Electron Microscopic Study of Tissue Cultures Infected with Simian Haemorrhagic Fever Virus

By O. WOOD,* NICOLA TAURASO‡ AND H. LIEBHABER*†

The Department of Epidemiology and Public Health* and the Department of Microbiology,† Yale University School of Medicine, New Haven, Connecticut, and the Laboratory of Virology and Rickettsiology, Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland‡

(Accepted 29 December 1969)

SUMMARY

The morphology and development of simian haemorrhagic fever virus was studied in tissue cultures infected with low multiplicities of virus. Early cytoplasmic lesions which were observed 24 hr after infection consisted of increased concentrations of polysomes and small vesicles 100 nm. in diameter filled with densely staining fibrillar material. As the lesions progressed, thickened membranous structures developed at the periphery of the vesicles. The membranes appeared to lengthen and thicken by a process of end-to-end and side-to-side fusion. Serial sections demonstrated that the fused, thickened structures were lamellae. Their role in the morphogenesis of simian haemorrhagic fever virus is not presently known.

Spherical virus particles measuring 40 to 45 nm. in diameter appeared in cells by 72 hr after infection. Infected cells contained vacuoles which enclosed accumulations of enveloped spherical particles. Some particles appeared to be budding into vacuoles.

INTRODUCTION

In November 1964 an outbreak of a severe febrile haemorrhagic disease occurred in newly arrived Rhesus monkeys held in the National Institutes of Health quarantine colony. The disease was limited to three macaca species; signs of infection were not observed in other monkeys in the colony. Few infected animals survived. Neither human disease nor antibody conversions have been noted in animal handlers tending the sick monkeys or in laboratory personnel working with infected tissues (Palmer et al. 1968). Several months prior to the N.I.H. outbreak a similar epizootic occurred in rhesus monkeys at the Sukhumi Institute of Experimental Pathology and Therapy, U.S.S.R. These monkeys were obtained from the same source as the N.I.H. (Shevtsova, 1967; Lapin et al. 1967). In 1967 a third outbreak resembling the previous epizootics occurred at the National Centre for Primate Biology in Davis, California, and a fourth outbreak has since occurred at a commercial primate facility in Sussex, Great Britain (Tauraso et al. 1969). Simian haemorrhagic fever, a highly communicable and generally fatal infection, appears to be a serious problem for laboratories and primate centres housing susceptible simian species. The epidemiological importance of this infection in nature is not known.

A virus was isolated from the blood of diseased animals from both the N.I.H. and Sukhumi
outbreaks. The isolates are serologically indistinguishable by complement-fixation, fluorescent antibody and neutralization tests (Tauraso et al. 1968). The virus isolated from the N.I.H. monkeys contains RNA and essential lipid and, by filtration studies, is < 50 nm. in diameter (Tauraso et al. 1968). To date, this virus is unrelated serologically to the arbo-viruses known to cause haemorrhagic disease (Casals, 1969). The host range of the agent is exceedingly narrow; isolation in tissue culture has been successful in only one cell line, the embryonic rhesus monkey kidney line MA 104. After six serial passages in MA 104 cells the virus was adapted to grow in an African green monkey kidney cell line BSC-1.

We have observed spherical particles 40 to 45 nm. in diameter in tissue culture cells infected with simian haemorrhagic fever virus which appear morphologically similar to members of the group B arboviruses (Wood, Tauraso, & Liebhaber, 1969). Infected cells contained in addition a novel membranous structure which we believed to be virus-related. The morphological characteristics of the virus particles and the membranous structures are described in this communication.

METHODS

Media. Minimal essential medium (MEM) supplemented by 5% calf serum and antibiotics was used throughout. For the growth of tissue cultures the medium contained Hanks' salts (HMEM-5 CS). For maintenance of the cultures and for all work carried out in the CO2 incubator the medium contained Earle's salts (EMEM-5 CS).

Cell cultures. Stock cultures of an embryonic rhesus monkey kidney line (MA 104) and a continuous African green monkey kidney line (BSC-1) were grown as monolayers in 32 oz. prescription bottles. The cultures were trypsinized weekly.

Virus. Simian haemorrhagic fever virus used in this study was originally isolated in the N.I.H. epizootic by Tauraso et al. (1968). Two strains of different tissue culture passage history were received in this laboratory; the MA 104 strain which had been through six serial passages in MA 104 cells (MA 6) and the BSC-1 strain which was derived from the MA 6 virus by eight subsequent passages in BSC-1 cells followed by two final passages in MA 104 cells (MA 6, BSC-1, MA 6).

