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An Electron and Immunoelectron Microscopic Study of Dengue-2 Virus Infection of Cultured Mosquito Cells: Maturation Events

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With 10 Figures

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Summary

The maturation process of dengue-2 virus in C6/36 mosquito cells was studied by electron microscopy at 12, 16, 24, 48, and 78 hours postinoculation (p.i.) and by immunoelectron microscopy at 48 and 78 hours p.i. Maturing virions appeared within cytoplasmic vacuoles and on the surface of infected cells from 24 hours p.i. onward in close topographical relationship to the dense particles that occurred concurrently in the cytoplasm. The dense particles measured 25 to 35 nm in diameter; the mature virions measured 50 to 55 nm in diameter, with a dense core measuring 30 to 35 nm in diameter covered by a 10 nm-thick membrane envelope. The morphological observations indicated that the dense particles were dengue nucleocapsids assembled in the cytoplasm and that they apparently budded into the vacuolar lumens and the extracellular space at the vacuolar and plasma membranes, acquiring membrane envelopes and becoming mature virions in the process. The virions that budded into the vacuolar lumens were released extracellularly by exocytosis. In the samples tested with dengue-2 polyclonal antibodies, intense immunostaining occurred at the sites of virus budding on the cell surface; host cell membrane and cytoplasm adjacent to the budding virions stained less intensely. In the samples tested with a dengue-2 monoclonal antibody specific for the envelope glycoprotein, budding virions stained rather exclusively, with no staining occurring in adjacent host membrane or cytoplasm.

Introduction

Morphogenesis of dengue (DEN) virus in various mammalian host cells has been studied ultrastructurally by various investigators (4, 6, 11, 12, 14, 15, 21, 22); yet, the mode of assembly of DEN nucleocapsids and the process of maturation and release of infectious virions remains obscure (16, 25). Available evidence indicates that a) the synthesis of viral macromolecules occurs on host polysomes, particularly on those associated with the rough endoplasmic reticulum, and the assembly of nucleocapsids probably takes place at or near the synthetic sites (4, 23); b) the nucleocapsids acquire their membrane envelopes at the time when or after they pass through the membrane of the endoplasmic reticulum in entering cisternae, and virions accumulate within the cisternae that eventually become virion-containing vacuoles (4, 15); and c) although confirmation is still lacking, it is speculated that the virion-containing vacuoles move to the cell surface and release their contents extracellularly by exocytosis (4, 5). The release of virions by budding at the cell surface or at internal membrane sites, which has been well documented with alphaviruses, has not been convincingly demonstrated with flaviviruses (16, 17, 24, 25).

One of the obstacles in carrying out morphogenetic studies of DEN virus in mammalian cell cultures is the inability to infect a sufficient number of host cells. This obstacle can be overcome by using a cloned mosquito cell line, C 6/36, established by IGARASHI (10) from Singh's cultured mosquito cells (19) originally derived from larvae of the *Aedes albopictus* mosquito. By studying dengue type-2 (DEN-2) infected C 6/36 cells by electron and immunoelectron microscopy, we have observed apparent budding of maturing DEN virions into the lumens of cytoplasmic vacuoles at the vacuolar membranes and into the extracellular space at the plasma membrane. This paper, therefore, describes the maturation process of DEN-2 virus in C 6/36 cells with morphological confirmation that DEN virions are released from infected mosquito cells by exocytosis of virion-containing vacuoles and by budding of individual virions at the cell surface.

Materials and Methods

Virus

DEN-2 virus, strain PR-159, was isolated from the serum of a dengue fever patient in Puerto Rico (7). The virus preparation used in this study was sequentially passed six times in primary green monkey kidney cells, four times in DBS-FRhL-2 cells and once in *A. albopictus* (clone C6/36) cells.

Host Cells

The C6/36 cell line of *A. albopictus* was chosen for this study because of its greater sensitivity to dengue virus infection compared to mammalian cells (10). The C6/36 cells were grown in 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 at 28° C.

Virus Infection

The C6/36 cells were infected at a multiplicity of infection (MOI) of 50 to 60. After adsorption for 1 hour at 35° C, maintenance medium containing 2 percent fetal bovine serum and antibiotics was added. The cultures were incubated at 28° C until cells were harvested for electron microscopy or supernatant culture fluid samples were taken for titration of extracellular virus. Virus plaque assays followed the procedure of ECKELS *et al.* (7).

Electron Microscopy

The culture medium was removed at selected times (12, 16, 24, 48, and 78 hours) after inoculation, and the cell monolayers rinsed once with PBS (pH 7.4) and then fixed *in situ* with 1/2 strength Karnovsky fixative for 1 hour at room temperature or at 4° C overnight. The cells were scraped off with a rubber policeman, washed with 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.

Immunoelectron Microscopy

The specimens obtained 48 and 78 hours p.i., which usually contained cells of varying degrees of infection, were used. The cell monolayers were rinsed once with PBS and fixed with 2 percent paraformaldehyde for 5 minutes and rinsed with acetone for 5 minutes at 4° C according to the procedure of CARDIFF *et al.* (4). The monolayers were rehydrated with PBS and treated at room temperature for 10 minutes with either anti-DEN-2 mouse hyperimmune ascites fluid (1 : 100) or DEN-2 monoclonal antibody 4G2 directed against a flavivirus group-specific epitope on the envelope glycoprotein (1 : 100). The control infected cell monolayers were treated with normal mouse serum (1 : 100). The monolayers were then reacted with affinity-purified biotinylated antimouse IgG and the ABC reagent according to the procedure provided by the manufacturer (Vector Laboratory, Inc., 1429 Rollins Road, Burlingame, CA). Color was developed with DAB for 3 to 5 minutes. After the immunostaining, the monolayers were fixed with 1/2 strength Karnovsky fixative for 1 hour at room temperature. The cells were scraped off with a rubber policeman and osmicated. Some specimens were stained in block with 0.5 percent barbiturate-buffered uranyl acetate. The cells were dehydrated and embedded in Poly/Bed 812 for electron microscopic observation.

