JOURNAL OF VIROLOGY, Jan. 1974, p. 222-225
Vol. 13, No. 1

Printed in U.S.A.

NOTES

Virus-Induced Cytoplasmic Membrane Structures Associated with Semliki Forest Virus Infection Studied by the Freeze-Etching Method

ISMO VIRTANEN AND JORMA WARTIOVAARA

The Electron Microscope Laboratory and The Third Department of Pathology, University of Helsinki, Helsinki, Finland

Received for publication 11 September 1973

Intracellular membrane structures associated with the Semliki Forest virus replication process were studied from freeze-etch replicas. Cleaved membrane structures inside the CPV I type vacuoles lacked the typical membrane particles present on most other fractured membranes. CPV II type vacuoles present in thin sections were obscured in the freeze-etch replicas by the cytoplasmic ground substance.

Various stages of the infection process of group A arboviruses are associated with membrane structures, e.g., the RNA replication (8-11), the virus-specific protein synthesis (2), and the budding stage (1).

Membrane structures named cytopathic vacuoles of type I by Grimley et al. have been suggested to be the site of the replication of the viral RNA (10). The function of another type of vacuole, named cytopathic vacuole of type II, has remained unclear, although it has been suggested to take part in the assembly of the viral nucleocapsids (10). In the present study the ultrastructure of these cytopathic vacuoles has been studied by the freeze-etching method.

Baby hamster kidney cells (BHK-21) were grown in culture bottles as described earlier and infected with Semliki Forest virus (SFV) at a multiplicity of 50 plaque-forming units per cell (12). After 5 or 6 h of infection the cells were harvested with a "rubber-policeman" and washed thoroughly with phosphate-buffered saline.

For thin sectioning, the pelleted cells were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. After post-fixation with 1.5% osmium tetroxide in 0.1 M phosphate buffer at pH 7.2, the cells were dehydrated with ethanol and propylenoxide and embedded in Epon 812. Thin sections were post-stained with uranyl acetate and lead citrate. For freeze-etching studies the cells were first prefixed in the above glutaraldehyde solution for 30 min and thereafter impregnated for 1 h with 20% glycerol in phosphate-buffered saline. Freeze-etching was performed in a Balzers BA 360M freeze-etching machine as described by Moor and Mühlethaler (13).

From thin sections two kinds of virus-induced membrane structures could be resolved, which have been reported also in previous studies on the replication process of Arbo A group viruses (Fig. 1 and 2).

Structures corresponding to cytopathic vacuoles of type I consisted of a large vesicle covered on the inside with membrane spherules about 50 to 70 nm in diameter (Fig. 1 and 2). In the center of some vacuoles fuzzy material could be seen, the nature of which remained obscure (Fig. 2). After 5 to 6 h of infection structures corresponding to cytopathic vacuoles of type II could also be visualized in thin sections often among the type I vacuoles (Fig. 2). These structures consisted mainly of tubules covered on the outside with dense spherules about 40 nm in diameter.

With the freeze-etching method, structures corresponding to the cytopathic vacuoles of type I could be observed among other intracellular membrane structures (Fig. 3 and 4). Membrane fracture faces were seen in the center of these vacuoles. It is noteworthy that the fractured central membrane material and spherules lining the inside of the cytopathic vacuoles of type I lacked the typical membrane particles reported to be present on most other cleaved

