Electron Microscopic Evidence of Nariva Virus Structure

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Nariva virus was first isolated in 1962 by Tikasingh et al. (1966) from Trinidadian rodents *Zygodontomys b. brevicauda* and was originally thought to be an arbovirus. It contains RNA, is ether-sensitive and haemadsorbs guinea-pig erythrocytes (Karbatsos, Buckley & Ardoin, 1969). Few of its properties are known, and since its structure and morphogenesis are also unknown, its taxonomic position is uncertain. We describe here observations on the structure and morphogenesis of Nariva virus propagated in BHK 21 cell cultures, thus providing some evidence for its classification. Monolayers of BHK 21 cells were grown in EM/TPB medium as described previously by (Macpherson & Stoker, 1962) and maintained in equal parts of EM/TPB and Basal Medium Eagle (Eagle, 1955) with 3% foetal bovine serum, 0.05% of 2 M-tris and antibiotics; kanamycin and tylosin tartrate was additionally added. Nariva virus (prototype strain TRVL-42520, 4th mouse brain passage) was obtained from Dr L. Spence (Trinidad Regional Virus Laboratory, Trinidad). Following two additional passages in newborn-mouse brain, the virus was adapted and thereafter grown in routine fashion in monolayers of BHK 21 cells. The virus was assayed in BHK 21 tube cultures by the haemadsorption (HAd) technique described by Vogel & Shelokov (1957). Brain suspensions from healthy mice were used as inocula for controls. The virus growth cycle was investigated using an input multiplicity of 0.1 ID$_{50}$/cell.

Infected and control cells were used for electron microscopic studies. Monolayers were fixed *in situ* using 2% glutaraldehyde in cacodylate buffer, pH 7.2, post-fixed in osmium tetroxide. Specimens were embedded in Maraglas (Ladd Research Industries Inc., Burlington, Vermont) by standard techniques. Thin sections were cut with a Porter-Blum, MT-2 Ultra-Microtome with diamond knife, and stained for 15 min. with a saturated solution of uranyl acetate diluted with an equal volume of ethanol followed by a solution of lead citrate (Reynolds, 1963). Negatively stained preparations were made by disrupting infected cells according to the technique described by Compans & Choppin (1967). Specimens were examined in a JEM-100B electron microscope.

The kinetics of Nariva virus replication were followed to correlate ultrastructural events with the development of virus-specific components (Fig. 1). Released and cell-associated virus increased simultaneously reaching maximum yields of virus 96 hr after infection. Thin sections of BHK 21 cells, 24 hr after infection showed frequent intracytoplasmic inclusion bodies. These inclusions were considered to be precursors, and contained dispersed electron-opaque tubular structures, of typical myxovirus-like morphology, which also constituted the inside of the virus particles (Figs. 6, 7b), and were therefore considered to be nucleocapsids. Beyond this early stage of infection these inclusions increased in volume and acquired a condensed conformation (Fig. 2a). A representative inclusion negatively stained is shown in Fig. 2b. The diameter of the nucleocapsids was estimated to be 20 nm. Ultrastructural changes, in addition to the synthesis of virus components, were irregular-shaped nuclei, widening of the perinuclear space, condensation and margination of the chromatin and grossly dilated endoplasmic reticulum. Furthermore, infected cells exhibited a unique bizarre arrangement of convoluted intracytoplasmic membranes and a proliferated outer nuclear membrane (Fig. 4a, b). There was also an alteration of the cell-surface membrane,
with an increase in its electron density and a fuzzy layer of surface projections (arrow, Fig. 5). Electron-dense nucleocapsid strands were often in close contact with the altered membrane, which bulged outwards as the virus budded (Fig. 6). The interaction of erythrocytes with cells lacking nucleocapsids in the adjoining cytoplasm was often the only recognizable sign of virus infection. In thin sections, extracellular virus particles were roughly spherical and very pleomorphic (Fig. 7a, b, c). They had an average external diameter (including the length of projections) of 200 nm. (range of diameters 130 to 250 nm.).

