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FILAMENTOUS STRUCTURES IN DENGUE TYPE 3 VIRUS INFECTED MOUSE NEURONES

SIRIPORN SRIURAIRATNA[1]

Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok 4, Thailand

NATTH BHAMARAPRAVATI and SHUNZO ONISHI[2]

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

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SUMMARY Dengue type 3 (H-87) virus was inoculated into suckling mouse brain and animals were sacrificed at 24 hr intervals. Parallel filamentous structures were found in infected neurones in close association with virus particles in the distended endoplasmic cisternae. They were usually arranged in a crystalloid pattern, oriented in different directions within the cisternae. Faint helical features were sometimes observed. These filamentous structures measured 15–25 nm in width and varied in length. Their possible involvement with viral material or a viral core is postulated.

INTRODUCTION

Studies on the development of arboviruses in experimental systems have clearly demonstrated viral nucleoid particles in animal cells and cell cultures infected with group A arboviruses (Higashi et al., 1967; Grimley and Friedman, 1970; Gil-Fernandez et al., 1973). However nucleoids have not been demonstrated convincingly in any systems infected with group B arboviruses. Group B arboviruses presumably propagate by budding of nucleoprotein from the intracellular vacuolar membrane or the endoplasmic cisternal membrane into the corresponding lumen, without a nucleoid or precursor particle phase (Ota, 1965; Matsumura et al., 1971; Blinzinger, 1972; Dalton, 1972; David-West et al., 1972). However, elongated structures, which were given different names, were sometimes observed in close association with virus particles in neurones infected with group B arboviruses, suggesting that they might participate in some way in the assembly of virus particles (Blinzinger et al., 1972; David-West et al., 1972; Sriurairatna et al., 1973).

1 Formerly research fellow of the Overseas Technical Cooperation Agency of Japan for 1967–1968, at the Research Institute for Microbial Diseases, Osaka University.

2 Present address: Department of Pathology, Osaka University Medical School, Joan-cho, Kita-ku, Osaka.

This paper reports the findings and significance of a filamentous structure associated with dengue virus and its involvement in morphogenesis is suggested.

MATERIALS AND METHODS

1. *Virus*

Dengu type 3 virus H-87 strain was obtained by serial passages in suckling mouse brain in our laboratory. The seed virus was suspended in 50% fetal bovine serum in phosphate buffered saline before use.

2. *Animals*

One to two day old albino suckling mice were used throughout. A group of 30 suckling mice was used for virus inoculation. Another group of 20 normal suckling mice, was kept in the same room as a control.

3. *Inoculation of the virus*

Suckling mice were inoculated intracerebrally with 0.02 ml of seed virus and the progress of their illness was observed. Two infected mice and one normal mouse were sacrificed every 24 hr for electron microscopic study.

4. *Electron microscopy*

The mice were anesthesized with anesthetic ether. The middle portion of the brain around the inoculation site was removed and minced into pieces of less than 1 mm^3 in 6.25% glutaraldehyde in 0.1 M phosphate buffer solution, pH 7.4 at 4 C. Tissues were kept in the fixative for 1 hr, washed well with 0.1 M phosphate buffer solution and postfixed in 1% osmium tetroxide in Millonig buffer (Millonig, 1961) for 1 hr at 4 C. Then they were dehydrated by passage through a graded series of concentrations of ethanol, infiltrated with propylene oxide and embedded in Epon 812 (Luft, 1961), polymerized at 60 C for 48 hr. Semi-thin sections of 0.5 μ to 1 μ thickness were cut and stained with toluidine blue O and pyronin (Trump et al., 1961) for orientation of the tissue blocks. Cerebrocortical areas were selected for electron microscopic examination. Thin sections were cut on a Reichert OmU_2 ultramicrotome with glass knives. Sections were mounted on 300 mesh uncoated grids, stained with 2% uranyl acetate solution (Watson, 1965) and lead citrate solution (Reynolds, 1963), and examined in a Hitachi HS-8 electron microscope at 50 KV.

RESULTS

1. *Illness and death of the animals*

Mice developed paralytic signs on the 9th day after inoculation. Eight mice died between the 9th and 11th day after inoculation. The other two mice were sacrificed in a moribund condition on the 11th day. Control mice did not show any abnormality and grew well during the experiment.

