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Abstract

An electron microscope study was performed on the ultrastructure and developmental proce of the Mukai strain of Japanese B encephalitis virus propagated in vitro on porcine kidney stable cells. The virus particle of Japanese B encephalitis is hexagonal in sections and approximately 40 mμ in the maximum diameter, composed of an outer membrane, 20Å thick, viroplasm, 30 Å thick and an electron-dense nucleoid, 25 mμ in diameter. The virus particles develop by a budding proce on the wall of the cytoplasmic vacuole. Thereafter, virus particles are densely packed in the vacuole usually in random arrangement and rarely in crystalline arrays. The vacuole containing virus particles gradually moves toward the cell surface and liberates the virus particles to the exterior of the cells through a narrow canaliculus. A structure suggestive of incomplete virus particles was also observed.

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ELECTRON MICROSCOPE STUDY ON THE DEVELOPMENT OF JAPANESE B ENCEPHALITIS VIRUS IN PS CELLS

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Since the work of HAYASHI[1] in 1935, it has been well established that Japanese B encephalitis (JBE) is caused by a virus, but its morphological characteristics and developmental processes in host cells have been unknown.

The size of JBE virus particles was reported to be 10 to 30 mμ in diameter, by the gradocol membrane method (YAOI *et al.*, 1939)[2]. A few electron microscope studies of JBE virus particles have been undertaken. Recently, electron microscopic observations on purified JBE virus particles by means of shadow-casting has revealed that the virus particles are spherical in shape, having an average diameter of 40 to 50 mμ (FUKAI, 1962[3]; NOJIMA, 1962[4]; KITAOKA et al., 1963[5]). Our electron microscope study of semipurified JBE virus particles has demonstrated that the shape of the virus particles is hexagonal (OTA *et al.*, 1963[6]), and in thin sections, the size of the particle was 30 to 40 mμ in diameter (OTA *et al.*, 1963[6]; HIGASHI, 1963[7]).

It is the purpose of this communication to present electron microscopic observations on the ultrastructure of JBE virus particles and their mode of development in porcine kidney stable (PS) cells. The development of virus particles in PS cells was also analysed in a one-step growth experiment.

MATERIALS AND METHODS

Both the JBE virus (the Mukai strain) and PS cells used were kindly supplied by Dr. Kanda Inoue at the Virus Institute of Kyoto University where they used these virus and cells for biological investigation.

Tissue culture method was essentially the same as that used by KANDA INOUE, and his co-workers (KANDA INOUE and OGURA, 1962[8], KATO and KANDA INOUE 1962[9]). Monolayers of PS cells were grown in bottles containing 10% calf serum and 0.5% lactalbumin hydrolyzate in Earle's balanced solution. For virus propagation, PS cells from 3-to 5-day old cultures (approximately 15×10^4 cells per ml) received virus inoculum of about 10^6 $TCID_{50}$ that was prepared by centrifuging culture fluid after nearly all of the cells had shown cytopathic effect.

Incubation temperature was 36°C.

The titer of cell-associated virus was estimated 5 days after infection by cytopathic effect. The result of a typical growth experiment is shown in Fig. 1a.

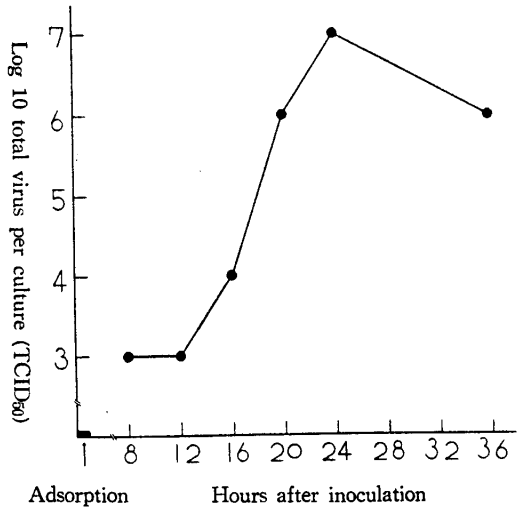

Fig. 1 a. Growth curve of cell-associated JBE virus in PS cells.

