structural proteins and releasing them into the cytosol; *G* Golgi apparatus; *JE* JE virus genome on host ribosomes, synthesizing the viral structural proteins and releasing them into the cisternae; *PM* plasma membrane; *RER* rough endoplasmic reticulum; *SV* secretory vesicle; *TV* transfer vesicle; *Va* cytoplasmic vacuole

this respect, presence or absence of a signal sequence in connection with the C protein-coding gene may provide cotranslational vectorial movement for newly synthesized viral structural proteins. Secondly, the effects of passage of JE virus through the Golgi apparatus need to be clarified. To our knowledge, this is the first study in which virus is shown within coated vesicles at the Golgi apparatus; it may suggest that, during the transport through the Golgi apparatus, virions acquire ligands that bind specifically to host membrane receptors. Thirdly, JE virions involved in trans-type maturation seem to be released rapidly and cleanly from infected cells, with the host cell membrane offering no barrier for their release. In contrast, budding DEN-2 virions accumulated in large numbers on the surface of host cells, suggesting that the virions had a long transit time through the host cell membrane in the budding process (7).

Most cases of flavivirus morphogenesis so far reported seem to fall into either cis-type or trans-type maturation. However, one type of maturation or the other does not seem to be specifically related to a given virus taxon. For example, by inoculating mice intracerebrally with a mouse-adapted DEN-2 virus (the New Guinea "C" strain, 23rd suckling mouse brain passage), Sriurairatna et al. (12) demonstrated virus particles exclusively in the endoplasmic reticulum cisternae and smooth membrane vesicles of neurons, suggesting a trans-type maturation for the DEN-2 virus infection. Likewise, by inoculating porcine kidney stable (PS) cells with JE virus (the Mukai strain, passage history unknown but passed several times through PS cells

by the investigator before use). Ota (11) demonstrated budding virions at cytoplasmic vacuolar membranes of infected cells, suggesting a cis-type maturation for the JE virus infection. It is possible, therefore, that a virus exhibits one or the other type of maturation based on passage history and adaptation to a host cell system. Further studies are needed to clarify this point.

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