2. *Light microscopic findings*

1) Control brains

Numerous neurones and glial cells were observed. A few dendritic processes or axons were generally seen extending from the thin cytoplasm that surrounded the large nucleus.

2) Infected brains

No specific change was observed in the experimental tissues compared with control brains, but focal neuronal necrosis were noted in tissues from mice showing definite paralysis on the 10th and 11th days and phagocytic glial cells were markedly increased in number and enlarged in size.

3. *Electron microscopic findings*

1) Control brains

There were many neuronal cytoplasmic organelles in the areas forming into neuronal processes and moderate numbers of polysomes and free ribosomes were observed. Short arrays of rough endoplasmic reticulum were seen arranged in groups and a few Golgi complexes and lysosomes were observed. Mitochondria were scattered and neurofilaments were often seen.

2) Infected brains

a) Days 1–4 after inoculation: No significant change was observed in this period. The fine structures of neurones and glial cells were similar to those of untreated mice.

b) Day 5 after inoculation: Nucleolar structural changes were noted in some neurones. The pars fibrosa and pars granulosa

were separated into two distinct parts (Fig. 1). The nuclei were slightly indented. Dispersion of the rough endoplasmic reticulum was observed in a few neurones. Frequently, one or more clusters of fibrogranular structures of various sizes were seen in the cytoplasmic matrix without a limiting membrane. At this stage other cellular structures were similar to those in control mice.

c) Days 6–9 after inoculation: From day 6 onwards, focal areas of infection were noted. The number of infected neurones and cytopathic changes increased with time after inoculation. In general, several infected neurones showing cytopathic changes were seen in one area whereas other neurones in the neighborhood were quite well preserved. Nucleolar segregation (Bernhard and Granboulan, 1968; Busch and Smetana, 1970) was persistent. Cytoplasmic changes were clear. There was a decrease in the number of polysomes and membrane associated ribosomes. Free ribosomes or ribosome-like particles were greatly increased in number and disintegrated particles were seen throughout the cytoplasm of neurones and some glial cells. Golgi complexes were often degenerating. The rough endoplasmic reticulum was mostly arranged in the perinuclear region, and cisternae were distended in some places (Fig. 2). A few round particles about the size of ribosomes were attached to the exterior of the