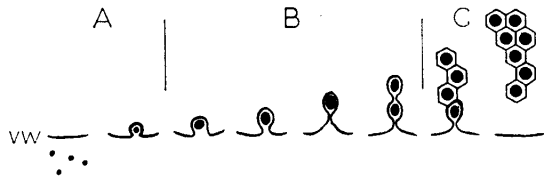

Fig. 1 b. Mode of the development of Japanese B encephalitis virus. Time elapses from left to right. Part A is not confirmed. Part C occurs in adequate conditions. VW: vacuolar wall

At 8 to 24 hours after infection, bottle-cultured PS cells were tripsinized and centrifuged. The resultant pellets were fixed for 40 minutes at 4°C in buffered 1% osmium tetroxide solution, and after dehydration, embedded in methacrylates. The sections were stained in saturated uranyl acetate solution. Observations were carried out in the Hitachi type HU 11 electron microscope.

RESULTS

1. Uninfected PS cells

PS cells from 3-to 5-day old cultures were used for virus propagation. During this period, uninfected PS cells are actively growing without displaying dege-

nerative changes in their ultrastructure. The nucleus is round and the nucleoli are prominent. In the cytoplasm, mitochondria, endoplasmic reticulum and vacuoles are scattered. There are a few cytoplasmic protrusions on the cell surface.

2. Infected PS cells and JBE virus particles

1) Liberation of JBE virus particles from PS cells

JBE virus particles could be observed 18 to 24 hours following infection.

JBE virus particles are most frequently observed as large aggregates in the cytoplasmic vacuoles of PS cells.

Figure 2 shows the earliest stage of development of JBE virus particles. One to three virus particles are seen in small cytoplasmic vacuoles located near the central region of the PS cells. Some of the virus particles in the process of budding from the wall of the cytoplasmic vacuoles are seen.

Figures 3 and 4 display the stage following Fig. 2. The cytoplasmic vacuoles are densely packed with numerous virus particles, each of which has the unequivocal characteristics of JBE virus. At this stage, virus particles can be observed arranged in a crystalline array as seen in Fig. 5. In crystals, the average spacing of virus measures 40 mμ. Crystallization of virus particles is, however, a rare phenomenon in this strain of JBE virus. Subsequently, the vacuoles packed with virus particles are enlarged, partially by the fusion of vacuoles and move in the cytoplasm towards the cell surface. Figures 6 and 7 represent this stage. Finally, the vacuole and the cell surface are connected by a narrow canaliculus and virus particles are liberated outside the cell, as illustrated in Fig. 8.

On liberation, the virus particles do not undergo any morphological changes, since the size and shape of virus particles within the vacuole and of extracellular particles appear to be the same. This indicates that the virus particles observed within the vacuoles are complete mature virus particles.

2) Morphology of JBE virus particles

The shape of the majority of JBE virus particles is found to be oval. Some of the virus particles which have sufficiently retained their original shape are hexagonal, as seen in Figs. 9 and 10. The maximum diameter measures 40 mμ and the minimum 32 mμ in average. The particles are composed of an outer membrane, viroplasm and an electron-dense nucleoid. The outer membrane and viroplasm surrounding the nucleoid, measure 20 Å and 30 Å, respectively, in thickness and the nucleoid 25 mμ in the maximum diameter. Some of virus particles seen in Fig. 9 suggest a double-layer structure of the outer membrane and the presence of an inner membrane around the nucleoid. Three virus

particles shown in Fig. 11 are connected to one another by a narrow canaliculus which is formed by continuation of the outer membrane and viroplasm of virus particles. This finding is closely related to the formation of virus particles.

3) Formation of JBE virus

Figure 12 is an enlarged micrograph of a part of Fig. 2. The virus particles are budding from the vacuolar wall. The outer membrane of virus particles is continuous with the vacuolar wall. Figures 13, 14 and 15 illustrate the stages following Fig. 12. The virus particles are connected with the vacuolar wall by a pedicle and in Fig. 14, two elongated particles are joined together. These micrographs are believed to show clearly that the development of JBE virus particles takes place on the vacuolar wall.

