INFECTION AND IMMUNITY, Dec. 1973, p. 1017-1028

Vol. 8, No. 6
Printed in U.S.A.

Dengue Virus Infection of Mice: Morphology and Morphogenesis of Dengue Type-2 Virus in Suckling Mouse Neurones

SIRIPORN SRIURAIRATNA, NATTH BHAMARAPRAVATI, AND ORANUJ PHALAVADHTANA

Department of Pathobiology, Faculty of Science, and Department of Pathology Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 4, Thailand

Received for publication 19 June 1973

In dengue type-2 virus-infected neurones of suckling mice, formation of single-membrane vesicles is observed in the distended cisternae of the endoplasmic reticulum mostly of the perinuclear zone around 72 h after inoculation. Electron-dense 50-nm virus particles are arranged in chains in these distended cisternae; some form small crystalloid aggregates. Aberrant particles of different shapes are also seen in the distended cisternae about the same time that the virus particles appear. Parallel filamentous structures are occasionally observed in the cisternae that contain very few virions, either characteristic or aberrant. Increasing cytopathic changes are present after 75 to 96 h. There is an intense vesicular formation. Large numbers of virions and aberrant particles are seen either in the endoplasmic reticulum cisternae or smooth membrane vesicles. They are spread throughout the neurocytoplasm, extending into the dendrites. Dengue virions which are enclosed in fairly intact membrane-bound vesicles are released during cytolysis of the neurones. Morphogenesis of dengue virus type 2 is discussed.

In neurones of suckling mice infected with dengue virus, Bhamarapravati et al. (6) demonstrated by direct fluorescent antibody method that dengue antigen was first detected in the perinuclear area in the form of small granules at about 72 to 96 h after inoculation before the antigen diffused all over the cytoplasm. Complement-fixing dengue antigen apparently behaved in the same manner during the early stage of cell infection as shown by Atchison et al. (2) by using a fluorescent complement fixation antibody method. Electron microscopy of isolated virus particles prepared from infected mouse brains and tissue cultured cells revealed that dengue virions are spherical particles measuring 48 to 50 nm with an envelope (8, 20, 23, 33). There are two prominent findings in Vero cells which are infected with dengue virus: the intense vacuolization of the cytoplasm and the presence of virus particles in membrane bound vesicles (23). The precise site and mode of replication, formation of the precursors and virus related structures, and the assembly of virus particles have not been as well documented as in some other group A and group B arboviruses, especially in cells of infected animals (14, 15, 18, 26, 27).

This study was undertaken to observe the morphology and morphogenesis of dengue-2 virus in the neurones and glial cells in the brains of suckling mice infected intracerebrally.

MATERIALS AND METHODS

Litters of 1- to 2-day-old suckling albino mice were intracerebrally inoculated with 0.02 ml of dengue-2 virus suspension (New Guinea "C", 20% suckling mouse brain, 23rd passage, titer 7×10^5 plaque-forming units per 0.3 ml) in 50% fetal bovine serum in phosphate-buffered saline. The infected mice were divided into two groups. In the first group of 20 mice, two mice were sacrificed at 24-h intervals until the end of the 5th day. In the second group of 36 mice, two were sacrificed at 3-h intervals starting from day 2 to the end of day 4 after inoculation. Some infected mice died before the end of the experiments in both groups.

Litters of uninoculated mice were kept in the same room, and one mouse was sacrificed at the same time interval as the infected mice.

Middle parts of the mouse cerebral hemispheres, about 1 to 2 mm proximal to the inoculation site, were removed and fixed in 4.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at 4 C, postfixed in 1% osmium tetroxide in Millonig buffer (24) for 1 h at 4 C, dehydrated in a graded ethanol series and propylene oxide, embedded in Epon 812 (21), and polymerized at 60 C for 48 h.

Semithin sections were cut at 0.5 to 1 μm thickness

and stained with toluidine blue and pyronine (36) for light microscopy examination. Cerebrocortical areas were selected for ultrathin sectioning, cut at about 50-nm thickness and mounted on 300-mesh, naked copper grids, stained with 2% uranyl acetate solution (37) and lead citrate solution (31), and examined in a Hitachi HS-8 electron microscope at 50 kV.

Immunofluorescent study. The presence of dengue antigen in the neurones was confirmed by direct immunofluorescent antibody technique. Imprints were prepared from the cut surfaces of the distal portion of the brain. Imprints were fixed in octanol at −20 C for 30 min, air-dried and stained with anti-dengue-2 mouse hyperimmune ascitic fluid conjugated with fluorescein isothiocyanate for 30 min, washed two times in 0.05 M phosphate-buffered saline for 5 min each, and mounted in buffered glycerine for fluorescent microscopy observation. The positive fluorescent staining was confirmed by procedures described before (6).

RESULTS

The infected mice showed definite paralysis on day 4 postinoculation with dengue-2 virus, and they were severely paralyzed on day 5. None of the infected mice survived to day 6. Control mice did not show any abnormalities.

Immunofluorescent findings. There was no specific positive staining observed in the control imprints. Dengue antigen appeared as fluorescent granules around 72 h postinoculation in the perinuclear area of a few scattered neurones in imprints from infected brains. Clumps of dengue antigen were seen in the general area of neurocytoplasm and of cytoplasm of glial cells between 75 to 96 h. Large clumps of dengue antigen were observed at 120 h, and were spread throughout the neurocytoplasm and cytoplasmic processes, and were occasionally seen in the interstitial tissue.

Light microscopy findings: control brains. No specific alteration was observed in toluidine blue stained Epon-embedded sections.

Infected brains. At 48 h postinoculation, some dispersion of Nissl substances was noted, but otherwise specific alterations could not be observed. Spotted toluidine blue stain in neurocytoplasm was seen during day 4 and 5. Neuronal necrosis was noted especially in mice sacrificed at the end of the 5th day after inoculation. Active phagocytic microglias were occasionally observed around the dying neurones.

Electron microscopy findings: control brains. Electron microscopy observation corroborated the findings in toluidine blue-stained sections. In the neurones, nuclear pores were clearly shown at intervals on the nuclear envelope. The nucleolus was observed as an anastomosing network of both the *pars granulosa* and *pars fibrosa* component (Fig. 1). Perichromatin granules and interchromatin granules were observed, and cytoplasmic organelles were rich in the predendritic areas. Nissl bodies (rough-surfaced endoplasmic reticulum; RER) were characteristic, and there were many polysomes and a moderate amount of free ribosomes. Mitochondria were scattered. Golgi complexes and lysosomes were present, and the glial cells showed small cytoplasmic processes. The general descriptions correspond with those described elsewhere (30).

Infected brains: nucleus. Nucleolar structural alteration was noted in some neurones 48 h postinoculation. The *pars granulosa* and *pars fibrosa* were not quite arranged in the form of an anastomosing network as in normal brains (5, 16). Striking nucleolar macrosegregation was observed in neurones around 72 h postinoculation (Fig. 2), and indentation of the nucleus was seen in the later period of infection. In neurones and glial cells that showed severe degenerative changes, the nuclei were usually pushed to a corner and were condensed into chromatin masses or fragmented in the necrotizing neurones and glial cells.

Hyaloplasm. A few clusters of fibrogranular material of variable size lay randomly in the neurocytoplasm at 48 h postinoculation. This material had no enclosing membrane and sometimes was arranged in rosette aggregates (Fig. 3). Free ribosomes and polysomes were located close to the margin of the material. This fibrogranular cluster was hard to find after 72 h, and none was seen at 120 h.