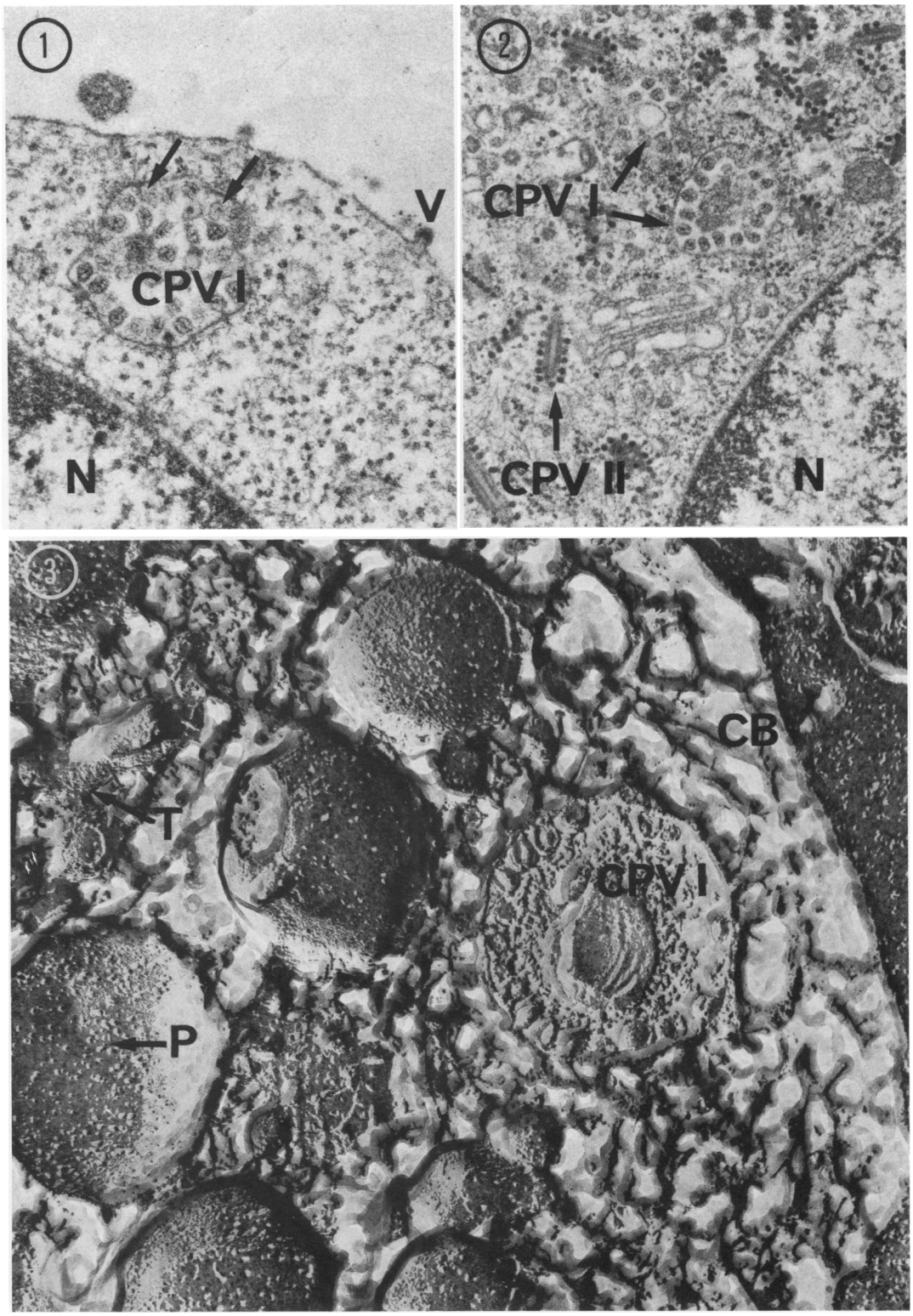

FIG. 1. *A cytopathic vacuole of type I (CPV I) near the cell surface in a thin-sectioned baby hamster kidney cell after 5 h of infection with SFV. Membrane spherules on the inside of the vacuole are indicated by arrows. Mature viruses are seen budding through the cell surface membrane (V). N, nucleus. Magnification: ×44,000.*

FIG. 2. *Both cytopathic vauoles of type I (CPV I) and II (CPV II) in the cytoplasm of a cell infected 5 h with SFV. Nucleocapsids are associated with the cytopathic vacuoles of type II. N, nucleus. Magnification: ×36,000.*

FIG. 3. *A freeze-etching micrograph of a cell infected 5 h with SFV. Fractured membrane spherules line inside of a cross-fractured cytopathic vacuole of type I (CPV I). Membrane particles (P) are seen on the cleaved membrane organelles surrounding the cytopathic vacuole of type I. A tubular structure resembling a cytopathic vacuole of type II is indicated (T). B, Cell boundary. Magnification: ×51,000.*