Results

DEN-2 Virus Growth Curves

Growth curves of DEN-2 virus in C6/36 cell cultures are shown in Fig. 1, in which the plaque titers of extracellular virus from unwashed cell cultures as well as cell cultures that were thoroughly washed after absorption of the virus inoculum are compared. A 12 to 16 hours latent period could be distinguished in the washed cultures, whereas residual virus inoculum obscured this phase of virus growth in the unwashed cultures. By 24 hours post-inoculation (p.i.), both sets of inoculated cultures had similar titers of extra-

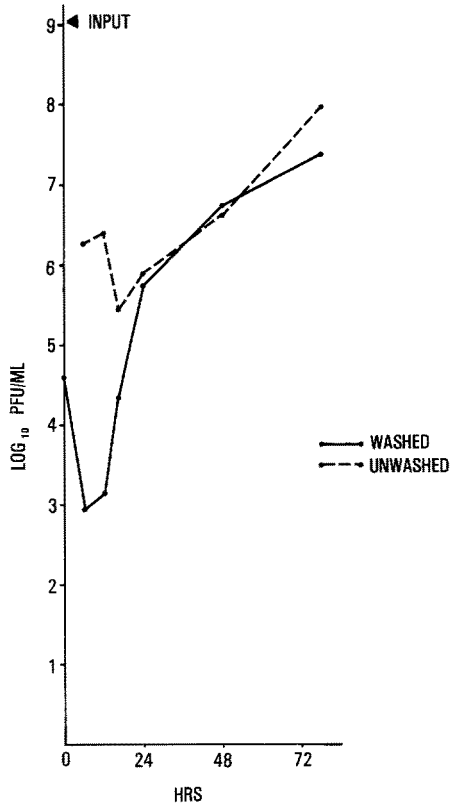

Fig. 1. Growth curves of extracellular DEN-2 virus from C6/36 cell cultures. The cells were inoculated at a 60 MOI and adsorbed for 1 hour at 35° C. After adsorption, infected cultures were either washed 6 times or unwashed, fed with maintenance medium, and incubated at 28° C. The zero time for the washed culture refers to the virus titer of the sixth wash sample

cellular virus which increased logarithmically to the final harvest. The kinetics of virus replication and electron microscopic observation indicated that a majority of cells in the DEN-inoculated cultures were infected during the experimental period.

Electron Microscopic Observation of DEN-2 Virus Infection of C6/36 Cells

No virions were found either extracellularly or intracellularly in preparations obtained 12 and 16 hours p.i. Maturing virions appeared for the first

Fig. 2. Infected cells, 24 hours p.i., showing dense particles (arrows) in the cytoplasm and maturing virions within the lumens of cytoplasmic vacuoles *(V)* and on the cell surface. *ES* extracellular space; *M* mitochondrion; *N* nucleus. Bar, 100 nm