Single-membrane-bound vacuoles of the sizes and at the sites of the CPV-1 and CPV-2 that have been described in Semliki forest virus (1, 12, 14, 15, 35) or in other group A arbovirus-infected cultured cells or neurones (10, 17, 18, 25, 28) may occasionally be observed in the hyaloplasm of a virus containing neurone. However, the vacuoles were empty and the cytoplasmic side was smooth (Fig. 4). Viral nucleoids or virions were not discernible in these structures, and there was no temporal relationship between these structures and other structures to be described.

Ribosomes. A moderate number of endoplasmic reticulum-associated ribosomes, free ribosomes, and polysomal ribosomes were observed in the neurones of infected mice examined at 24 and 48 h postinoculation. Degranulation of ribosomes from the endoplasmic reticulum was observed around 72 h postinoculation; polysomes were decreased in number and free ribosomes were disintegrated (Fig. 4). At 96 and 120 h, polysomes were almost absent and free ribosomes were scattered throughout the cytoplasm of almost every cell. In the necrotizing neurones,

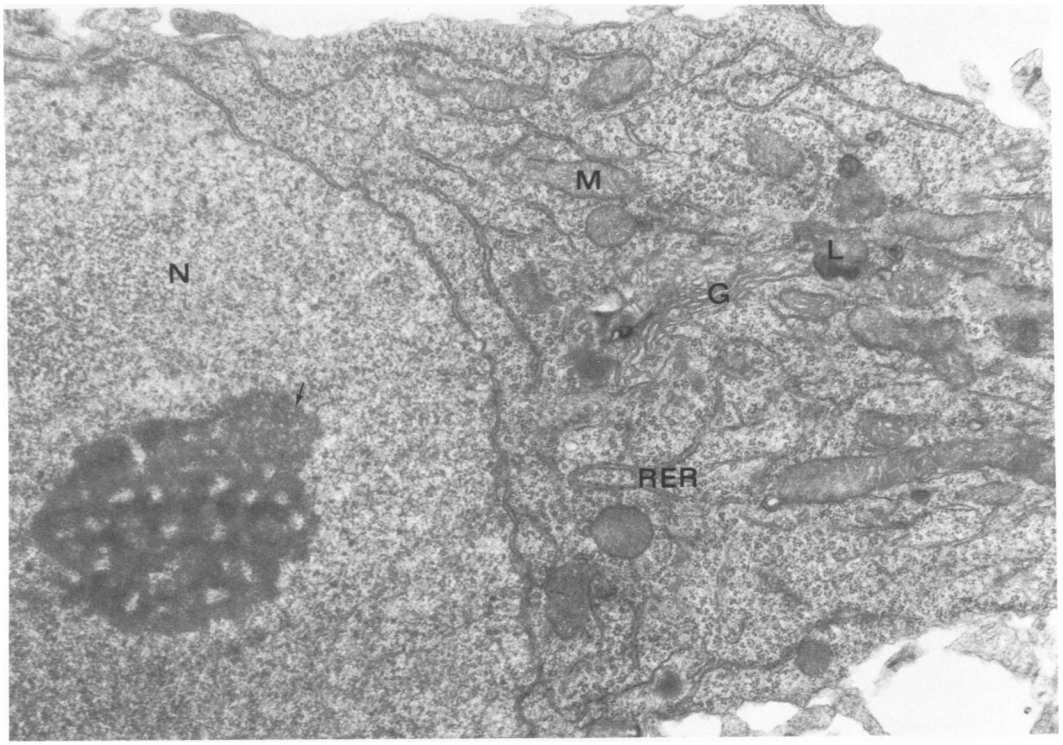


FIG. 1. *A neurone of an untreated suckling mouse sacrificed at the same interval as the infected neurone shown in Fig. 7. The nucleolus is at the center of the nucleus. A Barr's body (arrow) is visible. Cytoplasmic organelles are rich in the predendritic portion. Terminal processes from other neuronal cells are noted at several parts on the plasma membrane. Abbreviations: M, mitochondrion; N, nucleus; G, Golgi complex; L, lysosome; RER, rough-surfaced endoplasmic reticulum.* ×18,950.

ribosome-like particles mixed with cellular debris.

Golgi complexes. There was an increase in the number of Golgi complexes, with increased membranous saccules in some neurones observed at 48 h postinoculation, when other cytoplasmic structures were comparable to uninfected neurones (Fig. 5). Later on, around 72 h, the Golgi complexes were more or less degenerating. In some neurones, where virions could be seen, virus particles were occasionally observed in a Golgi vesicle.

Endoplasmic reticulum. There were no striking changes observed in Nissl bodies at 24 h postinoculation. Rearrangement of arrays of RER were noted in some neurones at 48 h. Several arrays of RER were packed in one area, whereas the other organelles were in the rest of the cytoplasm (Fig. 6). Sometimes, these RER cisternae exhibited more density than normal. Definite changes in the RER were observed in neurones around 72 h postinoculation, and the changes were progressive after 72 h. The endoplasmic reticulum was prominent in the perinuclear zone, and the cisternae were distended at random. Within these distended cisternae, vesicles of varying sizes were seen (Fig. 7). They were round to oval in shape and 80 to 280 nm in diameter. Some of the vesicles were cut tangentially and appeared as cylinders of different lengths. These vesicles contained flocculent or homogeneous electron translucent material with a clear center, and were located singly in the cisternae or in groups of two or more; they were rarely observed in the distended portion of the perinuclear cisternae. Frequently, the vesicles overlapped into each other with distinct individual outlines. These structures are considered by us to be different from those vacuoles seen in the hyaloplasm and are definitely relative to formation and maturation of the virions.

Dengue virions. Electron-dense round particles measuring 50 nm were first observed in the distended cisternae of the endoplasmic reticulum in the perinuclear region of isolated neurones at 69 h postinoculation, and increasing numbers of tnese particles were seen in the later stages. We believe that these were dengue virus particles. They were often found along the axis of the cisternae forming a long, single row of

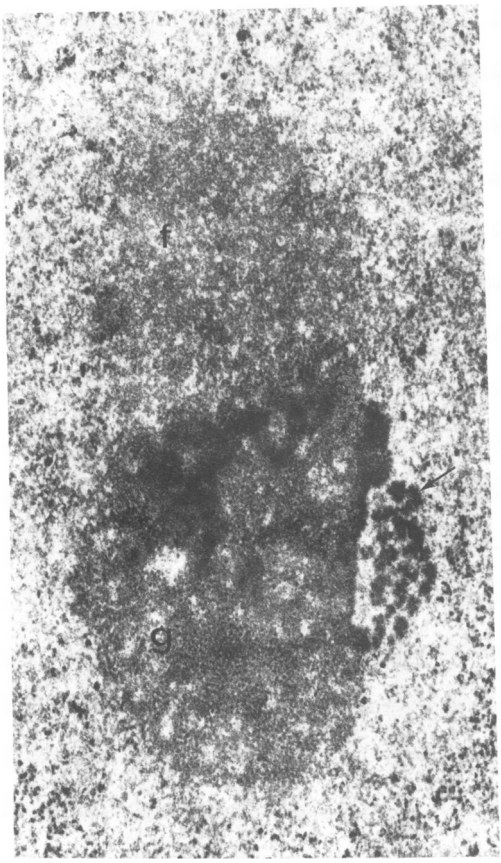


FIG. 2. *A segregated nucleolus of an infected neurone. The pars fibrosa (f) and pars granulosa (g) components are separated into two distinct parts. A dense zone of mixed component is seen in between. The Barr's body (arrow) remains characteristic.* ×25,200.