4) Other Findings

a. Intracytoplasmic fine granules

Numerous fine, electron-dense granules are present near the vacuoles containing virus particles as seen in Fig. 16. They are 180 Å in diameter. They are frequently arranged in an orderly array. In the central part of Fig. 12, small protrusion is noticed on the vacuolar wall. In the center of the protrusion, a small electron-dense granule, 180 Å in diameter is visible, and around the vacuole a few fine granules are scattered. Although these findings may lead us to presume that the fine granules are precursors of the virus particles, such a protrusion is seldom encountered.

b. Filament

A large amount of filaments is frequently seen together with fine granules in the cytoplasm of PS cells having virus-containing vacuoles, as illustrated in Fig. 16. It is supposed that the filaments are closely related with virus development.

c. Empty particles composed of double membrane

In Figs. 6, and 17, empty particles composed of double membrane are seen within the vacuole. In Fig. 6, this structure is connected with the vacuolar wall by a pedicle. In Fig. 17, it is hexagonal in shape and a similar structure is arising beneath a budding virus particle. Accordingly, this structure is similar in shape and developmental process to the virus particle. It differs in size, however, and lacks the nucleoid observed in JBE virus particles.

It is probable that this structure represents an incomplete virus particle.

d. Cytological changes

Enlarged mitochondria, onion skin-like stucture and honeycomb structure (Fig. 18) are seen in the cytoplasm and aggregates of small, electron-dense granules in the nucleus (Fig. 19).

DISCUSSION

The size of JBE virus is found to be approximately 40 mμ in diameter. This value agrees with our previous study (OTA *et al.*, 1963[6]) on JBE virus particles and several other observations[3,5] previously described and is similar to the size of Western equine encephalitis virus (45—48 mμ, MORGAN *et al.* 196I[10]) and Venezuelan equine encephalitis virus (40—48 mμ, MUSSGAY and WEIBEL, 1962[11]) of the arthropod-borne encephalitis group.

Intracellular crystallization of virus particles has been observed in animal viruses as well as in plant viruses. Notable examples are adenovirus, KJELLÉN *et al.*, 1955[12], MORGAN *et al.*, 1956[13]), herpes simplex virus (MORGAN *et al.*, 1959[14]), poliomyelitis virus (STUART and FOGH, 1959[15]) etc. In the Mukai strain of JBE virus, crystallization is rather a rare phenomenon. The average spacing of JBE virus particles in the crystal is approximately 40 mμ which is nearly equal to the average diameter of the virus particle. Therefore, the virus particles forming crystals are supposed to be mature virus particles.

In JBE virus, numerous virus particles are packed in the cytoplasmic vacuoles of PS cells after budding from the vacuolar wall. This type of formation and localization of virus particles has also been observed in Western equine encephalitis virus (MORGAN et al. 1961)[10] and Venezuelan equine encephalitis virus (MUSSGAY and WEIBEL 1962)[11]. In Western equine encephalitis, intracytoplasmic granules mature into virus particles at the wall of cytoplasmic vacuoles. In the present study on JBE virus, such a phenomenon has not been sufficiently demonstrated except for some suggestive findings. It is certain, however, that the synthesis of viral components or precursors of mature virus particles takes place possibly in the nucleus, since by fluorescent antibody method, specific fluorescence has been observed in the cytoplasm and in the nucleus, as previously reported (OTA *et al.*, 1963)[6]. Intranuclear aggregates of fine, electron-dense granules observed in the present investigation might represent the precursors of JBE virus particles. Recently, MORGAN *et al.* (1962)[16] have shown with influenza virus by the ferritin-conjugated antibody method that similar intranuclear granules contain viral antigen.

Fig. 1 b. is the schematic illustration of the developmental process of JBE virus particles observed in the present study. First, a virus particle buds on the vacuolar surface and then, other virus particles arise after it at the same site of the vacuolar surface, forming a rosary-shaped arrangement of virus particles. If the conditions are adequate, virus particles seem to arrange in a small unit of viral crystal immediately after separation.