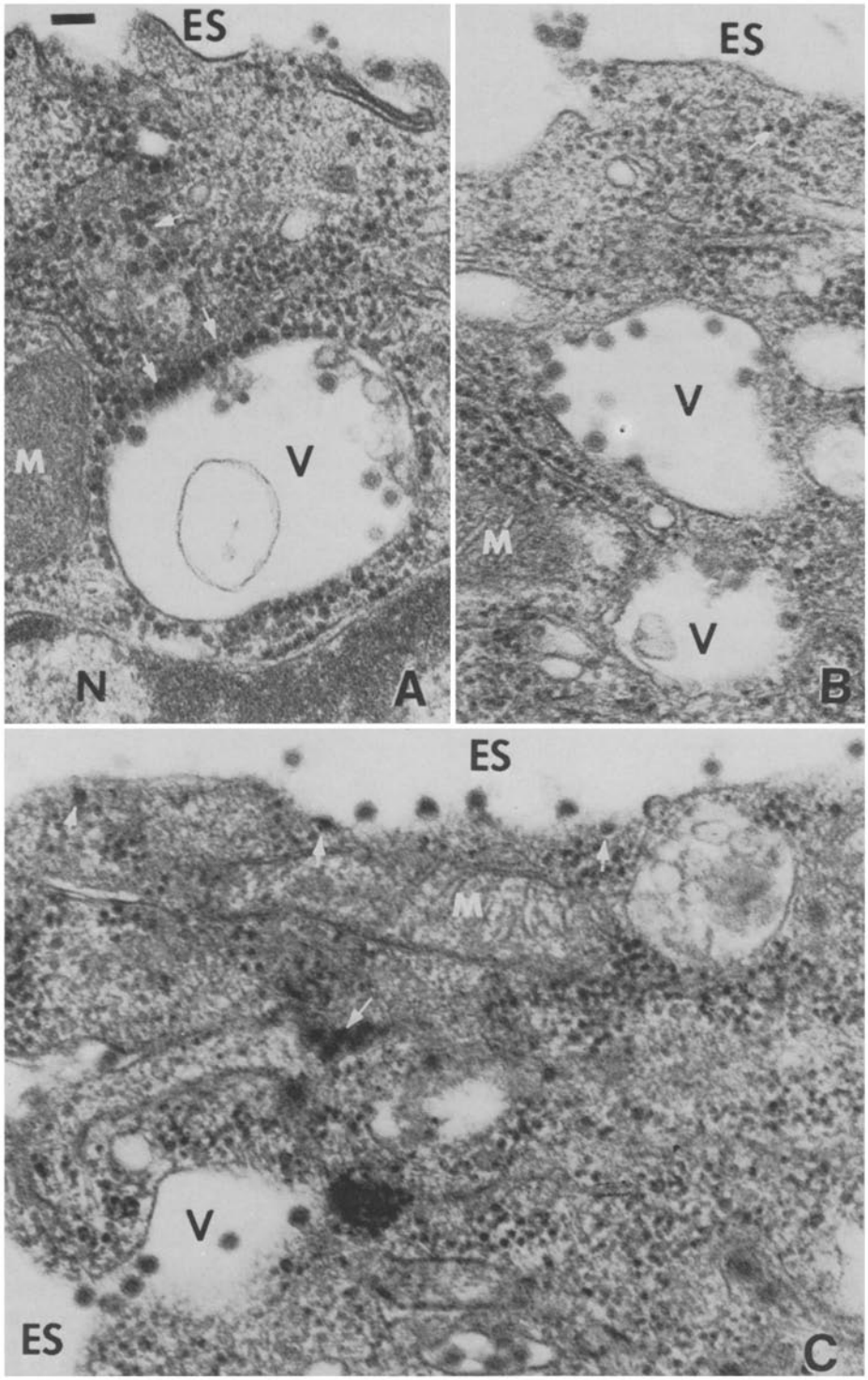

Fig. 2

time in infected cells at 24 hours p.i. They were found within cytoplasmic vacuoles and on the cell surface in close topographical relationship to characteristic dense particles that occurred concurrently in the cytoplasm (Figs. 2 A, B, C). The dense particles in the cytoplasm varied considerably in size, measuring 25 to 35 nm in diameter, but they were distinctly larger than host cell ribosomes that measured 17 nm in diameter, and showed a round, smooth contour in contrast to ribosomes that showed an irregular contour. Frequently these particles were oriented in a row on the cytoplasmic side of vacuolar membranes (Fig. 2 A). The virions within vacuoles and on the cell surface measured 50 to 55 nm in diameter, and exhibited a dense core, 30 to 35 nm in diameter, and a delicate, 10 nm-thick membrane envelope; radiating spikes were recognizable on the membrane envelope (Figs. 2 A, B, C). The dense particles and the mature virions were often situated on the cytoplasmic and luminal sides of vacuolar membranes and on the cytoplasmic and outer sides of the plasma membrane respectively. Virus particles were seen, budding into the vacuolar lumens and the extracellular space at the vacuolar and plasma membranes. On close observation, however, host cell membrane at virus budding sites was usually obliterated, and connection between host cell membrane and the viral membrane envelope at the budding sites could not be definitively confirmed. Nonetheless, morphological observations seemed to confirm that the dense particles budded at the host cell membranes, appearing in vacuolar lumens and on the cell surface as virions, acquiring viral membrane envelopes in the process. Virion-containing vacuoles were seen, opening to the cell surface and discharging virions externally (Fig. 2 C). Infected cells at this stage demonstrated varying degrees of plasma membrane change, ranging from "dotted-line" type membrane defects to membrane-absent areas.

At 48 hours p.i., the dense particles were seen prominently in the cytoplasm of infected cells, perhaps partly as the result of a proportional decrease in ribosomes (Fig. 3 A). The dense particles showed a tendency to align on the cytoplasmic side of vacuolar membranes. Maturing virions protruded from the vacuolar wall into the vacuolar lumen, trailing fuzzy membranous material from the wall (Fig. 3 A). Multiple maturing virions also appeared on the cell surface (Fig. 3 B). On the cell surface, virions were seen either positioned directly on the plasma membrane (Fig. 4 A), adhered to the plasma membrane-absent cell surface (Fig. 4 B), or scattered over the membrane-absent cell surface (Fig. 4 C). Some virions at the cell surface carried small particles that seemed to be attached to viral membrane envelopes (Figs. 4 A, B, C). These satellite particles were morphologically identical to host cell ribosomes, and some arranged themselves in a "beads on a string" formation, not unlike polysomes found intracellularly (Fig. 4 C). In addition to the satellite particles that were intimately associated with maturing virions, particles that were morphologically identical to the dense

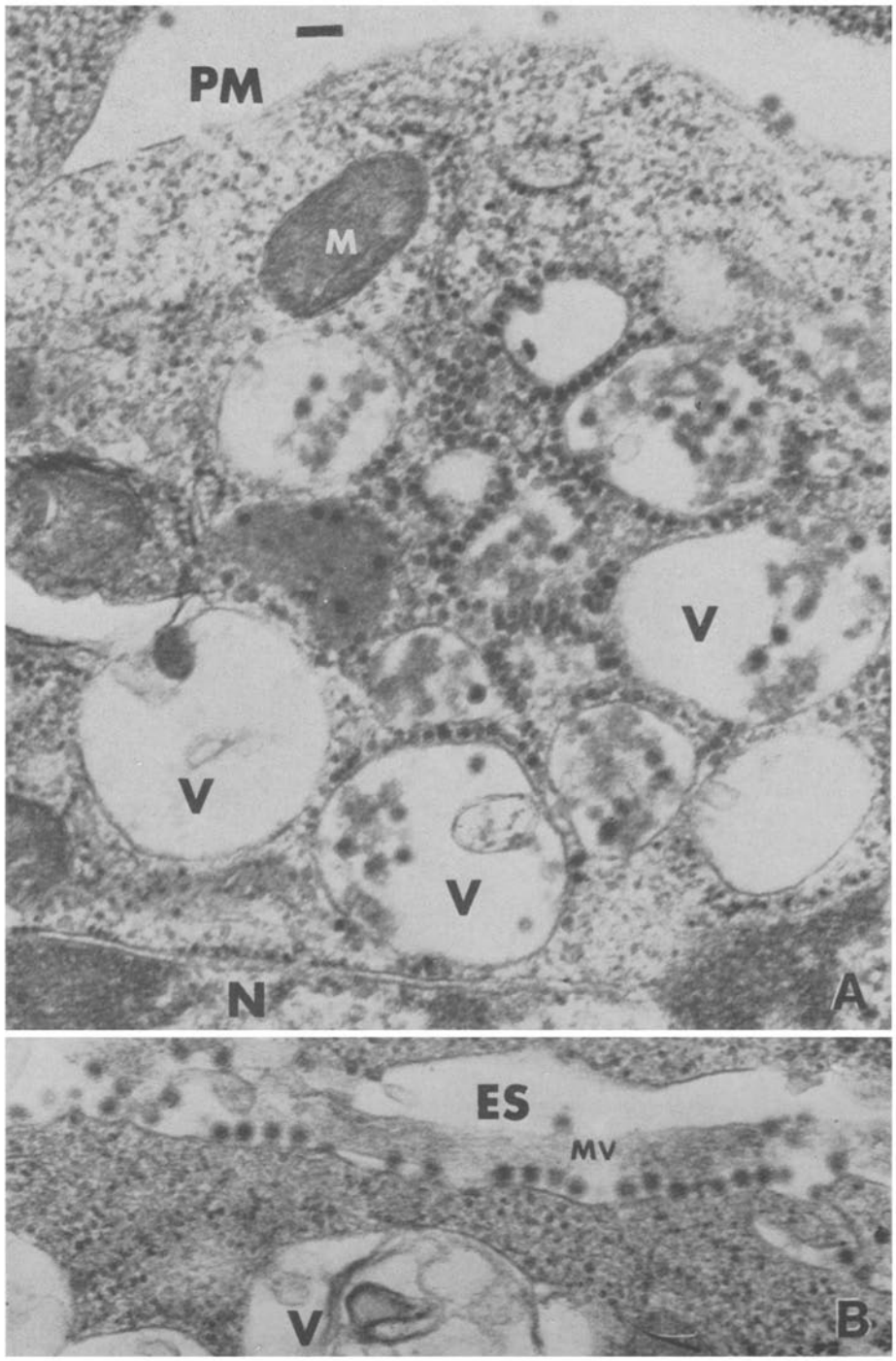

Fig. 3. Infected cells, 48 hours p.i., showing dense particles in the cytoplasm and maturing virions within vacuoles *(V)* and on the cell surface. *ES* extracellular space; *M* mitochondrion; *MV* microvillus; *N* nucleus; *PM* plasma membrane. Bar, 100 nm

particles also appeared among virions on the cell surface (Figs. 4 A, C). These extracellular dense particles did not show close association with maturing virions, and were probably incomplete virions that had "leaked" out to the extracellular space from the damaged surface.