particles with regular spacing of about 52 nm from center to center. Some particles were arranged in small crystalloid aggregates in the distended cisternae (Fig. 7). Other small and dense particles were attached to the cisternal membrane at the cytoplasmic side. They can not be differentiated from the ribosomes that were scattered in the hyaloplasm. In neurones where endoplasmic reticulum was not strikingly changed, individual virus particles were observed in small vesicles in the hyaloplasm, unrelated to any cytoplasmic structures. In rare neurones, a few virus particles were present in the perinuclear cisternae. By high magnification observation, the virus particles appeared to have a dense center about 40 nm in width. There was an outer light zone of 3 to 4 nm and an envelope next to this light zone (Fig. 8). Some virus particles did not have an envelope. No particular pattern of distribution of non-enveloped or enveloped virus particles was observed; both were present in the cisternae or in aggregates within a vesicle. Large aggregates of virus particles were seen in practically every neurone seen at 120 h. Some 30 to 50 virions were observed in certain aggregates surrounded by a thin, smooth membrane of the vesicle in the hyaloplasm. The membrane surrounding the viral aggregates can not be prominently stained, and it is usually seen by careful examination. In many instances, the membrane-bound viral aggregates were observed to be very close to the plasma membrane (Fig. 9), but we could not observe any budding or shedding of the virus particles in any of the sections. In the necrotic neurones the virus particles were always well bound in the enclosures.

Dense bands. Some electron-dense bands (Fig. 9) were observed in certain endoplasmic reticulum cisternae of the virus-producing neu-

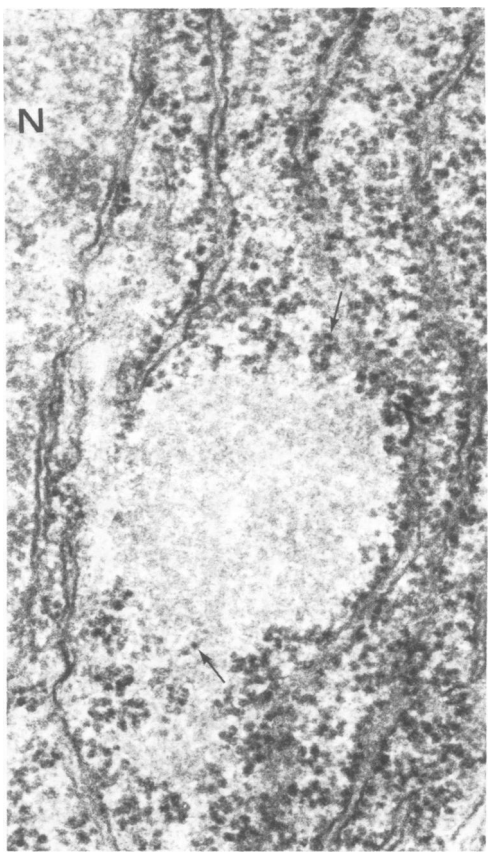


FIG. 3. *Neurocytoplasm at 51 h after inoculation. A cluster of fibrogranular material is seen in the arrays of endoplasmic reticulum. It has no limiting membrane. Free ribosomes and polysomes are seen at the margin of the cluster (arrows); nucleus (N).* ×62,500.

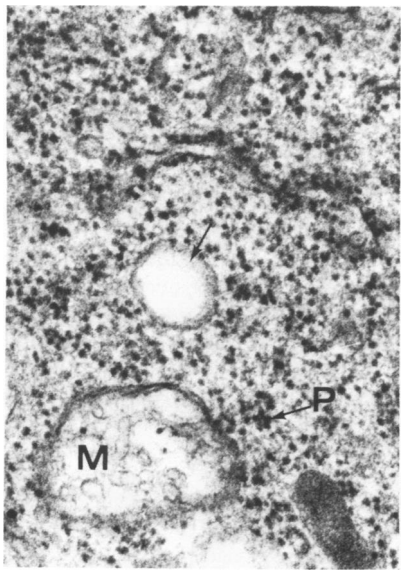


FIG. 4. *An empty cytopathic vacuole (arrow) is seen in the hyaloplasm of a neurone 75 h after inoculation. Polysomes (P) are greatly decreased in number. Free ribosomes are scattered throughout. The mitochondrion (M) has widened cristae with pale matrix.* ×48,000.

rones. They were beady in appearance and measured about 50 nm in width; a virus particle could be observed at one or both ends of these bands.

Aberrant dengue virions. Around 72 h postinoculation, in addition to the appearance of virus particles, electron dense particles of variable shapes in the endoplasmic reticulum cisternae of some neurones also could be seen. They were arranged in a disorderly way, and after 75 h, they usually were packed in a vesicle either with or without the spherical virus particles (Fig. 10) and could also be found in a distended perinuclear cisterna. Some of them appeared as variants of triangular structures whereas others resembled "tadpole" forms described by Murphy et al. in their study of St. Louis encephalitis virus (26). The aberrant particles measured 50 nm on the short axis and 75 to 90 nm on the long axis.

Filamentous structures. Other noteworthy structures occasionally observed in the endoplasmic reticulum were filamentous structures that were usually found in parallel arrays in the distended cisternae. Long and short filaments were present in the cisternae, which usually contain very few virus particles, and under this condition, numerous bundles of parallel filaments were seen in other cisternae with the virions or aberrant particles (Fig. 11, 12, 13). The filaments were all confined within the cisternae and ran in different directions. Sometimes in cisternae of the same neurone, a number of small round particles were observed and were smaller than the virions. Both the filaments and small, round particles were seen within the same cisterna. Once the filaments were seen either in the perinuclear cisterna or passing through the nuclear pore; they measured 15 to 25 nm in width. Each filament was composed of a central dense line and two lighter bands on the outside. The small, round particles also measured 15 to 25 nm in diameter, and each had a dense central core. The morphology, size, and location of both the filaments and the small, round particles make us believe that they are the same element, but cut in longitudinal and cross sections.

Lysosomes. There were usually a few lysosomes present in the neuronal and glial cyto-

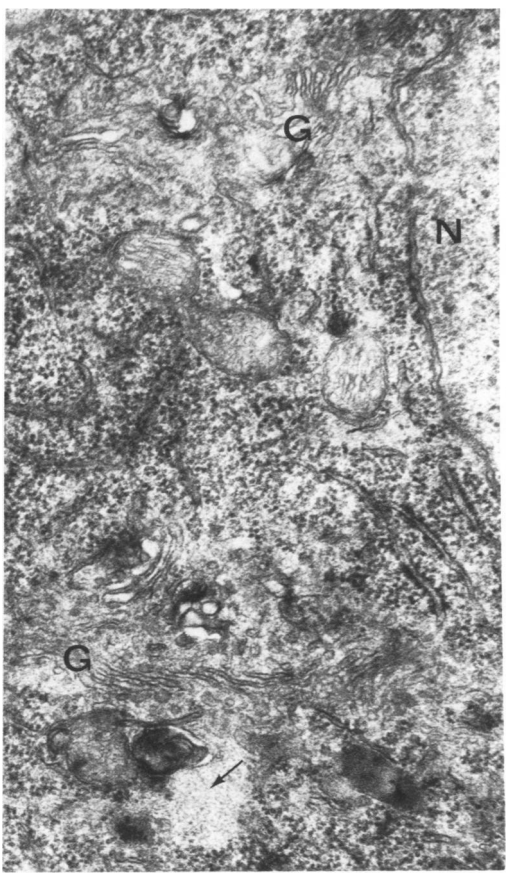


FIG. 5. *A neurone 48 h after inoculation. Golgi complexes (G) are increased in number. A small cluster of fibrogranular material is embedded in the hyaloplasm (arrow). Other organelles are comparable to the control; nucleus (N), mitochondrion (M).* ×32,000.