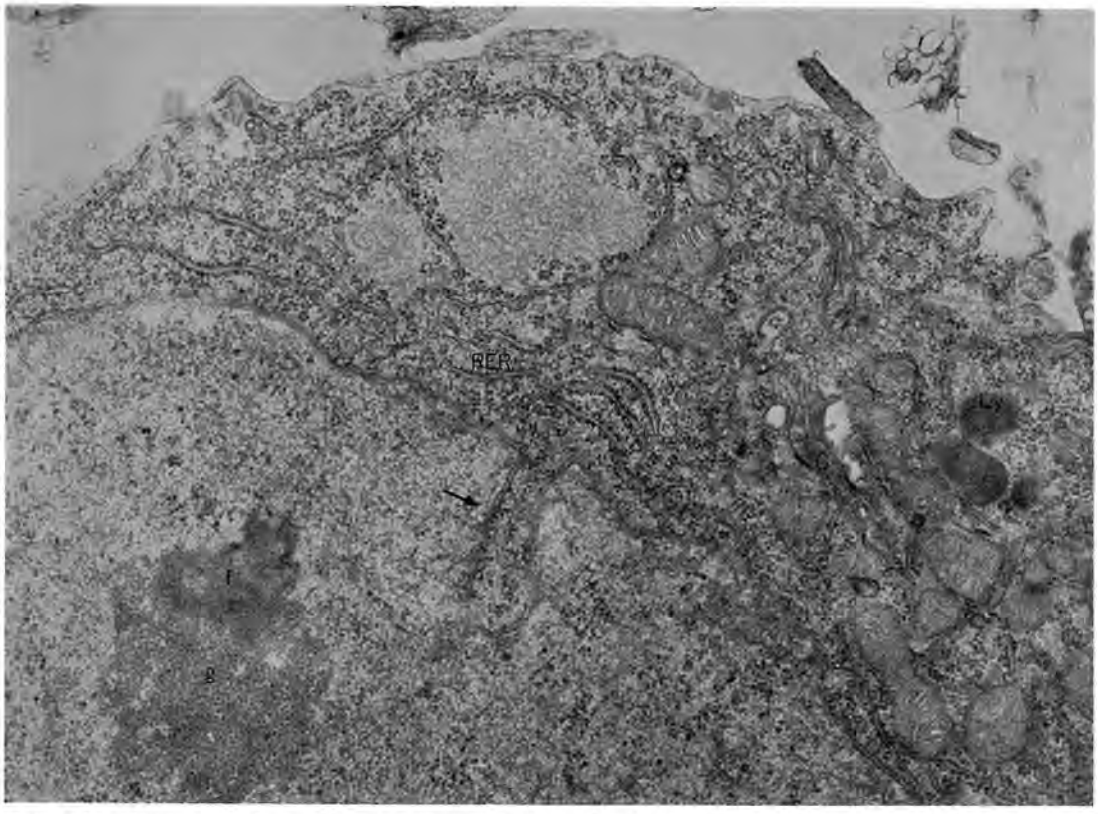

FIGURE 1. *A neurone 6 days after inoculation of dengue-3 virus. The nucleus is indented (arrow); the nucleolus is segregated into two distinct components, the pars fibrosa (f) and pars granulosa (g). Two clusters of fibro-granular structures are seen in the crytoplasmic matrix. Other organelles are similar to those in normal neurones. RER, rough endoplasmic reticulum; G, Golgi complex; Ly, lysosome.* ×30,000.

cisternal membrane, but they could not be excluded from the ribosomes or other particles scattered in the hyaloplasm. Four different structures were generally observed within distended cisternae of the endoplasmic reticulum. These were:

(1) *Cytopathic vesicles* Round to oval, smooth single membrane vesicles, varying from 80 to 250 nm in diameter were seen in the distended parts of the endoplasmic cisternae (Fig. 2). Frequently 2–5 vesicles were seen together in a cisterna, and they often overlapped each other. Sometimes they had short or long cylindrical forms. Some vesicles contained material of low density while others were clear.

(2) *Dengue virions* Round, electron dense particles with an average size of 50 nm were present in the cisternae with or without cytopathic vesicles (Fig. 2). These particles