In influenza virus, incomplete virus particles have been well investigated. The presence of incomplete virus particles in JBE is not so far demonstrated.

As stated above, empty particles which are found in JBE virus-containing vacuoles and are composed of double membrane indicate the possibility of the presence of incomplete virus particles in JBE virus. The reasons are as follows. In influenza virus, incomplete virus particles are larger in an average diameter than complete virus particles (WERNER and SCHLESINGER, 1954)[17] and contain less amount of RNA (ADA and PERRY, 1955)[18]. These observations have been confirmed by MORGAN (1962)[16]. In tobacco mosaic virus, the length of virus particles is determined by RNA (CRICK and WATSON, 1956)[19]. The structure seen in the present study is at least believed to be composed of viral protein.

SUMMARY

An electron microscope study was performed on the ultrastructure and developmental process of the Mukai strain of Japanese B encephalitis virus propagated *in vitro* on porcine kidney stable cells.

The virus particle of Japanese B encephalitis is hexagonal in sections and approximately 40 mμ in the maximum diameter, composed of an outer membrane, 20 Å thick, viroplasm, 30 Å thick and an electron-dense nucleoid, 25 mμ in diameter. The virus particles develop by a budding process on the wall of the cytoplasmic vacuole. Thereafter, virus particles are densely packed in the vacuole usually in random arrangement and rarely in crystalline arrays. The vacuole containing virus particles gradually moves toward the cell surface and liberates the virus particles to the exterior of the cells through a narrow canaliculus.

A structure suggestive of incomplete virus particles was also observed.

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LEGEND FOR ILLUSTRATIONS

Fig. 2 JBE virus particles, developing at multiple foci (arrows), are contained in small cytoplasmic vacuoles. The earliest stage of viral development. 21.5 hours after infection. Magnification: ×50,000

Fig. 3 Numerous virus particles (Vp) packing a cytoplasmic vacuole. 21.5 hours after infection. Magnification: ×53,000

Fig. 4 Virus particles in a cytoplasmic vacuole. 21.5 hours after infection. Magnification: ×140,000

Fig. 5 Crystalline arrays of virus particles (C) in cytoplasmic vacuoles. 21.5 hours after infection. Magnification: ×53,000

Fig. 6 Scattered virus particles (Vp) and empty particles (Ep) in enlarged vacuoles. Arrow points to a pedicle of the empty particle. 21.5 hours after infection. Magnification: ×50, 000

Fig. 7 Virus-containing vacuole located near the cell surface. Arrows indicating extracellularly liberated virus particles. 24 hours after infection. Magnification: ×50, 000

Fig. 8 Virus particles being liberated outside a PS cell. Arrow points to a canaliculus connecting the vacuole and cell surface. 21.5 hours after infection. Magnification: ×50, 000

Fig. 9 The outer membrane, viroplasm and nucleoid of the virus particle can be observed clearly. Arrows indicating virus particles hexagonal in shape. Portion of Fig. 6 at higher magnification. 21.5 hours after infection. Magnification: ×170, 000

Fig. 10 Virus particles hexagonal in shape. 21.5 hours after infection. Magnification: ×170, 000

Fig. 11 Arrows point to narrow canaliculi connectiug three particles. 21.5 hours after infection. Magnification: ×170, 000

Fig. 12 Virus particles (Vp) budding at the vacuolar wall. Note the outer membrane of the virus particle is continuous with the vacuolar wall. Small protrusion (Sp) and fine granules (Fg) also seen. Portion of Fig. 2 at higher magnification. 21.5 hours after infection. Magnification: ×170, 000

Fig. 13 Subsequent step of viral development (arrow). 21.5 hours after infection. Magnification: ×170, 000

Fig. 14 Two elongated virus particles joining together. Arrows indicating narrow canaliculi connecting the particles and vacuolar wall. 19 hours after infection. Magnification: ×130.000