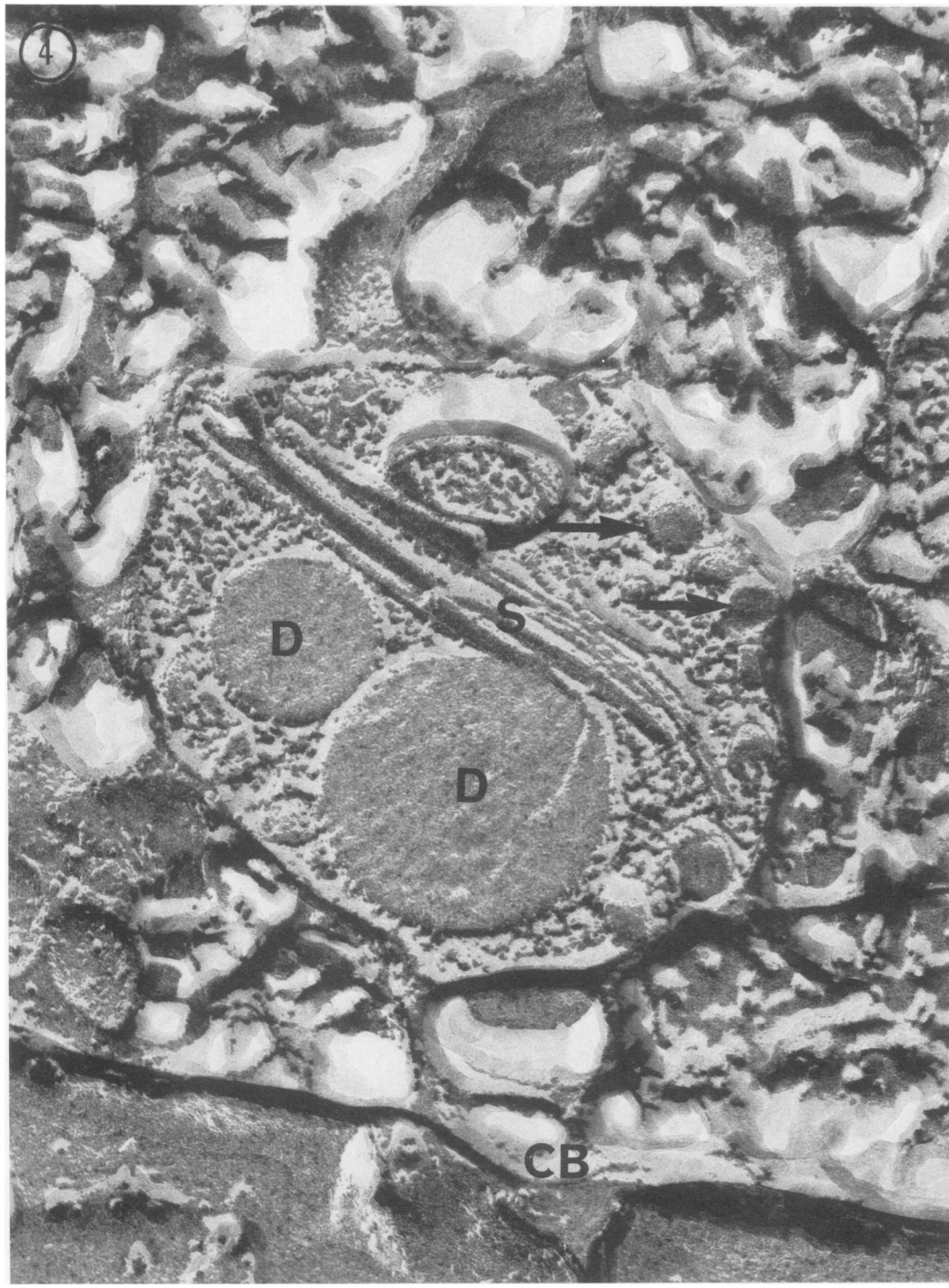

FIG. 4. *A cytopathic vacuole of type I near the cell boundary (CB) of a cell infected 5 h with SFV. The cross-fractured vacuole contains large cleaved membrane disks (D) and transversely fractured stacked membranes (S). The vacuole is lined on the inside with smaller membrane spherules (arrows). Membrane disks and spherules lack the typical membrane particles. Magnification: ×94,000.*

cellular membranes (3, 5, 14). The fractured surfaces of the surrounding membrane organelles contained such particles (Fig. 3). Whether some of these surfaces belonged to cleaved limiting membranes of cytopathic vacuoles of type I cannot be resolved, although it seems likely. All membrane structures excluding myelin (4) and artificial phospholipid membrane (7) have been shown to contain membrane particles on their fractured surfaces, and it has been claimed that the particles correspond to membrane proteins or localized lipid-protein complexes that are situated within the lipid bilayer and are exposed by the fracturing process (5, 7, 15).