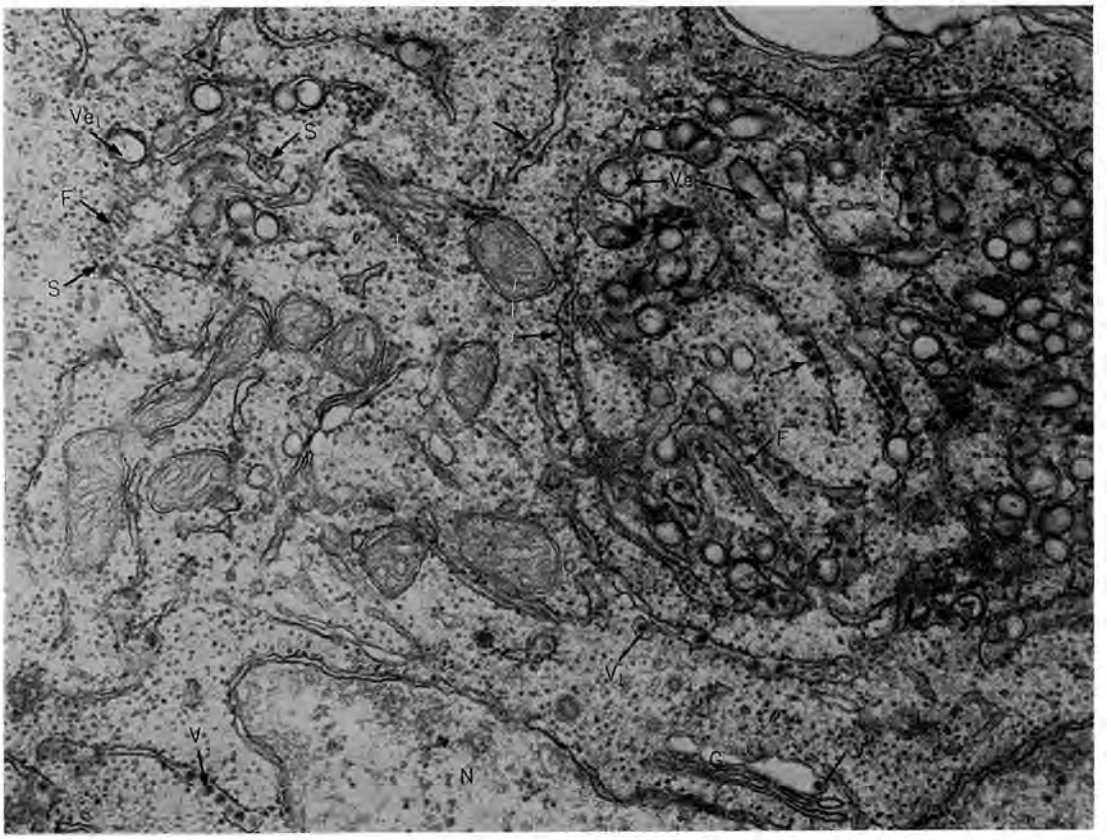

FIGURE 2. *A neurone 6 days after inoculation. The endoplasmic cisternae are aggregated in the perinuclear zone. Free ribosomes or ribosome-like particles have disintegrated throughout the cytoplasm. Sparse round particles, similar to ribosomes are observed on the cisternal membrane. Round to oval, and cylindrical vesicles (Ve) are seen within the cisternae of the endoplasmic reticulum. Spherical virus particles are observed along the axis of some cisternae (V). One virus particle is located singly in a small vesicle (V_1) and another (V_2) is seen in a dilated cisterna of a Golgi complex. Short and long filamentous structures (F) are observed in the cisternae in the area where vesicles and virus particles are seen, and they are especially numerous in the area in the upper left. Small round particles (S) are also seen in neighboring cisternae. The filamentous structures are seen with small round particles in some cisternae, or with the vesicles (Ve_1) and virus particles (arrow). G, Golgi complex; N, nucleus.* ×*36,000.*

were arranged along the cisternal axis, sometimes in aggregates of 3-10 particles or more (Fig. 3), and sometimes singly in a small single membrane vesicle or a Golgi vesicle. They were morphologically identical to the dengue virus particles reported by others (Oyama et al., 1967; Brandt et al., 1970; Nii et al., 1970; Smith et al., 1970; Kitaoka et al., 1971; Matsumura et al., 1971; Cardiff et al., 1973; Sriurairatna et al., 1973) and dengue type 1 virus particles in mouse neurones (the authors' unpublished observation). The virus particle had a central dense area about 40 nm in diameter with a less dense zone of 3–4 nm, and a thin envelope. Many virus particles had no envelope. No specific pattern of distribution of the virions with and without envelopes was observed in the sections examined.

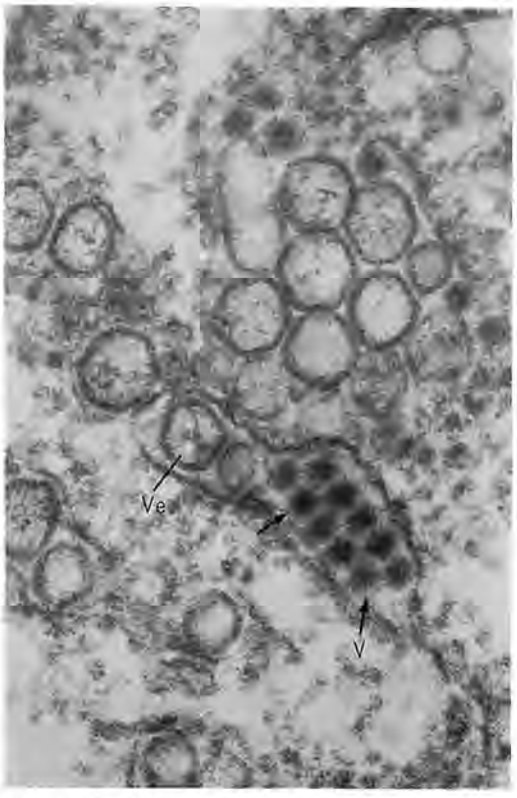

FIGURE 3. *Dengue-3 virus particles (arrow) in a distended cisterna with two cytopathic vesicles (Ve). An envelope is visible on a virus particle (V).* ×*110,000.*

(3) *Aberrant particles* Dense particles with projections from the sides, forming triangle-like structures were seen irregularly arranged in distended endoplasmic cisternae in a few neurones. Sometimes they were seen together with spherical virions in a single cisterna (Fig. 4). These aberrant particles measured approximately 50 nm along the short axis, and 75–90 nm on the long axis.