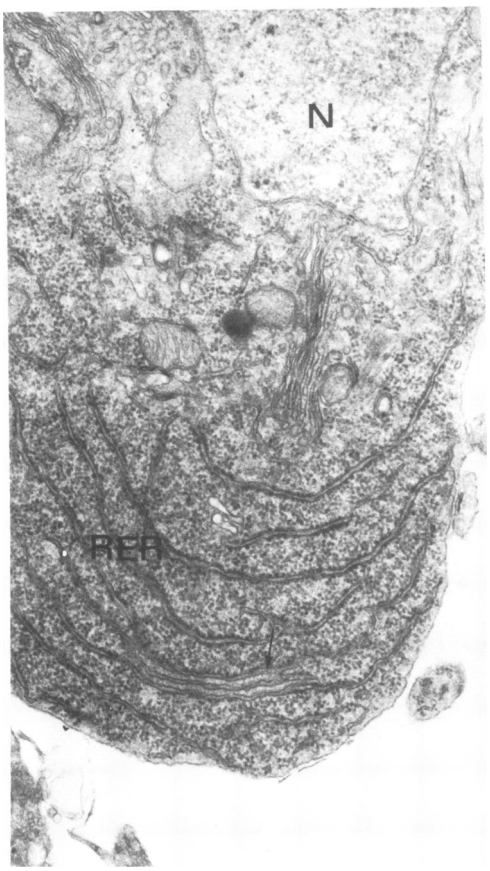


FIG. 6. *A neurone 48 h after inoculation. The endoplasmic reticulum clustered peripherally in one area. Degranulation of ribosomes from the cisternal membrane is noted (arrow). Other organelles are seen in the rest of the cytoplasm; nucleus (N).* ×24,200.

plasm of brains observed at 24 to 48 h postinoculation. A great increase in the number of lysosomes was observed in 96- and 120-h brains. Autophagic vacuoles filled with altered mitochondria, endoplasmic reticulum, ribosomal debris, or cytopathic vesicles were often seen in the neurocytoplasm. In the glial cells that showed active phagocytosis, several phagosomes containing heterogeneous globoid materials, myelin figures, or viral aggregates were prominently seen (Fig. 14).

Mitochondria. Mitochondrial alterations were first observed at 72 to 75 h postinoculation. They showed dilated cristae in the virus-producing neurones and were severely swollen in the necrotizing neurones observed at 96 and 120 h postinoculation (Fig. 15).

The organelle changes described above were seen in a small number of neurones at 72 h. More and more neurones showed viral particles and other changes at 96 and 120 h postinoculation. Vesicle formation during this period was intense, and sometimes the vesicles themselves looked distorted. Only a few enveloped virions were rarely observed in the intercellular substance or close to a neuronal plasma membrane or neuronal processes. Viral adhesion and penetration were never observed. Degenerative changes of neurones and glial cells were prominent at 120 h, and in the necrotizing neurones, the plasma membrane was disrupted. Virus particles and cytopathic vesicles were generally well bound in the enclosures, and a few virions lay free in the hyaloplasm among the organelle debris. Coalesced elements of organelles could be scattered in the areas of viral and cytopathic vesicular enclosures, and there were numerous phagocytic glial cells present at 120 h. The cytoplasmic vacuoles of these cells were loaded with large phagocytosed inclusions. Virus particles could be seen in these large phagocytic vacuoles of glial cells. A few virions were occasionally engulfed by the glial cell or were observed in a glial cell vesicle.

DISCUSSION

In the present study, the first cellular alteration induced by dengue virus is the appearance of a cluster of fibrogranular structures, lying free in the cytoplasm without the limiting membrane (Fig. 3). This structure is probably a virus-directed structure, because it is not observed in neurones of normal mice nor in neurones of suckling mice infected for up to 48 h. The presence of nucleolar segregation also at 48 h after inoculation makes it difficult to say whether nucleolar segregation preceded the formation of cytoplasmic fibrogranular structures or not. Degranulation of the endoplasmic reticulum and ribosomal disintegration were noted in neurones and some glial cells of mice sacrificed at 48 h. These changes imply that some alterations of the cellular ribonucleic acid system caused by the infecting ribonucleic acid virus are happening prior to the observation of virus particles themselves. Replication of the virus is then observed in neurones and certain glial cells.

The enveloped, spherical dengue-2 virus particles that measured 50 nm, which are seen in the cisternae of the endoplasmic reticulum, are morphologically identical to the descriptions of isolated dengue virus particles or virions in cultured cells made by other investigators (8, 9, 20, 23, 33; A. Oyama et al., Abstr. 15th Annu. Meet. Soc. Jap. Virol., p. 316–317, 1967). The