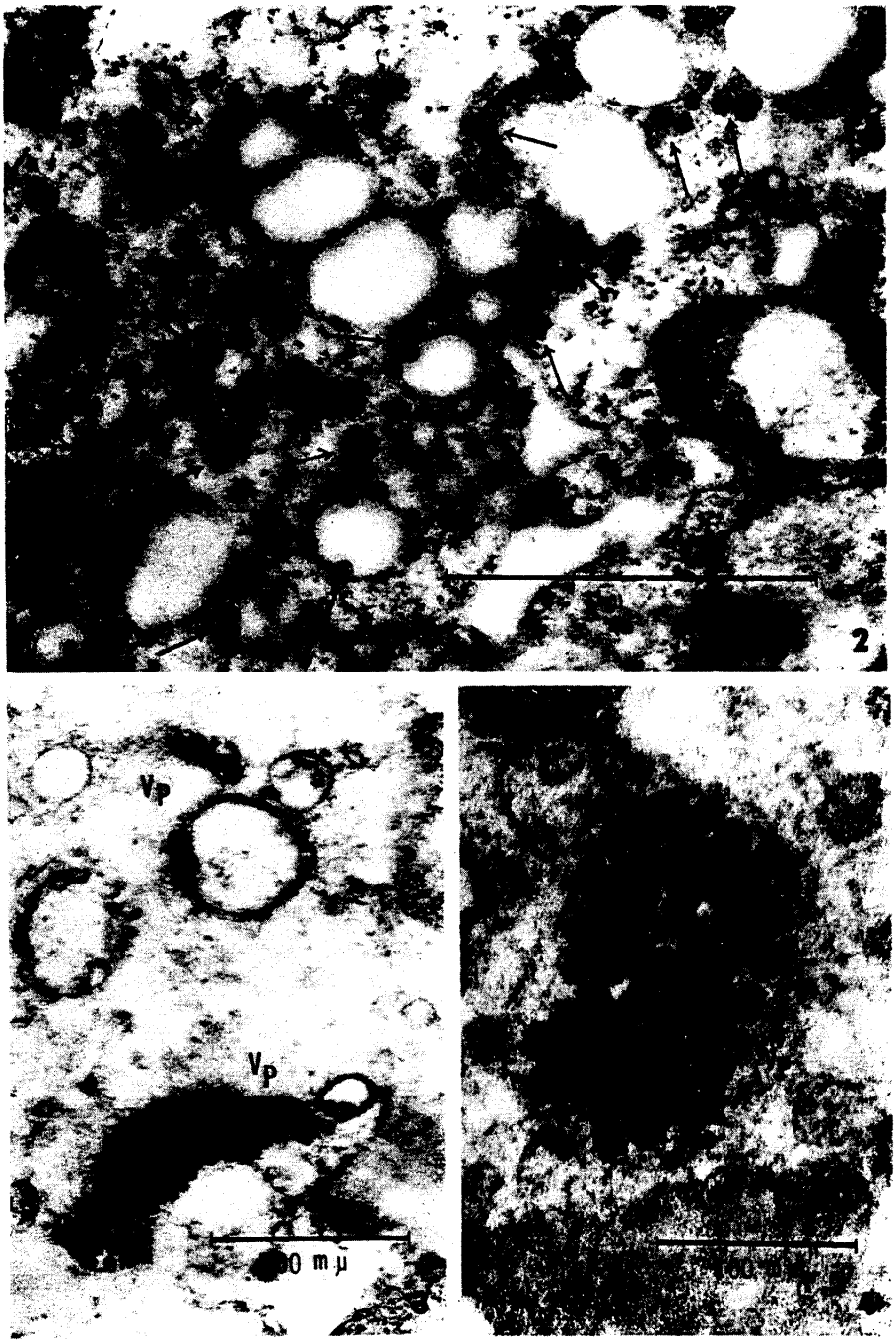
Fig. 15 Arrows point to canaliculi connecting a virus particle and cell surface. 19 hours after infection. Magnification: ×130, 000

Fig. 16 Numerous fine granules (Fg) and filament (F) seen in an infected PS cell. Some of granules arranged in parallel rows (arrows). 19 hours after infection. Magnification: ×50, 000

Fig. 17 Empty particle (Ep) hexagonal in shape. Similar structure arising beneath a virus particle (arrow). 21.5 hours after infection. Magnification: ×100, 000