(4) *Filamentous structures* Moderately electron dense filamentous structures were seen in the endoplasmic cisternae, especially in neurones showing mild cytopathic changes or producing a few virus particles. They usually ran in parallel along the cisternae but sometimes they were arranged laterally or tangentially. These filamentous structures measured 15–25 nm in width and varied in length. Small and round particles were observed in other distended cisternae in areas where filamentous structures were seen or with the filamentous structures in cisternae. These particles also measured 15–25 nm in diameter. The filaments, and the small, round particles were sometimes both seen with the cytopathic vesicles, or spherical virions and aberrant particles in a single distended cisterna (Fig. 2, 4). At higher magnification, the small round particles were seen to have a dense core and the filament showed a central dense line with two outer less dense bands (Fig. 5). Sometimes, faint helical features were observed (Fig. 6).

In addition to the striking changes described above, a few tortuous tubular bodies were seen in the cytoplasm of neurones containing virus and aberrant particles; between the cisternae filled with cytopathic vesicles, the virus and the aberrant particles (Fig. 4). Several single vesicles containing individual virions were sometimes seen located close to the periphery of a tubular body, but no intimate relation between the two was observed.

d) Days 10–11 after inoculation: Great changes were observed. Almost every

neurone and glial cell was affected. The changes varied in extent. In neurones producing virus, virus and aberrant particles were frequently seen in large vesicles with smooth membranes. The aberrant particles were much more numerous than 6–9 days after inoculation and were seen in a ratio of about 2: 1 to spherical virions. In many instances, neurones heavily loaded with virus were seen close to a capillary or a large blood vessel, but no evidence of virus production or virus particles was found in the endothelial cells or capillary lumen. Necrotised neurones were seen in scattered areas. The cytoplasmic organelles showed severe degenerative changes. The mitochondria were swollen. The plasma membrane was often disrupted. Many virus particles, aberrant particles and cytopathic vesicles were bound by large or small single membrane among the debris of organelles. Phagocytic glial cells were numerous and were very active. These cells often surrounded a necrotic neurone with long, thin cytoplasmic extensions, so that the necrotic neurone appeared like an inclusion in the cytoplasm of the glial cell. Areas of dense heterogeneous material were frequently seen in the cytoplasm of large or hypertrophic phagocytic glial cells.