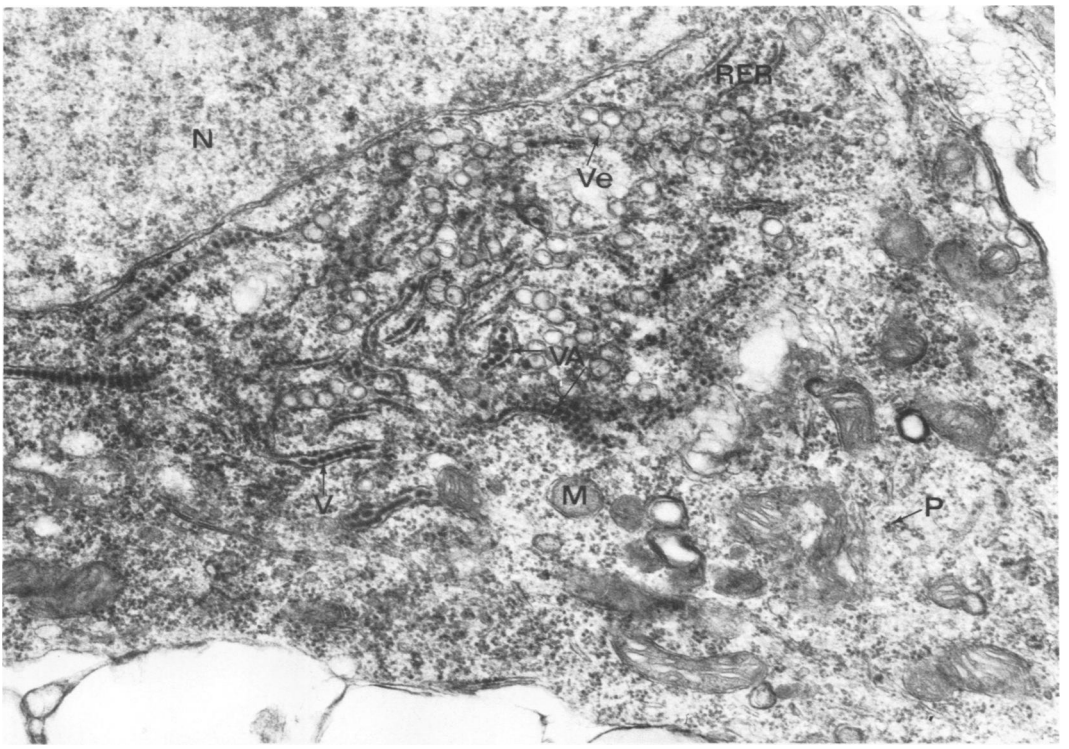


FIG. 7. *At 72 h after inoculation, the endoplasmic reticulum (RER) is prominent at the perinuclear area. The cisternae are distended at random. Within these distended cisternae varying numbers of cytopathic vesicles (Ve) and the dengue virions (V) are seen. The virions are arranged in chains along the cisternal axis. Viral crystalloid aggregates (VA) are formed in other distended cisternae. Polysomes (P) are markedly decreased. Nucleus (N), mitochondrion (M).* ×25,740.

presence of assembled virions within the cisternae conforms with the general observation in other group B arbovirus infections (3, 4, 13, 26, 27, 32, 34). Aberrant particles found in the cisternae had no envelopes (Fig. 10). The location and the absence of envelopes among these particles are suggestive of malformed or pleomorphic dengue virions, similar to what happens in the case of rubella and Bunyamwera viruses (22). Dengue virions in neurones appear to be slightly different from those in Vero cells, where virus particles were seen in the cytoplasmic vacuoles, and the center to center distances between particles were shorter (23). The beady, dense bands shown in Fig. 9 appear to be closely packed virus particles cut through the equator rather than tangentially cut.

Cytopathic vesicles observed in the infected neurones (Fig. 7) are unique structures in cells where group B arboviruses are replicating (7, 13, 26). These vesicles are somewhat different from the vacuoles in cells infected by group A arboviruses (1, 10, 12–15, 17, 18, 25, 28). Vesicles of group B arboviruses are mostly located in the cisternae, but the vacuoles of group A arboviruses are in the hyaloplasm.

Although budding of viral nucleoids from, or formation of nucleoids on, the vacuolar membrane appears to be easily demonstrable in group A arboviral infection (14, 15, 17, 25, 28), it is rarely demonstrable in group B arboviral infection. To the best of our knowledge, such an example has been clearly shown only in Bunyamwera group virus infection of mice by Murphy et al. (27) and in Japanese B encephalitis virus in porcine kidney stable cells by Ota (29). It was assumed by Matsumura et al. (23) that the mature, completed virions found within membrane-enclosed structures were presumably "budded" through the vacuolar membrane and resembled what had been described for cells infected with group A arboviruses in their study of dengue-infected Vero cells. They also mentioned that budding of the virus particles from the plasma membrane of the cell was rarely seen and did not show any convincing

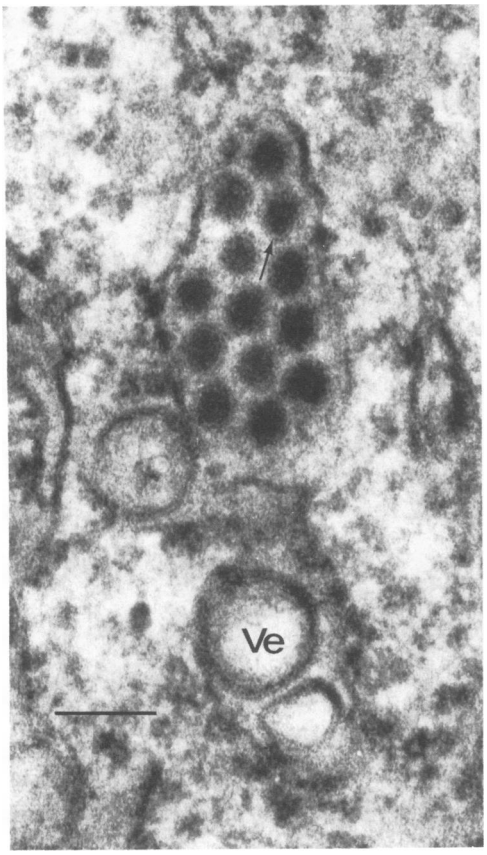


FIG. 8. *High magnification micrograph of dengue virions. They are discrete particles arranged in crystalloid pattern and are spaced regularly, center to center. The viral envelope (arrow) is seen on some virions; cytopathic vesicle (Ve). The bar indicates 100 nm. ×135,000.*

illustration in their article. Their Fig. 5 showed an isolated virion in the intercellular space, and Fig. 6 revealed an aggregate of viral particles extracellularly near the plasma membrane. Oyama et al. (Abstr. 15th Annu. Meet. Soc Jap. Virol., p. 316–317, 1967) did not describe budding at all in their study of dengue-4-infected mouse brain. We did not see nucleoid formation on, or budding of nucleoid from, the cisternal membrane. We only say that the cytopathic vesicles appear at about the same time that individual virus particles are seen or before the presence of virus particles along the endoplasmic reticulum cisternae.

The filaments which appear either in longitudinal or cross-sectional profiles, or both, are very interesting and have not been described in experimental models of both group A and B arboviral infections. Two viruses, however, induce the formation of structures in some way reminiscent of the filaments seen in this study. Erlandson et al. (12) described tubular structures 30 to 40 nm in width and up to 1.7 μm in length in the cytopathic vacuoles in HEp-2 cells infected with Semliki forest virus. Grimley and Friedman (15) also demonstrated several ring-formed or rod-formed profiles in single-membrane compartments in neurones of mice infected with the same virus. Blinzinger et al. (7) studied mouse brain infected with Zimmern virus (group B arbovirus) and described "microhelices" in the cisternae of the endoplasmic reticulum in the immediate vicinity of the newly formed virions. The microhelices were 28 nm in width and some cross section of the helices could also be seen. The filaments seen in our dengue-infected neurones are different from

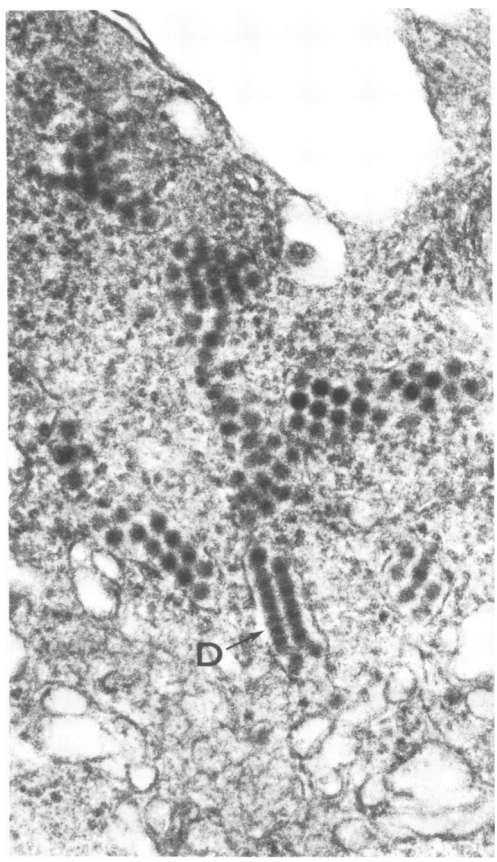
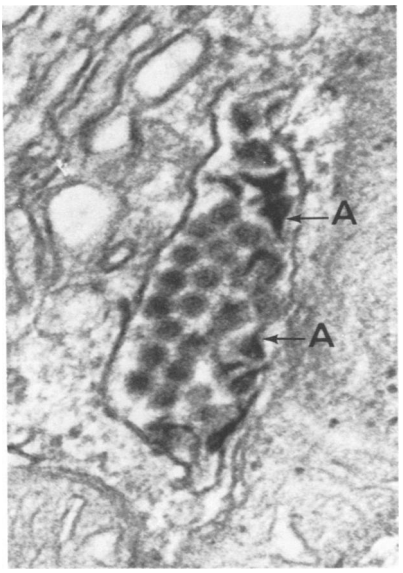