Despite the appearance of multiple maturing virions at the cell surface, the connection between the membrane envelopes of budding virions and the host plasma membrane was rather difficult to confirm. First of all, the membrane envelope of a budding virion showed a considerable difference in morphological appearance from the contiguous host plasma membrane (Figs. 4 A, B). Moreover, the host plasma membrane at virion budding sites was frequently obliterated. Maturing virions were also found on the membrane-absent cell surface, where the tilting technique failed to identify plasma membrane in relation to viral membrane envelopes (Figs. 4 B, C). "Dotted-line" type membrane defects and membrane absent areas frequently occurred at the cell surface where virion budding was taking place; however, whether these membrane changes revealed actual membrane loss or resulted from a particular angle of sectioning could not be definitively determined in this study even by the use of the tilting technique.

At 78 hours p.i., the cytoplasm of infected cells was variably disorganized. Many cytoplasmic vacuoles contained mildly electron-dense, fuzzy material that seemed to extend into the vacuolar lumen from ribosomes situated in the vacuolar wall (Fig. 5 A). Membrane lamellae were frequently seen within the fuzzy material, indicating a membranous nature of the material. Cytoplasmic vacuoles showed varying extents of obliteration of their limiting membranes, and the dense particles and virions existed side by side without clear demarcation by vacuolar membranes (Figs. 5 A, B). In addition to individual virions, filamentous forms appeared, probably through linear, helical arrangement of DEN virus genomes and their co-envelopment by common membranes (Figs. 5 A, B). Many infected cells exhibited increasingly prominent degenerative changes, and had membrane-absent areas on their surface; in these areas virions were emerging into the extracellular space, attaching trails of cytoplasmic material from the exposed surface (Figs. 6 A, B). Some virions on the cell surface carried one or more satellite particles as were seen in the preceding period. The surface of infected cells often had irregular cytoplasmic projections, probably as the result of the opening of virion-containing vacuoles to the surface, and these cytoplasmic projections attached virions to their surfaces (Fig. 6 B). Occasionally, multiple dense particles were seen in the extracellular space, emerging from membrane-absent areas of the cell surface (Fig. 6 C). Some infected cells at this stage were covered by budding virions (Fig. 7). Some showed break-down or peeling-off of the peripheral cytoplasm as laminae, with opening of dilated cisternae into the extracellular space and releasing of the cisternal contents, including virions, extracellularly.

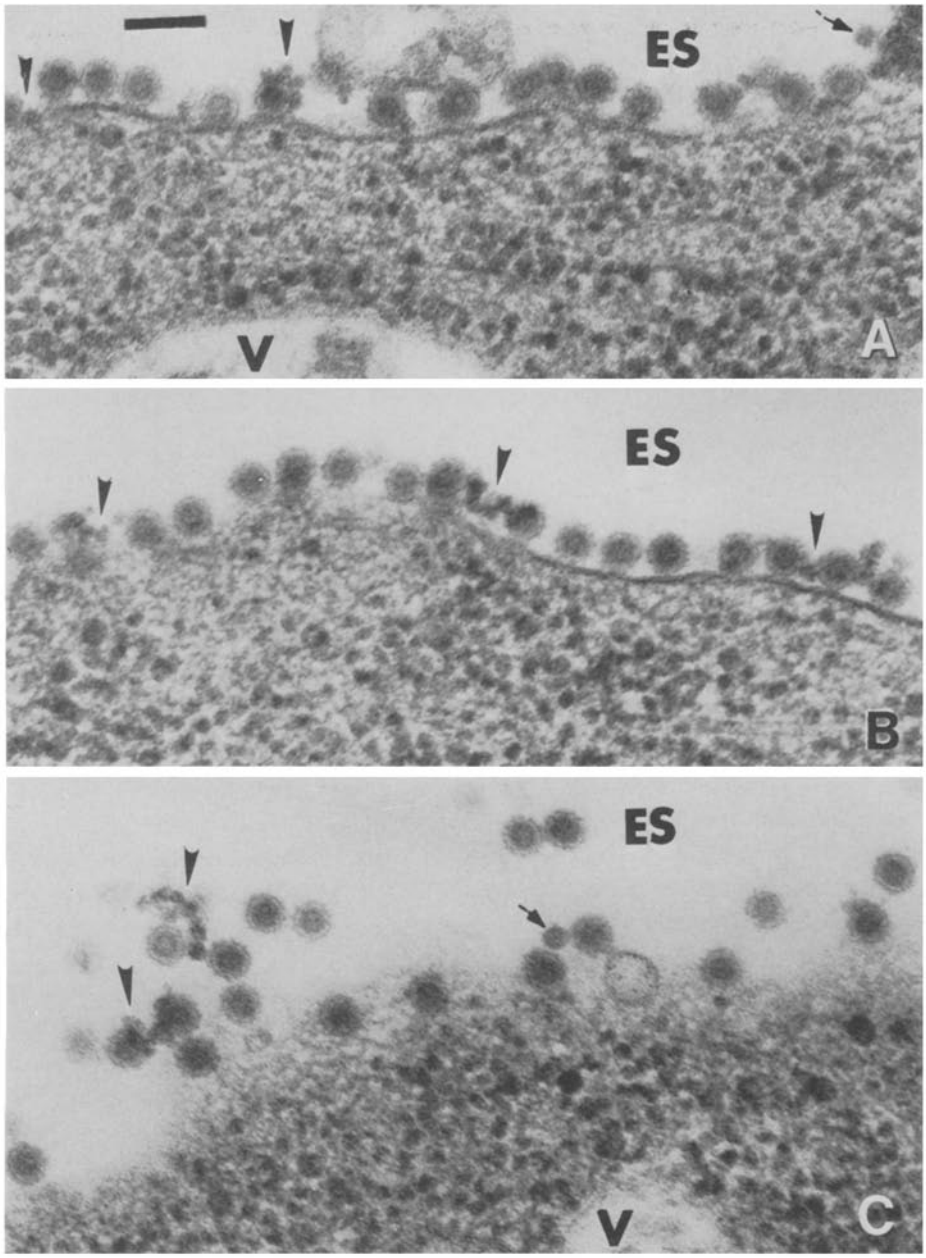

Fig. 4. Maturing virions on the surface of infected cells, 48 hours p.i. *ES* extracellular space; *V* vacuole. Bar, 100 nm. *A* Virions sitting on the plasma membrane. Arrowheads point to the satellite particles; an arrow points to an extracellular dense particle. *B* Virions sitting on the plasma membrane and on the membrane-absent cell surface. Arrowheads point to the satellite particles. *C* Virions scattered over the membrane-absent cell surface. Arrowheads point to the satellite particles; an arrow points to an extracellular dense particle