Fig. 18 Honeycomb structure (H) observed in the cytoplasm near the nucleus. 20 hours after infection. Magnification: ×50, 000

Fig. 19 Intranuclear aggregate of fine, electron-dense granules (G). N: Nucleus. Magnification: ×50, 000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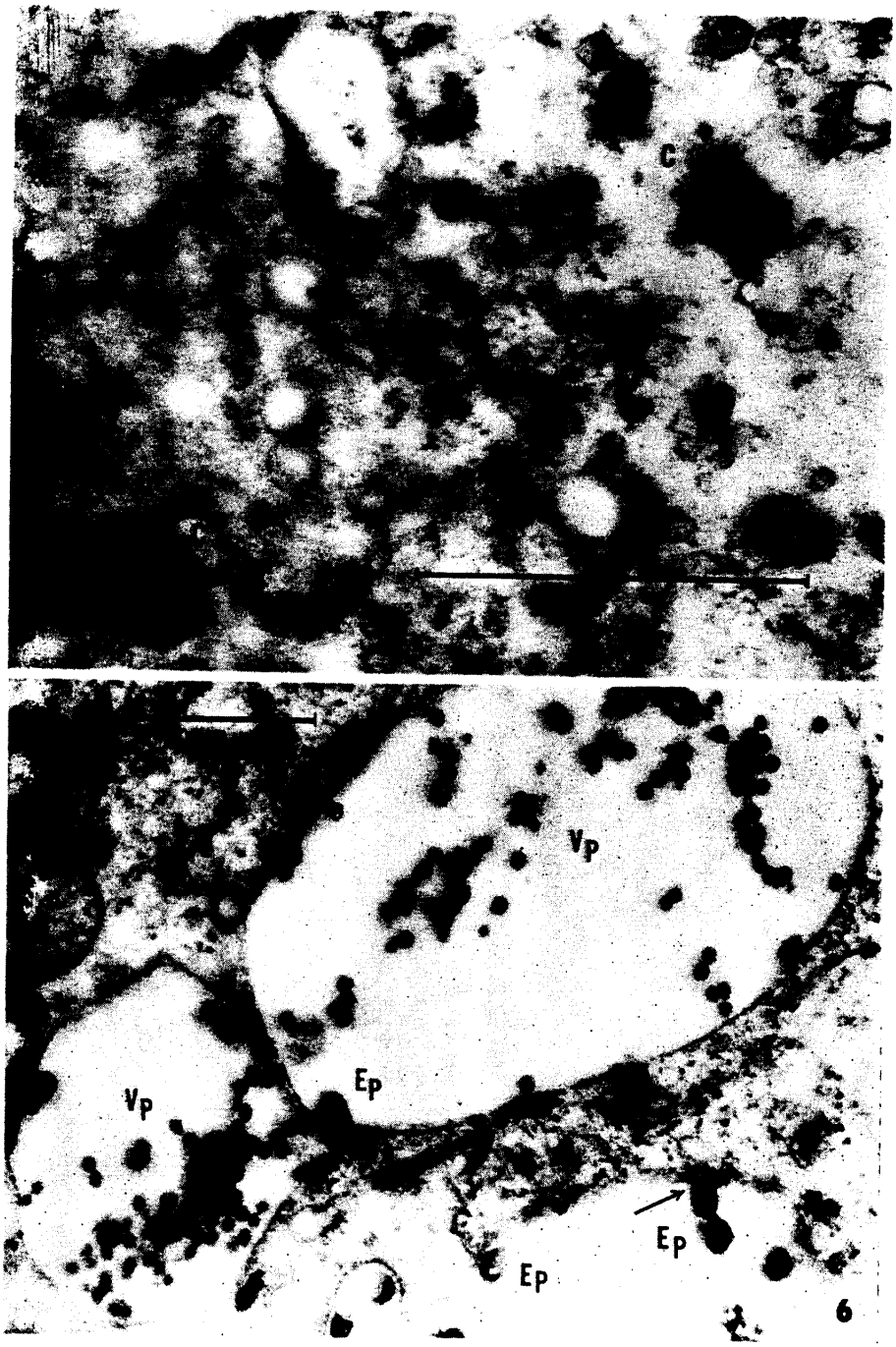
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