Infection of cells for electron microscope studies. Cells from stock cultures were grown as monolayers in 8 oz. prescription bottles. When confluent the cell sheets were drained of medium and inoculated with virus at an input multiplicity of 0.5 p.f.u./cell. The inoculum was left in contact with the cell sheet for 1 hr at 37°, when it was removed. The infected monolayer was washed once with warm phosphate-buffered saline (PBS) and was then re-fed with 10 ml. of warm maintenance medium and incubated at 37°. At 24 hr intervals an infected and control culture were removed from the incubator. The culture fluids were harvested and stored frozen at -70° until they were assayed for infectivity. The entire cell sheet was immediately prepared for electron microscopy.

Preparation of cells for electron microscopy. Tissue cultures were scraped from the glass into maintenance medium with a Teflon policeman. After a low-speed centrifugation the cells were resuspended in 4% (v/v) glutaraldehyde in phosphate-buffered saline (pH 7.2) and fixed for 30 min., after which they were washed three times in PBS, postfixed for 1 hr in 1% (w/v) osmium tetroxide. The cells were then dehydrated in graded alcohols and embedded in Epon Araldite (Mollenauer, 1964). Cell blocks were cut on a Porter Blum MT-2 ultramicrotome. Silver and gray sections were stained with uranyl acetate and lead citrate and examined in a Philips 200 electron microscope.

Partial purification of virus. Infected tissue culture fluid was concentrated approximately 50-fold in a Diaflo ultrafiltration cell fitted with an XM-50 membrane. Virus was further
Structure of simian haemorrhagic fever virus

concentrated by sedimentation onto a 65% sucrose cushion in the SW 25-2 Spinco rotor centrifuged for 2 hr at 25,000 rev./min. After being suitably diluted the cushioned virus was layered on a 15 to 65% linear sucrose density gradient and centrifuged in the Spinco SW 25-2 rotor at 25,000 rev./min. for 16 hr. Fractions were collected dropwise from a hole punctured in the bottom of the tube. Each fraction was assayed for infectivity by the plaque method. Ten microlitre samples from every fifth fraction were weighed to determine solution densities.

Fig. 1. (a) Section of MA 104 cell 24 hr after infection. The cytoplasmic focus representing an early cytopathic change appears relatively electron-dense. It contains within it clusters of ribosomes (r) and vesicles (v) filled with deeply staining filamentous material. (b) A higher magnification of an early lesion demonstrating the clusters of ribosomes (r) and filament filled vesicles (V).
Fig. 2. Infected MA 104 cell 48 hr after infection. Vesicles separated from surrounding cytoplasm by distinct limiting membrane contain ribosome-like particles (a). Long filaments of membrane lying open in the cytoplasm (b) appear to undergo side-to-side fusion (c), giving rise to a paired structure.

Fig. 3. Higher magnification of paired membrane. Three major electron-dense zones are present: one central 16 nm. thick (b) and two peripheral each 8 nm. thick (a). Electron-transmitting zones each 9 nm. thick (c) between the central and peripheral dense zones are bisected by a thin electron-dense line (d) 2 to 3 nm. thick.
RESULTS

Electron microscopy of infected cells

Under the conditions of infection described, cytopathology was observed 24 hr after infection. As seen in the electron microscope, early lesions related to virus infection appeared as electron-dense cytoplasmic foci containing aggregates of ribosomes and many small vesicles with a modal diameter of 100 nm. The vesicles were filled with densely staining filamentous material. Some vesicles also contained electron-dense particles which could not be differentiated from ribosomes (Fig. 1a, b). Over the next 24 hr the vesicles acquired a rather striking limiting membrane, the inner surface of which was lined with clusters of ribosomal-like particles. The densely staining filamentous material which earlier filled the vesicles was no longer present (Fig. 2). The membranes lining neighbouring vesicles appeared to open and fuse end to end, giving rise to long filaments of membrane lying open in the cytoplasm (Fig. 2). The membrane which lined the vesicles was 25 nm. thick and symmetrical about its long axis. It was composed of two peripheral electron-dense layers 8 nm. thick separated by an electron-transmitting zone 9 nm. thick. The electron-transmitting zone was bisected by a thin electron-dense line 2 to 3 nm. thick (Fig. 2). The long filaments of membrane appeared to undergo further change involving side-to-side fusion. Along one of these filaments was a region over which two single membranes had fused to form a paired structure (Fig. 2). The morphology of a paired membrane is seen at a higher magnification in Fig. 3. It was 50 nm. thick and symmetrical about its long axis. Three major electron-dense layers were observed; one central was 16 nm. thick and two peripheral were each 8 nm. thick. An electron-transmitting zone 9 nm. thick between the central and peripheral dense layers was bisected by a thin electron-dense line 2 to 3 nm. thick. The formation of paired membranes appeared to be a general phenomenon, for as the infection progressed essentially all the membranes which earlier had lined the vesicles appeared as paired open structures.
Fig. 5. Complete virus particles within cytoplasmic vacuole. Virus particle (p), which measures 40 to 45 nm. in diameter, contains a core (c) measuring 22 to 25 nm. in diameter.