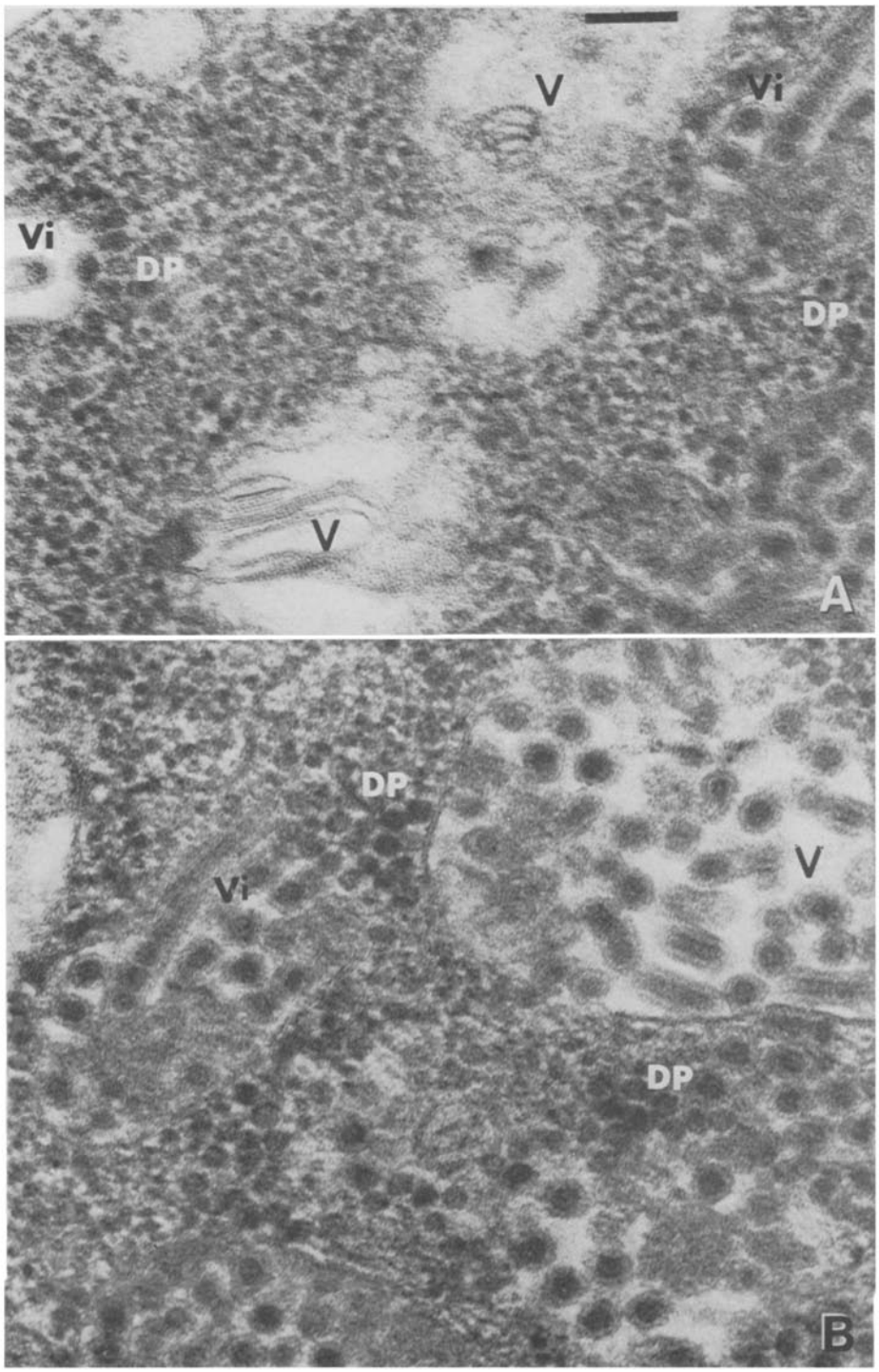

Fig. 5

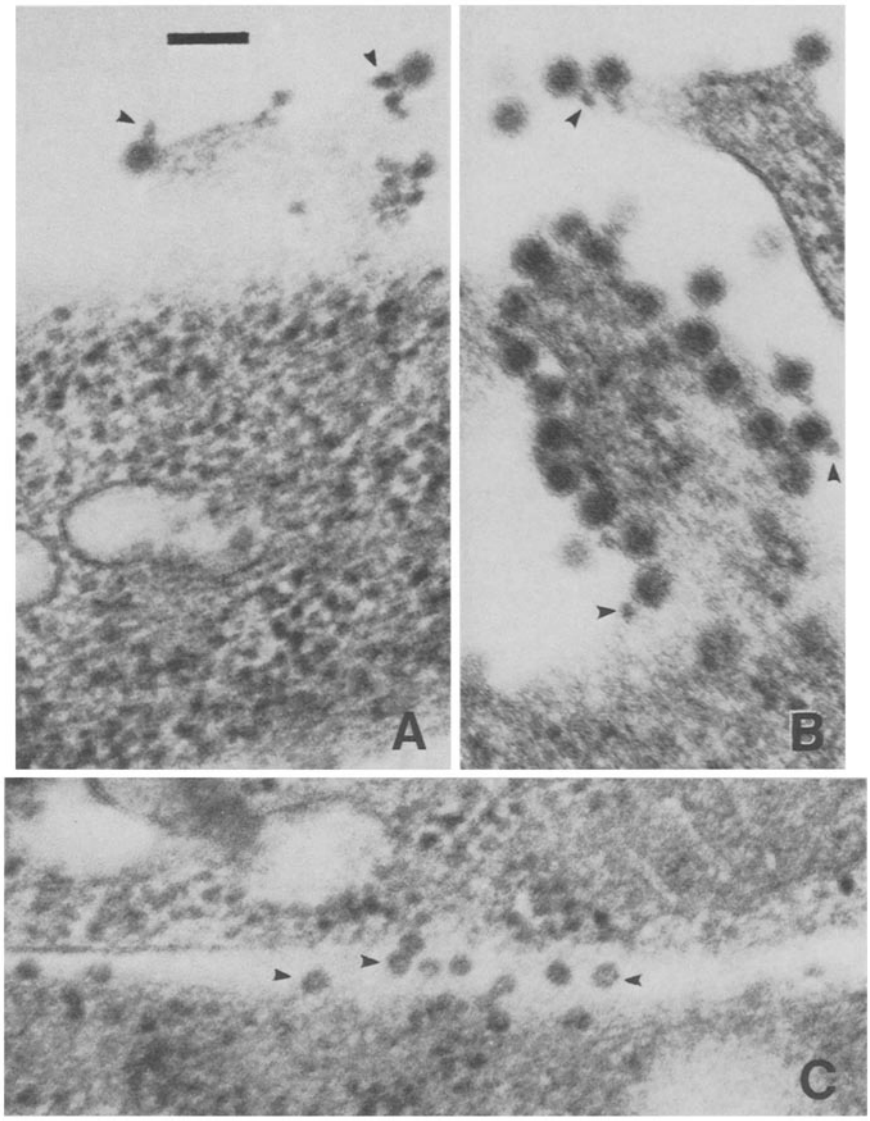

Fig. 6. Virions and dense particles appearing extracellularly at the membrane-absent cell surface of infected cells, 78 hours p.i. Bar, 100 nm. *A* Virions carrying trails of cytoplasmic material and satellite particles (arrowheads). *B* Virions appearing on the surface of cytoplasmic projections. Arrowheads point to the satellite particles. *C* Dense particles (arrowheads) between the two infected cells

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Fig. 5. The cytoplasm of an infected cell crowded with dense particles *(DP)* and virions *(Vi)*, 78 hours p.i. *V* vacuole. Bar, 100 nm. *A* Note the vacuoles containing membrane lamellae. *B* Note the dense particles, virions, and filamentous forms showing close topographical relationship to each other