FIG. 9. *Portion of a neurone 96 h after inoculation. Viral aggregates are seen in single membrane bound vesicles. The cytopathic vesicles appear somewhat distorted. Two parallel beady bands (D) are noted in a cisterna. ×65,000.*



the tubular-rod formation or microhelices in respect to size and cross-sectional profiles. The location and chronological events relating to the three structures suggest that they may be similar to the entity involved in virus formation, rather than structures which are formed as a consequence of cell injury. The presence of numerous parallel filaments in the neurones with relatively few scattered virions suggests that the filaments are formed before virus multiplication. The filaments always are arranged in a crystalloid pattern (Fig. 12, 13). The presence of filaments in a single cisterna along with virions and the arrangement of filaments and viral crystalloid aggregates suggest to us that the filaments may be the direct precursor of dengue virions or definitely a virus-directed structure.

FIG. 10. *The aberrant particles (A) are in a vesicle together with the spherical virions. ×85,000.*

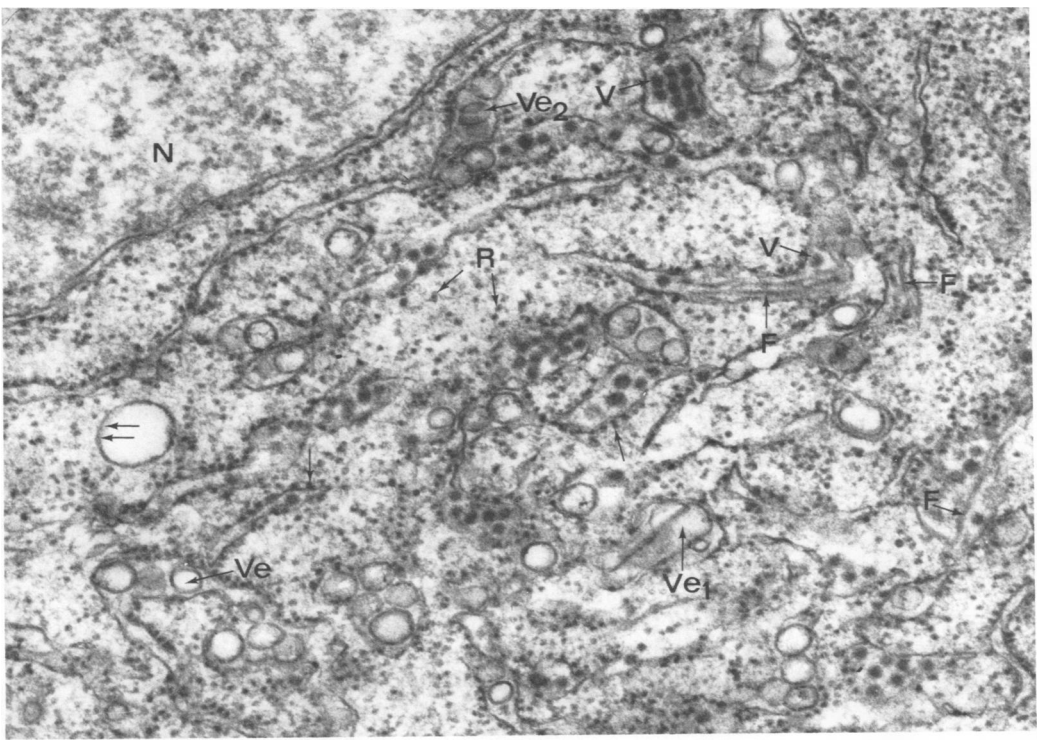


FIG. 11. *At 75 h after inoculation, dengue virus particles (V) are mostly arranged in small crystalloid aggregates. Some cisternae contain parallel filamentous structures (F). The cytopathic vesicles (Ve) are prominent and sometimes appear in longitudinal profile (Ve_1) or overlapped into each other (Ve_2). Double arrow shows an example of a vacuole which is often devoid of any particles, and the lumen is always empty. It is considered to be different from those vesicles that are seen in the distended cisternae. Free ribosomes (R) are scattered throughout the cytoplasm, and some are observed on the outer perinuclear membrane. Particles of the same size as the ribosomes are also seen on the cisternal membrane (arrows); nucleus (N). ×45,000.*

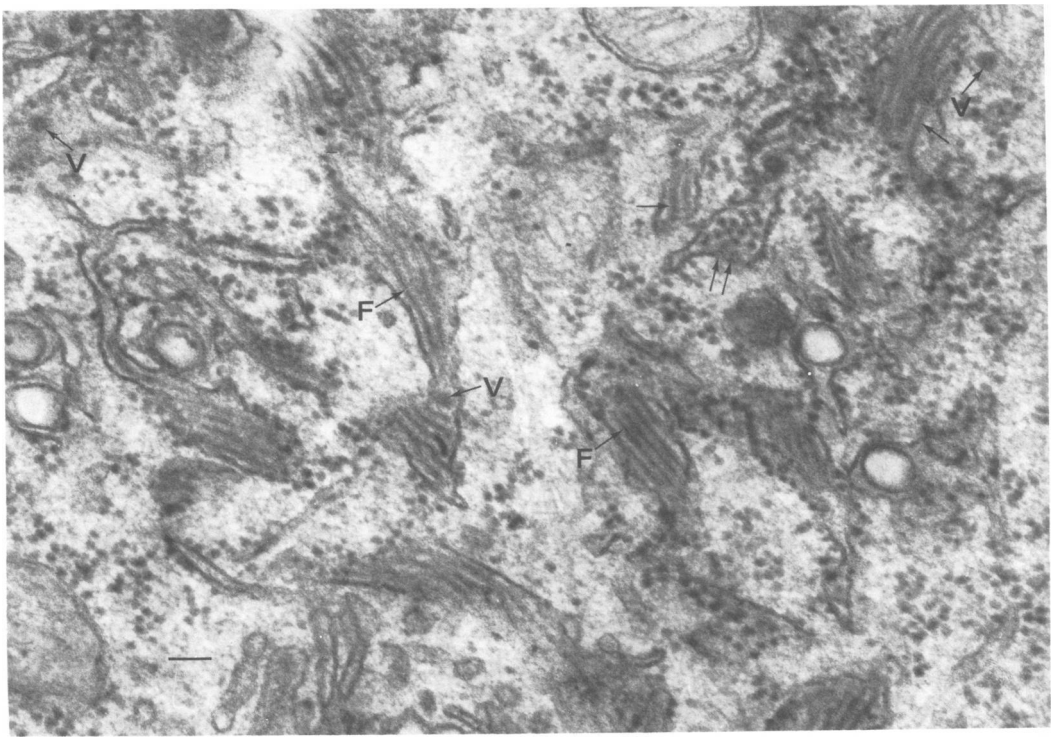


FIG. 12. *A neurone contains only a few virions (V) in scattered cisternae. Numerous filaments (F) are present in the perinuclear zone. They are arranged in parallel in the distended cisternae of the endoplasmic reticulum. Each filament has a central dense line. Some filaments appear in larger sizes (arrows). Small round particles with a dense central core are seen in a cisterna in the vicinity of the filaments (double arrow). The bar indicates 100 nm. ×55,300.*