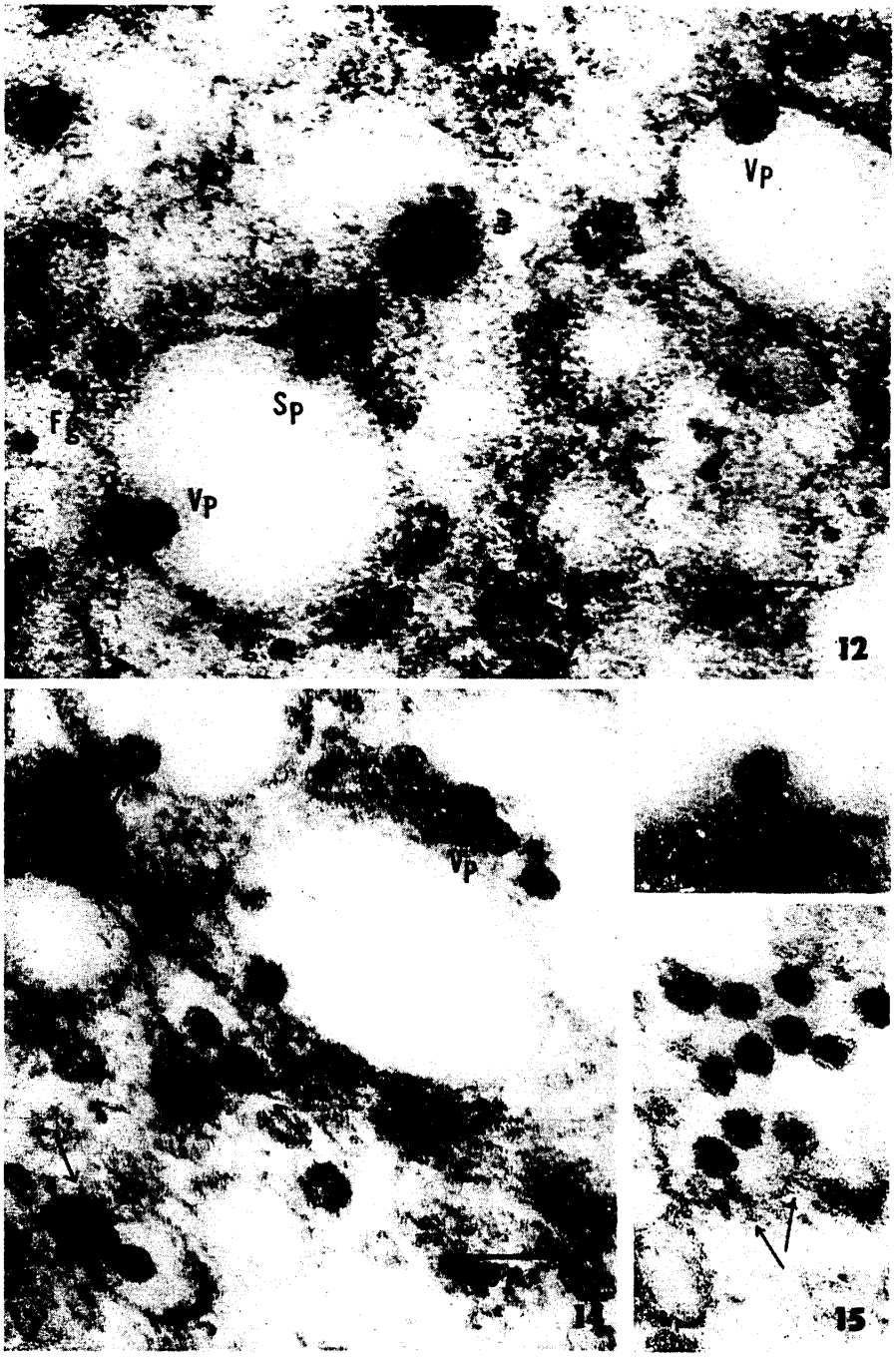
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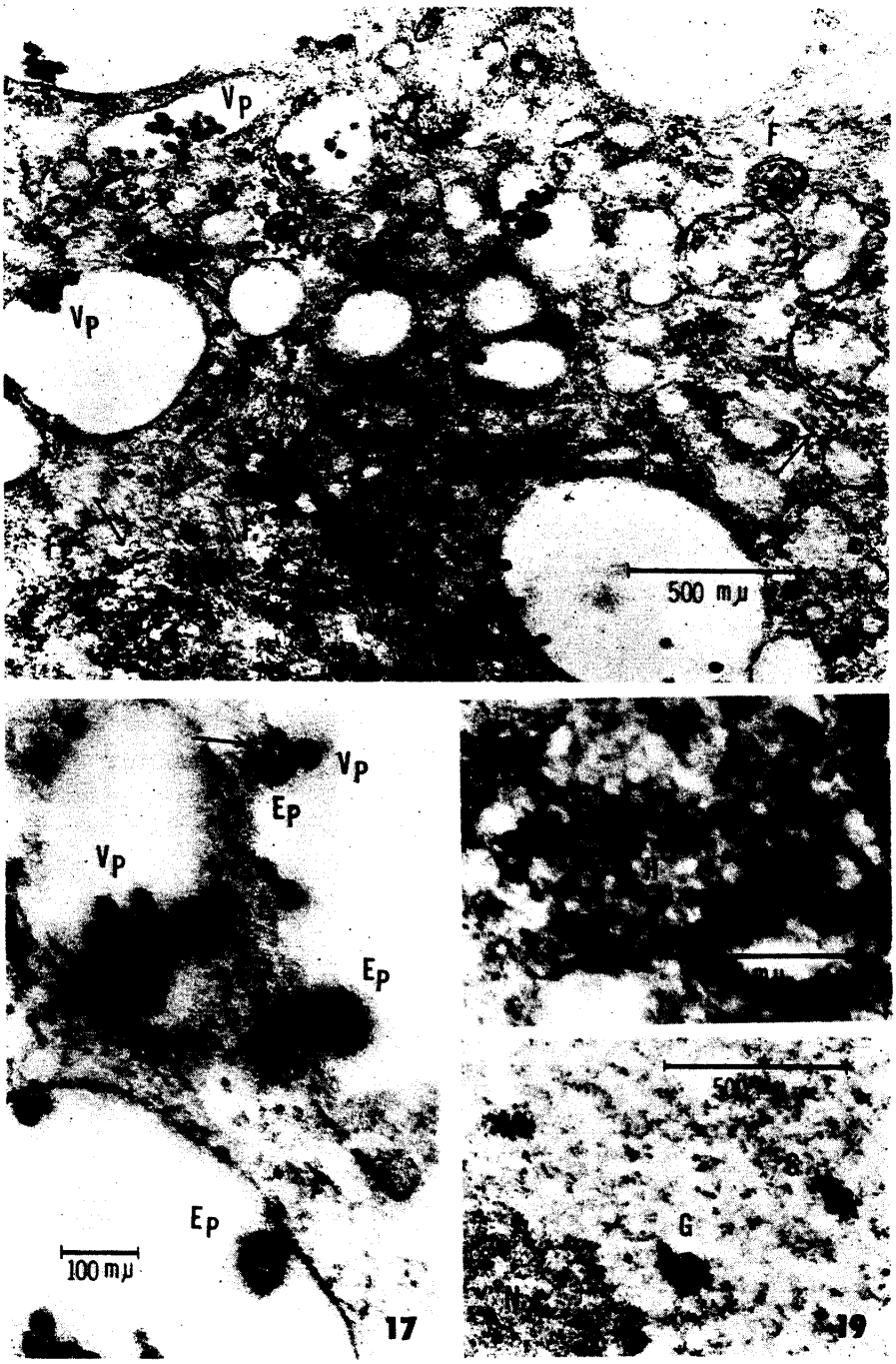
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