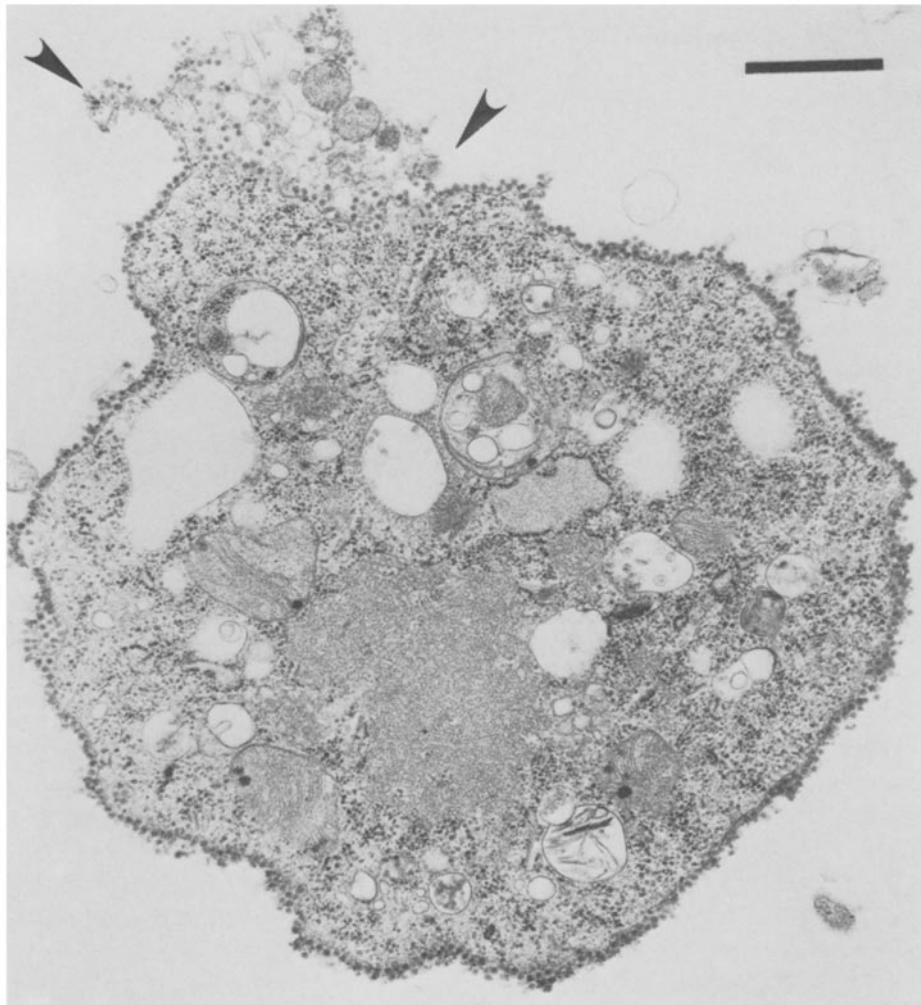

Fig. 7. A severely infected cells whose surface is lined with virions, 78 hours p.i. Bar, 1 μm. Arrowheads point to the ruptured membrane. The nucleus is not in the section plane

Immunoelectron Microscopic Observation of DEN-2 Virus Infection of C6/36 Cells

To confirm that viral antigens are found at the plasma membrane and may be involved in virion budding, infected cells at 48 and 78 hours p.i. were examined by immunoelectron microscopy. In samples reacted with a DEN-2 hyperimmune antiserum, minute, immunopositive spots measuring 50 to 100 nm or more were found in some areas on the relatively flat surface of infected cells (Fig. 8 B). These immunopositive spots seemed to correspond to early budding sites of maturing virus particles at the plasma membrane. The remaining plasma membrane and underlying cytoplasm in