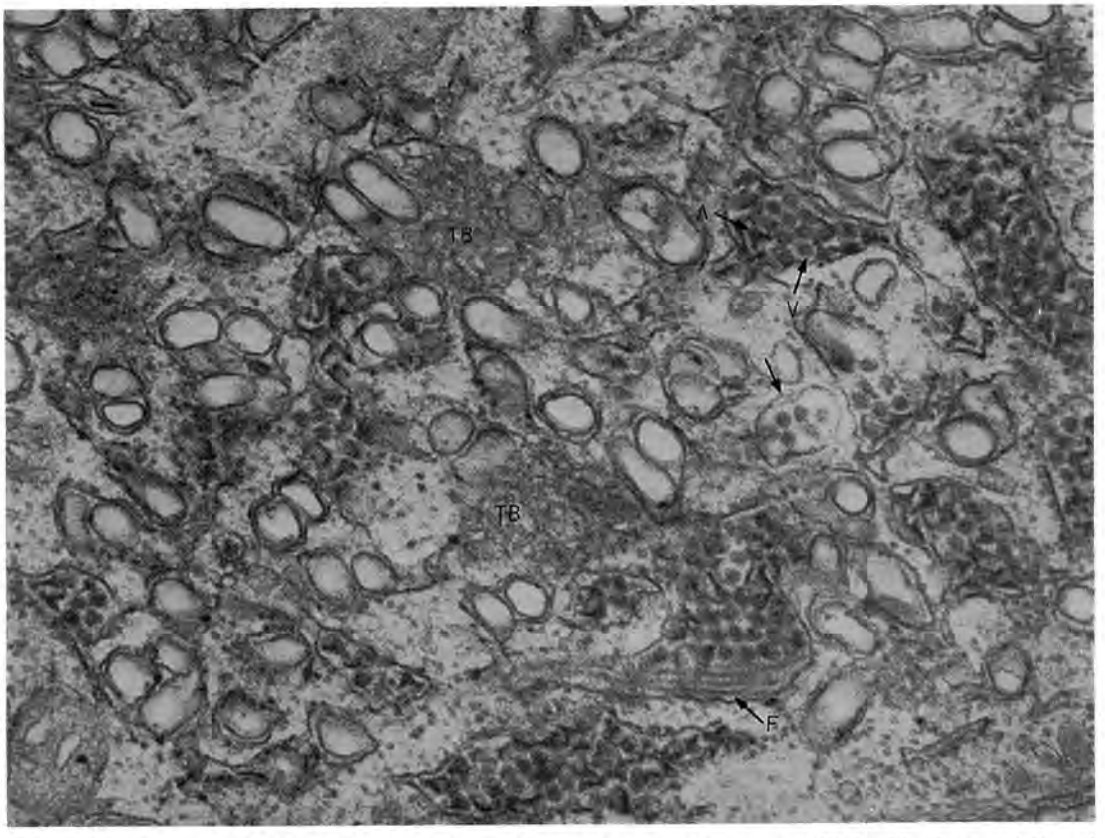

FIGURE 4. *A neurone 11 days after inoculation. The endoplasmic cisternae are filled with aberrant (A) and spherical virus (V) particles. One vesicle with a smooth membrane contains 5–6 enveloped virions (arrow). Four parallel filaments (F) are seen in a distended cisterna with virus and abserrant particles. Two tortuous tubular bodies (TB) are seen in this field among the cytopathic vesicles and cisternae containing virus.* × *48,000.*

Virus particles, aberrant particles and other dense amorphous elements were also seen in them.

DISCUSSION

The incubation period, onset of illness and scattered foci of neuronolysis observed in the present work are in good agreement with results of light microscopic studies on dengue type 3 viral infection of mouse brain by Craighead et al. (1966). These workers reported that the incubation period was prolonged when compared with infections by dengue types 1, 2, and 4 viruses.

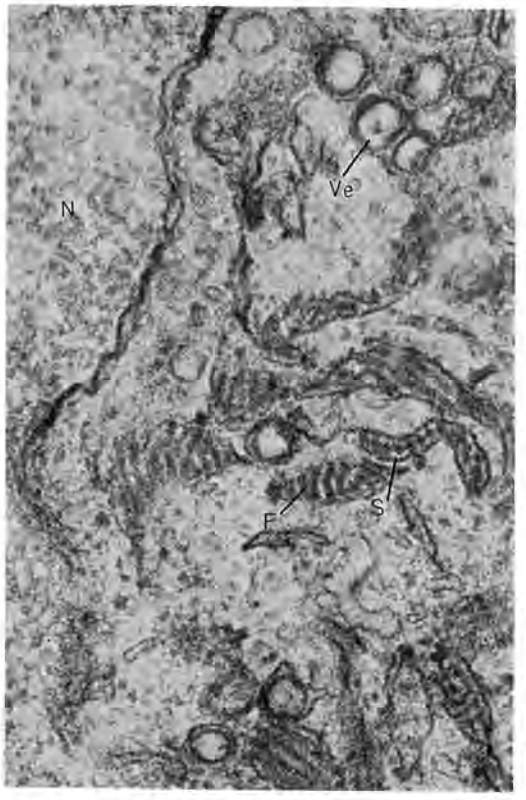

FIGURE 5. *Filamentous structures in the perinuclear area of a neurone. The filamentous structures (F) consist of a dense central line with two outer less dense bands. The small round particles (S) have a dense core. Cytopathic vesicles are bounded by smooth membranes of the endoplasmic cisternae making them appear like double membrane vesicles (Ve). N, nucleus.* ×50,000.

Our observations on the development of virus particles and neurocytoplasmic responses, including aberrant particles, filamentous structures and cytopathic vesicles are quite parallel to events observed in neurones infected with dengue type 2 virus (Sriurairatna et al., 1973) except the tortuous tubular bodies, which were not seen previously. Structures similar to these tubular bodies have been reported in a variety of cells infected with arboviruses, as well as in diseased cells without virus and normal cells (Murphy et al., 1968; Filshie and Rehacek, 1968; Nii et al., 1970; Tandler, 1973; Chandra, 1968; Uzman et al., 1971). In our findings, the tortuous tubular bodies seemed to represent a cellular reaction of the host cell to infection, rather than to be structures involved in the synthesis of viral materials.