Tubular membrane structures of the same size as cytopathic vacuoles of type II seen in thin sections could be resolved with the freeze-etching method (Fig. 3). In glycerol-impregnated specimens fractured membranes are obtained (3, 5). Therefore, structures like cytopathic vacuoles of type II, whose main characteristics are viral nucleocapsids lying on the outside of tubular structures, cannot be undisputedly distinguished from the cytoplasmic ground substance with the freeze-etching method. The successful visualization of these structures would require deep-etching of glycerol-free isolated vacuoles to uncover the membrane associated nucleocapsids, as has been done with membrane-bound ribosomes (17).

Cytopathic vacuoles of type I have been postulated to originate from Golgi-derived vesicular material (11). However, fracture planes of Golgi-membranes have been shown to contain membrane particles (14). Thus, the transformation of Golgi-vesicles or some other cytoplasmic membrane organelles into particle-free inner membranes of the cytopathic vacuoles of type I would therefore involve reorganizations in the molecular structure of the membranes.

The freeze-etching method has also been used to study the budding process of arbo A group viruses through the cell surface membrane (6, 16). The fractured surfaces of budding Sindbis viruses, another arbo A group virus, lack the membrane particles which were shown in the present study to be lacking also from the inner membranes of the cytopathic vacuoles of type I, an interesting correlation which needs further investigation.

This investigation was supported by a grant from The Sigrid Jusélius Foundation.

LITERATURE CITED

1. Acheson, N. H., and I. Tamm. 1967. Replication of Semliki Forest virus: an electron microscopic study. Virology **32**:128-142.
2. Ben-Ishai, Z., N. Goldblum, and Y. Becker. 1968. The intracellular site and sequence of Sindbis virus replication. J. Gen. Virol. **2**:365-375.
3. Branton, D. 1966. Fracture faces of frozen membranes. Proc. Nat. Acad. Sci. U.S.A. **55**:1048-1056.
4. Branton, D. 1967. Fracture faces of frozen myelin. Exp. Cell Res. **45**:703-707.
5. Branton, D. 1971. Freeze-etching studies of membrane structure. Phil. Transact. R. Soc. Serol. B. **261**:133-138.
6. Brown, D. T., M. R. F. Waite, and E. R. Pfefferkorn. 1972. Morphology and morphogenesis of Sindbis virus as seen with freeze-etching techniques. J. Virol. **10**:524-536.
7. Deamer, D. W., R. Leonard, A. Tardieu, and D. Branton. 1970. Lamellar and hexagonal lipid phases visualized by freeze-fracture. Biochim. Biophys. Acta **219**:47-60.
8. Friedman, R. M., and I. K. Berezesky. 1967. Cytoplasmic fractions associated with Semliki Forest virus ribonucleic acid replication. J. Virol. **1**:374-383.
9. Friedman, R. M., J. G. Levin, P. M. Grimley, and I. K. Berezesky. 1972. Membrane-associated replication complex in arbo virus infection. J. Virol. **10**:504-515.
10. 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.
11. Grimley, P. M., J. G. Levin, I. K. Berezesky, and R. M. Friedman. 1972. Specific membranaceous structures associated with the replication of group A arboviruses. J. Virol. **10**:492-503.
12. Kääriäinen, L., K. Simons, and C.-H. von Bonsdorf. 1969. Studies in subviral components of Semliki Forest virus. Ann. Med. Exp. Biol. Fenn. **47**:235-248.
13. Moor, H., and K. Mühlethaler. 1963. Fine structure in frozen-etched yeast cells. J. Cell Biol. **17**:609-628.
14. Reith, A., and R. Oftebro. 1971. Structure of HeLa cells after treatment with glycerol as revealed by freeze-etching and electron microscopic methods. Exp. Cell Res. **66**:385-395.
15. Tillack, T. W., R. E. Scott, and V. T. Marchesi. 1972. The structure of eryhtrocyte membranes studied by freeze-etching. II. Localization of receptors for phytohemagglutinin and influenza virus to the intramembranaceous particles. J. Exp. Med. **135**:1209-1227.
16. Waite, M. R. F., D. T. Brown, and E. R. Pfefferkorn. 1972. Inhibition of Sindbis virus release by media of low ionic strength: an electron microscopic study. J. Virol **10**:537-544.
17. Wartiovaara, J., and D. Branton. 1970. Visualization of ribosomes by freeze-etching. Exp. Cell Res. **61**:403-406.