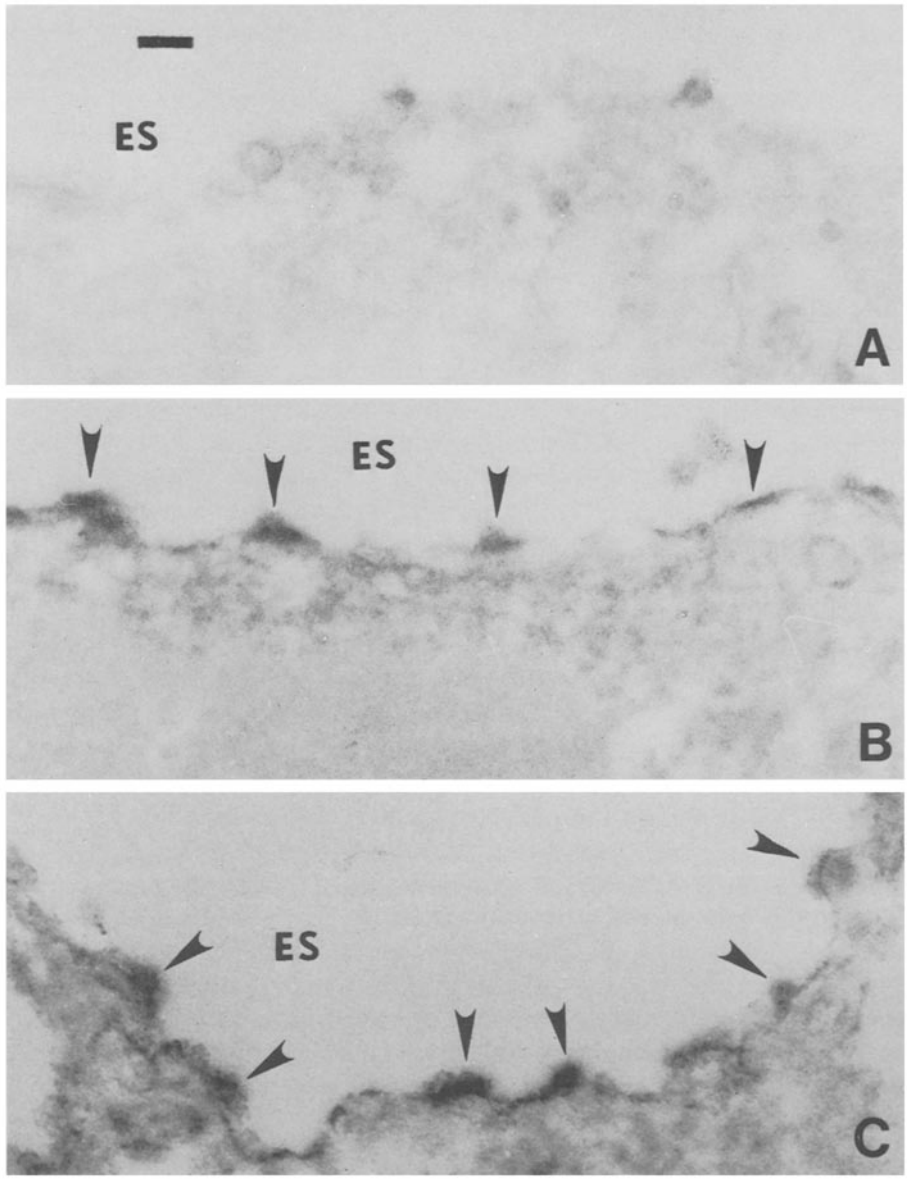

Fig. 8. Immunoelectron microscopy of infected cells, 48 hours p.i. *ES* extracellular space. Unstained with uranyl acetate or lead citrate. Bar, 100 nm. *A* Reacted with normal mouse serum (control). *B, C* Reacted with anti-DEN-2 mouse hyperimmune ascites fluid. Arrows point to the immunostaining spots

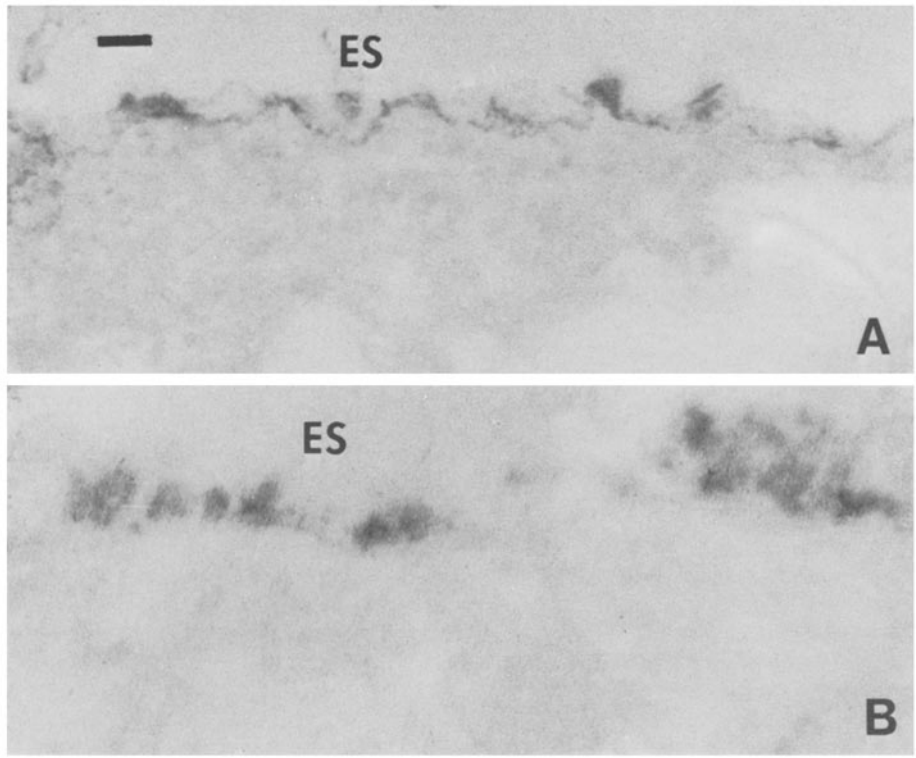

Fig. 9. Immunoelectron microscopy of infected-cell surfaces where apparent virus budding is taking place, 48 hours p.i. *ES* extracellular space. Unstained with uranyl acetate or lead citrate. Bar, 100 nm. *A* Reacted with anti-DEN-2 mouse hyperimmune ascites fluid. Note the mild immunostaining of the plasma membrane and possibly of the underlying cytoplasm. *B* Reacted with a monoclonal antibody against the DEN-2 envelope glycoprotein. Note the absence of immunostaining of the plasma membrane and the underlying cytoplasm

nearby areas were relatively unstained (Fig. 8 B). In areas of the cell surface where multiple budding virions were recognizable, these virions stained intensely; plasma membrane and cytoplasm adjacent to the apparent budding virions showed less-intense staining (Figs. 8 C, 9 A). In samples reacted with a DEN-2 monoclonal antibody preparation specific for the viral envelope glycoprotein, virions at the cell surface and within superficial vacuoles stained rather specifically with no staining occurring of host cell membrane and cytoplasm (Figs. 9 B, 10 A, B).