The filamentous structures and the small round particles are very interesting. Their presence in the cisternae in our series of micrographs indicates that they are sections of the same element at different planes. These filaments were also observed in mouse neurones infected with dengue type 2 (Sriurairatna et al., 1973). No evidence for budding of naked viral nucleoids into the cisternal lumen was found in any of our sections or in the work of others on neurones and cell cultures infected with several group B arboviruses (Blinzinger, 1972; David-West et al., 1972; Tandler et al., 1973). Structures similar to the filamentous structures were seen in the vicinity of newly formed virus particles in mouse neurones infected with Zimmern encephalitis virus and named "microhelices" (Blinzinger et al., 1971), and "elongated tubular structures" were found in close association with virus particles in neurones infected with yellow fever virus (David-West et al., 1972). These structures appear to us to represent viral precursor elements, although the transitional stage from the filamentous phase to spherical virions has not yet been observed. The

concomitant existence of the filaments with the virions and aberrant particles in distended cisternae suggests that these elements are in the process of assembly, with some completed virions, some aberrant and some incompleted forms. Further characterization of each component is needed to clarify its nature and function.

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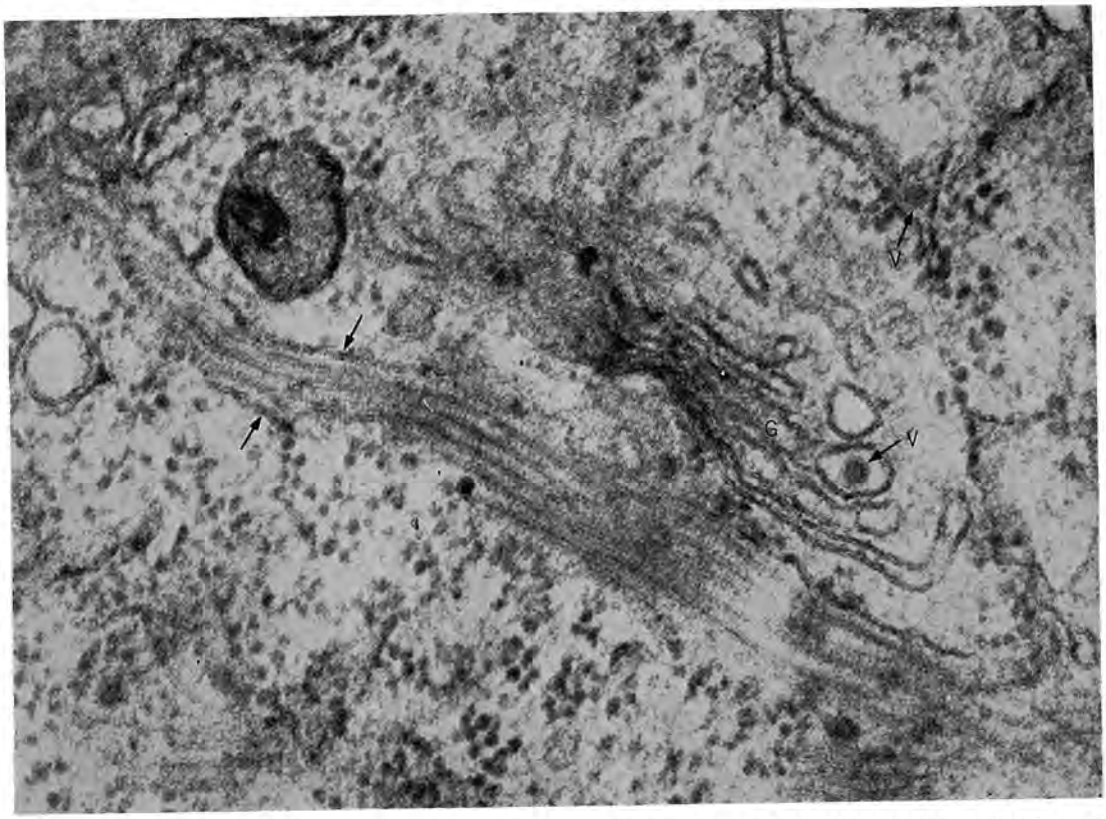

FIGURE 6. *Micrograph of filamentous structures at high magnification showing faint helical features. The cisternal membrane is visible in parts (arrows). An enveloped virus particle is seen in a Golgi vesicle and another in an endoplasmic cisterna (V). G, Golgi complex.* ×*85,000.*

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