From the study of many serial sections of the same neurones, which show evidences of replication of dengue virus as described earlier, it appears unlikely that the morphogenesis of dengue virus will take shape by budding of viral nucleoid on the vacuolar-cisternal membrane into the vacuolar or cisternal spaces to complete their maturation in the manner that has been described in Semliki forest virus (1, 12, 14, 15, 35), Chikungunya virus (10, 17, 18), Western equine encephalitis virus (25), Venezuelan equine encephalitis virus (28), St. Louis encephalitis virus (26), Bunyamwera group virus (27), and Japanese B encephalitis virus (29, 38). Whenever complete dengue virions are seen they already are inside the membrane bound vesicles or in the cisternae of the endoplasmic reticulum. No foci of early viral formation could be seen in the hyaloplasm. We would like to propose that the filaments are most likely to be dengue viral precursor, a RNA nucleoid, and are formed in the cisternae. Viral protein coat is somehow acquired in relationship to the cytopathic vesicles which are intensely formed near the filaments. The next stage may be represented by the beady bands which could be closely packed viral particles in rows (Fig. 9). The final stage of viral maturation occurs after the cleavage of particles in beady bands, resulting in the formation of individual particles arranged in rows or aggregates.

Release of dengue virus occurs after cytolysis, whereas most of the viral particles remain well enclosed in the vesicles which may be broken up later. This may be a protective mechanism to prevent premature lysis of the virus by lysosomal enzymes released during cytolysis. Virus-containing vesicles are heavily present in dendrites and to a lesser extent in axons which usually do not contain endoplasmic reticulum. Propagation of virions through the dendritic processes is a possibility as suggested in an immumofluorescent study (6). Even though Craighead et al. (11) described vascular changes in the cerebrum of suckling mice inoculated with dengue virus, we do not see any virions in the endothelial cells. Choroid plexus is not examined in this study, thus we could neither exclude nor confirm the early replication of virus in this location as proposed by Johnson

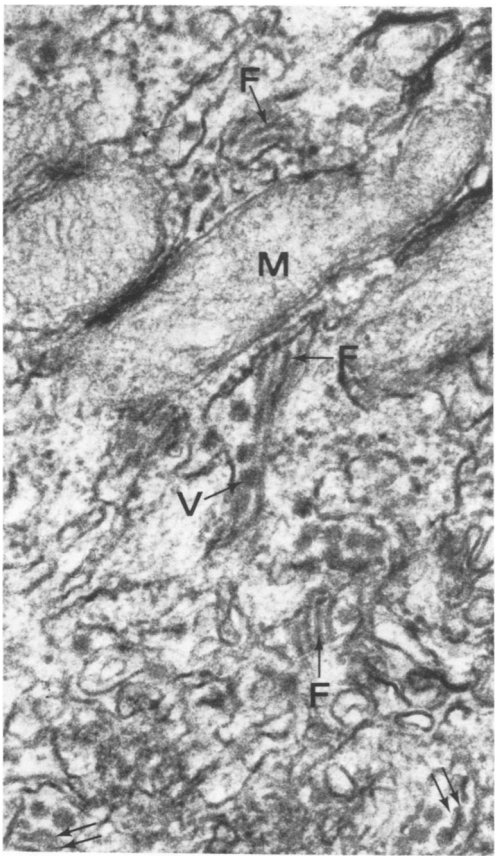


FIG. 13. *Portion of a neurone 96 h after inoculation. Filaments (F) are in the cisternae similar to those that appear in Fig. 11 and 12 either alone or with the virus particles (V). Double arrow shows aberrant-shaped particles that assume spherical and filamentous forms; mitochondrion (M).* ×61,000.

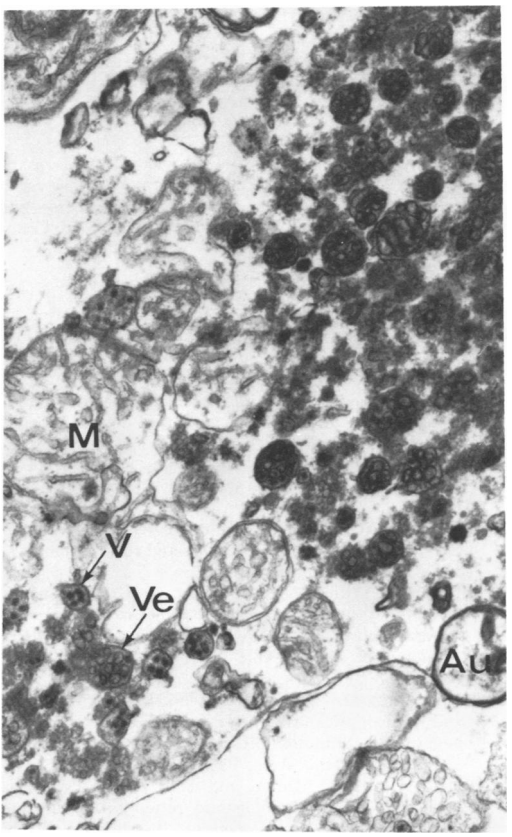
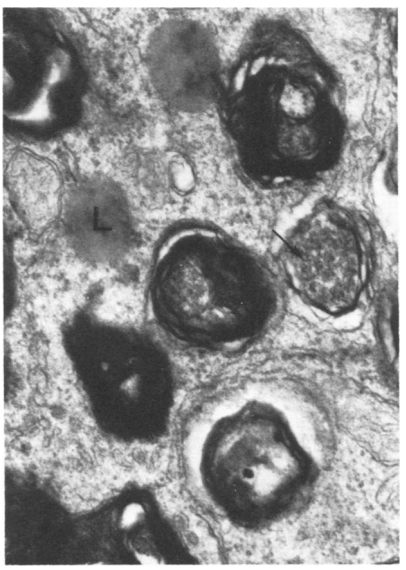


FIG. 15. *Cytoplasmic portion of a necrotic neurone 120 h after inoculation. The mitochondria (M) are swollen. The mitochondrial membrane shows degenerative change. An autophagic vacuole (Au) is noted. The hyaloplasm is relatively clear. Viral (V) and cytopathic vesicular enclosures (Ve) are seen among the organelle debris. At the upper left corner is part of a glial cell cytoplasm.* ×25,200.

FIG. 14. *Portion of a microglia 120 h after inoculation. Many phagosomes are present in the cytoplasm. They are filled with heterogeneous dense materials, myelin bodies, or phagocytosed viral aggregates (arrow). Two lipid droplets (L) are seen in this area.* ×34,200.



(19) in an immunofluorescent work on Sindbis virus.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant R-22, AI 10509-01 from the National Institute of Allergy and Infectious Disease. The electron microscope is a gift from the Japanese government through the Overseas Technical Cooperation Agency.