Discussion

It is well established that the alphaviruses assemble their nucleocapsids in the host cell cytoplasm and mature at the cell surface by budding (1, 2, 16). On the other hand, the developmental sequence of the flaviviruses remains

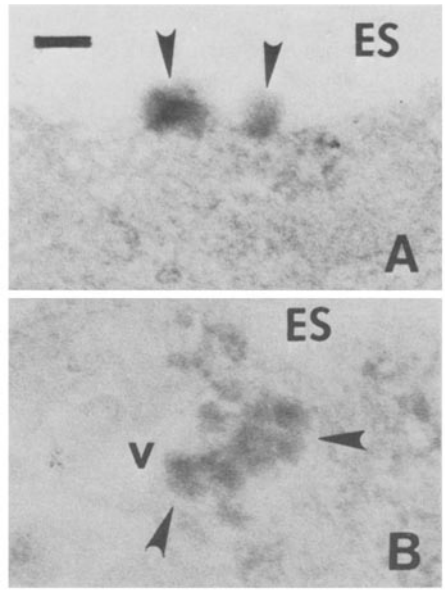

Fig. 10. Immunoelectron microscopy of virions (arrows) reacted with a monoclonal antibody against the DEN-2 envelope glycoprotein, 48 hours p.i. *ES* extracellular space; *V* vacuole. In-block stained with 0.5 percent uranyl acetate. Bar, 100 nm. *A* At the cell surface. *B* Withing a superficial vacuole

uncertain mainly because of the absence of a stepwise budding process which links nucleocapsids in the cytoplasm to maturing virions at the plasma membrane. Mature virions of flaviviruses have been observed principally within the lumens of cytoplasmic vacuoles during infection of cultured mammalian cells (4, 11, 12, 15, 16); consequently, it is generally presumed that flaviviruses mature by passage into cytoplasmic vacuoles (8). There is at least one report that demonstrates DEN virions appearing from the surface of infected mammalian cells (14). The present study of DEN-2 virus-infected cells demonstrated the appearance of characteristic dense particles and mature virions respectively on the cytoplasmic and luminal sides of the vacuolar membranes and on the cytoplasmic and outer sides of the plasma membrane from 24 hours p.i. onward. Although a transitional sequence of the dense particles into virions at the vacuolar and plasma membranes could not be definitively confirmed because of the obliteration of host cell membrane at the virus budding sites, it was apparent from morphological observations that the dense particles were DEN nucleocapsids which became virions by budding at the vacuolar and plasma membranes into the vacuolar lumens and the extracellular space, acquiring viral membrane envelopes in the process. The virions that accumulated within the vacuolar lumens were released extracellularly by exocytosis; the virions that appeared on the cell surface were released directly into the extracellular space.

The manner and extent of participation of host cell membrane in the acquisition of viral envelopes by maturing DEN-2 virions remains unclear. In this study, "dotted-line" type membrane defects and membrane-absent areas of the plasma membrane were frequently observed on the infected cell surface where budding virions were present. Nonetheless, whether these membrane changes revealed actual membrane damage incurred by viral maturation or merely represented artificial variations arising through sample processing and sectioning for transmission electron microscopy is difficult to evaluate. As the infection progressed, however, severely infected cells displayed ruptures of their plasma and cytoplasmic membranes in association with the appearance of large numbers of virions on the cell surface and within the cisternae.

In DEN virus-infected mammalian cells, STOHLMAN *et al.* (23) found that a majority of virus-specific proteins were bound to host cell membranes, including plasma membrane, and suspected that host cell membranes might serve as sites for viral maturation. By an immunofluorescent microscopic method, CARDIFF *et al.* (4) could not demonstrate DEN antigens on the plasma membrane of DEN virus-infected mammalian cells. However, CATANZARO *et al.* (5) and CARDIFF and LUND (3) could demonstrate viral antigens on the plasma membrane of infected mammalian cells by more sensitive immunoelectron microscopic methods. No viral budding was observed at the plasma membrane in either study. CATANZARO *et al.* hypothesized that the viral antigen staining became evident as part of an exocytotic process of virion-containing vacuoles. In the present study, DEN-2 specific antibody reactivity was observed to be localized in areas of virus budding. Host cell membrane and cytoplasm adjacent to the budding virions showed less intense staining when the cells were reacted with DEN-2 polyclonal antibodies and no staining when the cells were reacted with DEN-2 monoclonal antibody specific for the viral envelope glycoprotein. The diffuse staining by the polyclonal antibody may be due to reaction of antibodies with virion and non-virion polypeptides which are known to be inserted in the plasma membrane (23). It is known that the DEN-2 polyclonal antibody used for the current studies is reactive with a non-virion glycoprotein (NS-1) as well as the virion envelope glycoprotein (E) (data not shown). When the anti-E glycoprotein monoclonal antibody was used, staining did not occur on the plasma membrane except at the budding sites of maturing virions. This may indicate that levels of E glycoprotein present in the host plasma membrane are too low to be detectable by the presently employed immunoelectron microscopic method or that this glycoprotein in the plasma membrane is inaccessible to its specific antibody. It has also been found that the anti-E monoclonal antibody fails to induce immune cytolysis in DEN virus-infected cells (F. ENNIS, personal communication); this would seem to substantiate the above findings. On the other hand, alternative modes of virion mem-

brane envelope acquisition may occur. For example, the possibility that the viral membrane envelope was assembled at the budding site of a maturing virion can not be ruled out by this study. It has been shown by chemical analysis that the viral membrane envelope composition changes significantly during morphogenesis and viral biosynthetic activities continue right up to the point of viral release extracellularly (18). In a chronological study of the maturation process of Japanese encephalitis virus in Vero cells, LEARY and BLAIR (13) reported that virions formed in the cisternae of rough endoplasmic reticum starting from what they termed smooth membrane structures; the immature virions then moved to the Golgi apparatus where glycosylation of the envelope and viral maturation took place; and mature virions were released by exocytosis as virion-containing vacuoles fused with the plasma membrane. The maturation process of Japanese encephalitis virus in Vero cells observed by these investigators, therefore, differs from that of DEN-2 virus in C 6/36 cells observed in this study. The possibility that flaviviruses in different virus-host cell combinations exhibit different maturation processes needs to be clarified.

Data for the morphogenesis of flaviviruses in cultured mosquito cells and in mosquitoes vary considerably among investigators. FILSHIE and REHACEK (9) studied the morphogenesis of Murray Valley encephalitis and Japanese encephalitis viruses in cultured mosquito cells, and reached the conclusion that the maturation of flaviviruses in mosquito cells occurred at the internal membranes as in mammalian cells. On the other hand, WHITFIELD *et al.* (26) observed in the secretory cells of the salivary glands of St. Louis encephalitis virus-infected mosquitoes large numbers of virus particles which accumulated within cisternae of endoplasmic reticulum and shed externally via a process of local disintegration of apical plasma membrane. Similar changes were observed in DEN-2 virus-infected *A. albopictus* mosquitoes (20). Another electron microscopic study of a low multiplicity infection of C6/36 cells with DEN viruses showed maturing virions in cytoplasmic vacuoles, but not on the cell surface (12). In the present study, by inoculating C6/36 cells with DEN-2 viruses at a high MOI of 50 to 60, we have observed an acute dengue infection in which a large number of virus particles were released from infected cells by exocytosis of virion-containing vacuoles and by exit of individual virions at the surface. This experimental system, therefore, seems to offer a useful model for the study of DEN virus morphogenesis and of DEN virus-host cell inter-relationships.

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