Fig. 6. Virus particles lining cytoplasmic vacuole. Many particles appear to be in various stages of completion (f).

Fig. 7. Virus particle appears to be budding into a vacuole (b).
The paired membranes often reached a length of several micrometres. The absence of oval and circular cross sections suggested that these structures were not tubular but lamellar. Serial sections approximately 50 nm. apart demonstrated the lamellar nature of these structures. An overall picture of a lamella was constructed from serial sections. It is diagrammatically illustrated in Fig. 4.

Complete virus particles were first seen 72 hr after infection when the cells had already undergone extensive degeneration. Virus particles were seen within cytoplasmic vacuoles (Fig. 5). The spherical particles measured 40 to 45 nm. in diameter; they contained 22 to 25 nm. cores often with electron-transmitting centres (Fig. 6). Occasionally cores were seen budding into a vacuole (Fig. 7). Particles in earlier stages of development were not observed with certainty; however, the lamellae were often coated with electron-dense particles which were most often in the size range of ribosomes though a few approached the 20 to 25 nm. size of virus cores (Fig. 3).

Association of specific infectivity with the virus particles

Virus was partially purified from infected tissue culture fluids by equilibrium density gradient centrifugation in a sucrose gradient. A single peak of infectivity was isolated from the gradient at a solution density of 1.19 to 1.20 g./ml. Particles present in these fractions were pelleted by ultracentrifugation. Convalescent serum specific for simian haemorrhagic fever virus neutralized 99% of the p.f.u. contained in such pelleted material. Portions of the same pellets which were fixed, embedded, sectioned and stained in the same manner as described for infected cells contained cell-free virus particles which were morphologically indistinguishable from those observed within infected cells.

DISCUSSION

Tissue culture cells infected with simian haemorrhagic fever virus, group A or group B arboviruses, appear to share a common striking morphological element. The proliferation of cytoplasmic membranes has been described in group A infections by several authors (Erlandson et al. 1967; Grimley, Berezesky & Friedman, 1968). Specific vacuoles have been shown to be the site of virus RNA replication, and membranes which line other vacuoles appear to serve as sites for the development of virus cores. In the later stages of infection large numbers of tubular structures are seen coated with virus cores. In group B arbovirus infection membrane proliferation has been observed at sites of active virus replication. In contrast to group A infections, the large three-dimensional masses of convoluted membranes which develop have no obvious physical relation to either virus cores or enveloped particles (Murphy et al. 1968). Cells infected with simian haemorrhagic fever virus contained proliferated membranes which appeared as broad undulating lamellae. The surfaces of these lamellae were often studded with electron-dense particles, the largest of which approximated the size of virus cores. They could not, however, be identified with virus cores.

Though morphologically similar to the group B arboviruses, simian haemorrhagic fever virus appears to differ from known arboviruses in several important respects. To date no serologic cross reactions have been found between simian haemorrhagic virus and arboviruses, especially those viruses known to cause haemorrhagic disease. Furthermore, simian haemorrhagic fever virus does not follow the usual arbovirus pattern of pathogenicity for laboratory mice, and its host range, which on the basis of current knowledge is limited to macaca and vervet monkeys and to two tissue culture lines derived from these species, differs from those of known haemorrhagic viruses. Moreover, since no evidence is yet available
for the biological transmission of simian haemorrhagic virus by an arthropod vector, and since this is in fact the only basis for the present classification of arboviruses, it cannot and should not be classified as such.

The authors wish to acknowledge the excellent technical assistance of M. Garrison. This work was supported by General Research Support Grant FR-05443-07 from the United States Public Health Service.

REFERENCES


(Received 16 October 1969)