LITERATURE CITED

1. Acheson, N. H., and I. Tamm. 1967. Replication of Semliki forest virus: an electron microscopic study. Virology **32**:128-143.
2. Atchison, R. W., J. V. Ordonez, G. E. Sather, and W. McD. Hammon. 1966. Fluorescent antibody, complement fixation method for detection of dengue virus in mice. J. Immunol. **96**:936-943.
3. Baruch, E. 1963. Electron microscopic study of spinal cord of mice infected with yellow fever virus. J. Ultrast. Res. **9**:209-224.
4. Bearcroft, W. G. C. 1960. Electron microscopic studies on the liver cells of yellow fever infected rhesus monkeys. J. Pathol. Bacteriol. **80**:421-426.
5. Bernhard, W., and N. Granboulan. 1968. Electron microscopy of the nucleolus in vertebrate cells, p. 81-149. *In* A. J. Dalton and F. Haguenau (ed.), Ultrastructure in biological system, vol. 3, The nucleus. Academic Press Inc., New York.
6. Bhamarapravati, N., S. B. Halstead, P. Sookavachana, and V. Boonpucknavig. 1964. Studies on dengue virus infection. I. Immunofluorescent localization of virus in mouse brain tissue. Arch. Pathol. **77**:538-543.
7. Blinzinger, K., W. Müller, and A. P. Anzil. 1971. Microhelices in the endoplasmic reticulum of murine neurones infected with a group B arbovirus. Arch. Gesamte Virusforsch. **35**:194-202.
8. Brandt, W. E., R. D. Cardiff, and P. K. Russell. 1970. Dengue virions and antigens in brain and serum of infected mice. J. Virol. **6**:500-506.
9. Cardiff, R. D., S. B. Russ, W. E. Brandt, and P. K. Russell. 1973. Cytological localization of dengue-2 antigens: an immunological study with ultrastructural correlation. Infect. Immunity **7**:809-814.
10. Chain, M. M. T., F. W. Doane, and D. M. McLean. 1966. Morphological development of Chikungunya virus. Can. J. Microbiol. **12**:895-900.
11. Craighead, J. E., G. E. Sather, W. McD. Hammon, and G. J. Dammin. 1966. Pathology of dengue virus infection in mice. Arch. Pathol. **81**:232-239.
12. Erlandson, R. A., V. J. Babcock, C. M. Southman, R. B. Bailey, and F. H. Shipkey. 1967. Semliki forest virus in HEp-2 cell cultures. J. Virol. **1**:996-1009.
13. Filshie, B. K., and J. Rehacek. 1968. Studies of the morphology of Murray valley encephalitis and Japanese encephalitis viruses growing in cultured mosquito cells. Virology **34**:435-443.
14. Grimley, P. M., I. K. Berezesky, and R. M. Friedman. 1968. Cytoplasmic structures associated with an arbovirus infection: loci of viral ribonucleic acid synthesis. J. Virol. **2**:1326-1338.
15. Grimley, P. M., and R. M. Friedman. 1970. Development of Semliki forest virus in mouse brain. An electron microscopic study. Exp. Mol. Pathol. **12**:1-13.
16. Hay, E. D. 1968. Structure and function of the nucleolus in developing cells, p. 1-79. *In* A. J. Dalton and F. Haguenau (ed.), Ultrastructure in biological system, vol. 3, The nucleus. Academic Press Inc., New York.
17. Higashi, N., A. Matsumoto, K. Tabata, and Y. Nagamoto. 1967. Electron microscope study of development of Chikungunya virus in green monkey stable (VERO) cells. Virology **33**:55-69.
18. Janzen, H. G., A. J. Rhodes, and F. W. Doane. 1970. Chikungunya virus in salivary glands of Aedes aegypti (L): an electron microscope study. Can. J. Microbiol. **16**:581-586.
19. Johnson, R. T. 1965. Virus invasion of the general nervous system: a study of Sindbis virus infection in the mouse using fluorescent antibody. Amer. J. Pathol. **46**:929-946.
20. Kitaoka, M., A. Shimizu, P. Tuchinda, and C. Kim-Anake. 1971. Electron microscopic observations on dengue type 2 virus. Biken J. **14**:361-364.
21. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9**:409-414.
22. Madeley, C. R. 1972. Virus morphology. Churchill Livingstone, Edinburgh.
23. Matsumura, T., V. Stollar, and R. W. Schlesinger. 1971. Studies on the nature of dengue viruses. V. Structure and development of dengue virus in Vero cells. Virology **46**:344-355.
24. Millonig, G. 1961. Advantages of a phosphate buffer for OsO_4 solution in fixation. J. Appl. Physiol. **32**:1632.
25. Morgan, C., C. Howe, and H. M. Rose. 1961. Structural development of viruses as observed in the electron microscope. V. Western equine encephalitis virus. J. Exp. Med. **113**:219-234.
26. Murphy, F. A., A. K. Harrison, G. W. Gray, Jr., S. G. Whitfield, and F. T. Forrester. 1968. St. Louis encephalitis virus infection of mice. Electron microscopic studies of central nervous system. Lab. Invest. **19**:652-662.
27. Murphy, F. A., A. K. Harrison, and T. Tzianabos. 1968. Electron microscopic observations of mouse brain infected with Bunyamwera group arboviruses. J. Virol. **2**:1315-1325.
28. Mussgay, M., and J. Weibel. 1962. Electron microscopic and biological studies on the growth of Venezuelan equine encephalitis virus in KB cells. Virology **16**:52-62.
29. Ota, Z. 1965. Electron microscope study of the development of Japanese B encephalitis virus in porcine kidney-stable (PS) cells. Virology **25**:372-378.
30. Peters, A., S. L. Palay, and H. F. Webster. 1970. The fine structure of the nervous system. The cells, and their processes. Harper and Row, Publishers, New York.
31. Reynolds, E. 1963. The use of lead citrate at the high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208-212.
32. Shestopalova, N. M., V. N. Reingold, A. V. Gagarina, E. A. Kornilova, G. V. Popov, and M. P. Chumakov. 1972. Electron microscopic study of the central nervous system in mice infected by Omsk hemorrhagic fever (OHF) virus. J. Ultrast. Res. **40**:458-469.
33. Smith, T. J., W. E. Brandt, J. L. Swanson, J. M. McCrown, and E. L. Buescher. 1970. Physical and biological properties of dengue-2 virus and associated antigens. J. Virol. **5**:524-532.
34. Southman, C. M., F. H. Shipkey, V. I. Babcock, R. B. Bailey, and R. A. Erlandson. 1964. Virus biographies. I. Growth of West Nile and Guaroa viruses in tissue culture. J. Bacteriol. **88**:187-199.
35. Tan, K. B. 1970. Electron microscopy of cells infected with Semliki forest virus temperature-sensitive mutants: correlation of ultrastructural and physiological observations. J. Virol. **5**:632-638.
36. Trump, B. F., E. A. Smuckler, and E. P. Benditt. 1961. A method for staining epoxy sections for light microscopy. J. Ultrast. Res. **5**:343-348.
37. Watson, M. 1958. Staining of tissue culture sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. **4**:475-478.
38. Yasuzumi, G., and T. Tsubo. 1965. Analysis of the development of Japanese B encephalitis (JBE) virus II. Electron microscope studies of neurones infected with JBE virus. J. Ultrast. Res. **12**:304-316.