Nariva virus particles exhibited internal nucleoprotein components of about 20 nm. in diameter. These were surrounded by an envelope displaying symmetrically distributed surface projections (arrows, Fig. 7e) which were approximately 20 nm. long, 10 nm. wide and were separated by a gap of roughly 10 nm.

**Fig. 1.** Growth curve of Nariva virus in BHK21 cells. Input multiplicity of 0.1 ID50/cell. Released virus, ○—○; cell associated virus, ○——○.

**Fig. 2.** (a) Intracytoplasmic inclusions in a Nariva virus-infected BHK21 cell. Accumulation of electron-dense tubular structures can be seen. (b) An aggregate of nucleocapsids released from a Nariva virus-infected BHK21 cell, stained with phosphotungstic acid.

**Fig. 3.** (a) A single Nariva nucleoprotein element stained with phosphotungstic acid. (b) Nucleocapsids in the cytoplasm of an infected cell. Note the regular periodic cross-striations and helical coiling within the strand (arrow). (c) Higher magnification of a Nariva nucleoprotein element revealing single-strand helical structure (arrow), stained with phosphotungstic acid.
The structure (Fig. 7a, b, c) and 200 nm diameter of the Nariva virus particle (as reported in this paper) is not consistent with the arbovirus-like structure (Venezuelan equine encephalomyelitis, Guaroa, Melao, Pacuistructural class 1) and 59 nm diameter previously published by Bergold, Graf & Munz (1969) on purified and negatively stained particles. High resolution electron micrographs of nucleocapsids revealed helical strands (arrow, Fig. 3b). Their estimated diameter of 20 nm and a regular periodic arrangement of cross-striations spaced at about 3 to 4 nm. agreed well with those of negatively stained nucleocapsids. Furthermore, studies of negatively stained preparations have confirmed the helical structure of the nucleocapsids, and showed that they most probably consist of a single strand of nucleoprotein (arrow, Fig. 3c). The Nariva nucleocapsids are flexuous as those of parainfluenza-DA (Walder, unpublished results), mumps and measles viruses (Finch & Gibbs, 1970). The mean length of negatively stained nucleocapsids (loosely coiled) was estimated to be 1.8 μm. (distribution of lengths 1.3 to 2.0 μm.) (Fig. 3a). These results indicate that the diameter and cross-striation spacings of Nariva nucleocapsids are similar to those described previously (Nakai & Imagawa, 1969; Compans et al. 1964; Feller, Dougherty & Distefano, 1969; Howe et al. 1967) for measles, Newcastle disease virus (NDV) and other parainfluenza viruses, although their estimated length (mean 1.8 μm.; modal 1.68 μm.) appears to be longer than that of NDV (Compans & Choppin, 1967) and of Sendai, mumps and measles (Finch & Gibbs, 1970). This difference may be because the Nariva nucleocapsids have more loosely bound adjacent turns of the helix. Their diameters, however, differed significantly from those of influenza virus (9 nm.) and respiratory syncytial virus (11 to 15 nm.) (Waterson, 1962; Norrby, Marusyk & Orvell, 1970). A unique ultrastructural change resulting from Nariva infection was the proliferated nuclear and cytoplasmic membranes (Fig. 4a, b) which were morphologically similar to the cytomembranes described by Sjöstrand (1968). Implications of these proliferated membranes to the assembly of Nariva particles or its components is currently unclear.

Despite the resemblance in morphology and mode of development, no serological cross-reactions have been found between Nariva and any of the known myxoviruses, nor is it known to be related to any other viruses (Karabatsos et al. 1969; Tikasingh et al. 1966). On the basis of the structural and morphogenetic evidence presented here, Nariva can clearly be classified as a new paramyxovirus. However, on the basis of nucleocapsid structure and the behaviour of its haemagglutinin receptor insensitivity to RDE or trypsin, it is rather more like the measles-distemper-rinderpest viruses, yet the nucleocapsids develop only in the cytoplasm as with NDV-mumps-parainfluenza viruses. It would therefore still be unwise to place it in either of the classes of paramyxoviruses mentioned. One can speculate that Nariva virus may be a natural recombinant.
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