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Morphological Variants of Sindbis Virus Obtained from Infected Mosquito Tissue Culture Cells

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Tissue-cultured *Aedes albopictus* cells infected with morphologically homogeneous Sindbis virus were found to produce progeny virions which could be divided into three classes based on size. The thickness of the envelope was constant on all three sizes of progeny virions suggesting that the variability in size rested with the viral nucleocapsid. It is suggested that the three classes of virions have icosahedral nucleocapsids composed of common subunits organized in decreasing triangulation numbers.

The arboviruses are a group of infectious agents which morphologically are composed of a molecule of single-stranded RNA which is enclosed within a protein-containing nucleocapsid which is, in turn, surrounded by a membraneous envelope (1, 4, 9, 15, 19). The morphology of the various components of the intact Sindbis virions have been described by using a variety of electron microscope preparative procedures. Simpson and Hauser (19) have shown the normal Sindbis virion to be 65.0 nm overall and to contain a nucleocapsid which is about 35 nm in diameter. The outermost surface of the virion is covered with an amorphous material which has been variously described as spikes or fuzz in studies utilizing negative staining (1, 19) and ultrathin sectioning (1). Brown et al. (4) have shown this outer layer to be composed of 4-nm subunits by using freeze-etching techniques. The outermost surface has been shown by Compans to contain the viral glycoprotein (7).

Beneath the surface is a layer which in ultrathin sections has the morphology of a classical membrane bilayer (1). Brown et al. (4) have shown that this bilayer is cleaved by freeze etching in the manner expected for a typical unit membrane but is free of the 6.5-nm intramembrane particles usually found in cleaved membranes. The morphology of the Sindbis nucleocapsid has been studied in negatively stained preparations by Horzineck and Mussgay (11) and in freeze-dried and freeze-etched preparations by Brown et al. (4). Horzineck and Mussgay (11) concluded that the nucleocapsid is an icosahedron 35.5 nm in diameter. These investigators further suggested that the nucleocapsid is a member of the class P = 3 icosadeltahedra, having a triangulation number of 3 (the smallest member of this class) and a total of 32 subunits each 14.0 nm is diameter. Brown et al. (4) confirmed the overall size of the nucleocapsid as 35 nm but suggested that it was a class P = 1 icosahedron with T = 9 and 92 subunits with a center to center spacing of 7.0 nm.

This investigation describes two new size classes of enveloped virions found in tissue cultured *Aedes albopictus* cells infected with Sindbis virus.

MATERIALS AND METHODS

Cells, virus, and media. Methods for growth and titration of Sindbis virus in primary chicken embryo fibroblasts (CEF) have been described (17). Sindbis virus was obtained from Elmer Pfefferkorn (Dartmouth College) and was plaque purified and propagated by passage at low multiplicity on CEF cells.

A. albopictus cells were obtained from Imogene Snyder (Walter Reed) and propagated in the medium of Mitsuhashi and Maramorsch (14) supplemented with 20% fetal calf serum and brought to pH 7.2 by addition of sodium bicarbonate. The growth of arboviruses in tissue-cultured mosquito cells has been described (21).

Infection of A. albopictus cells with Sindbis virus. Confluent monolayers of *A. albopictus* cells were infected with Sindbis virus at a multiplicity of 30 to 50 plaque-forming units per cell. The preparation of input virus was found to be morphologically homogeneous containing no obvious aberrant forms on examination in the electron microscope. Plaque assays of the input virus on CEF cells revealed no distinct variability in plaque morphology.

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After infection the cells were incubated at 28 C for periods varying from 40 h to 5 days (21). These infected cells show no cytopathic effect, but rather a chronically infected culture is obtained which produces virus whole continuing cell division (16, 21). Some of the cells were serially passaged at 3- to 4-day intervals. Each time the cells were passed, some of the growth media was examined by electron microscopy for progeny virus. The growth media containing the newly produced virus was removed, and after a low-speed spin (3,000 $\times$ g for 10 min) to remove any debris, it was spun at 25,000 rpm for 5 h (72,000 $\times$ g). The pellet containing newly synthesized virus was resuspended in a small volume of phosphate-buffered saline and studied in the electron microscope by negative staining. The infected cells were fixed and prepared for thin sectioning as described below.

Preparation of specimens for electron microscopy. Negative staining was carried out by the procedure of Anderson (2). Phosphotungstic acid was prepared as a 2% solution which was brought to pH 7.2 by addition of sodium hydroxide.

Cells were prepared for ultrathin sectioning after fixation for 1 h in 2% glutaraldehyde in Millonigs phosphate buffer (13). The glutaraldehyde-fixed cells were washed twice in phosphate buffer and then postfixed with 2% osmium tetroxide in phosphate buffer. Dehydration and embedding in Epon 812 was by the procedure of Luft (12).

Specimens were examined in a Siemens 101B electron microscope which was calibrated with a grating having 2,160 lines per mm (Fullam).

RESULTS

Virions recovered from the growth media of Sindbis-infected Aedes albopictus cells. Negatively stained preparations of the virus produced from Sindbis-infected *A. albopictus* cells are shown in Fig. 1. Three distinct size classes of virions were seen. The dimensions of these particles are presented in Table 1. Measurements were made either of the diameter of the complete particle or of the particle excluding the outer layer of spikes or fuzz. The latter arrangement allowed us to measure and compare particles from which the spike layer was missing (Fig. 1E, F) or otherwise poorly preserved. This system of measurement was particularly essential in dealing with the smallest of the virions seen in the infected cell supernatants. These particles were found only rarely with complete surrounding envelope (Fig. 1D) suggesting that their small size increased their lability relative to the other particles. Measurements made of particles from which the spike layer was completely missing were identical to those made on intact particles excluding the spike layer.

The largest of the progeny virions (normals, Fig. 1A) were identical in size and general morphology to the virions in the inoculum and it is assumed that these represent normal Sindbis particles. A second class of virions (mediums) were found to be distinctly smaller than normals (Table 1), (Fig. 1B, C). These virions had a cross-sectional diameter 80% that of the normal particles when the total diameter was taken, and a diameter 74% of normal excluding the spikes or fuzz from the measurement. The third class of virions (smalls) (Fig. 1D, E, F) had a total diameter 59% that of normals and a "spike excluded" diameter 52% that of normals. The mediums and smalls were found to differ from the normals primarily in their internal structure; the spikes or fuzz found on the surface was of constant thickness regardless of the particle size class measured. Medium and small particles with and without spikes were occasionally penetrated by stain in such a way as to reveal a membrane 7 to 8 nm thick which probably represents the unit membrane seen in thin-sectioned virions (Fig. 1B, E). Occasionally, the internal structures of the virions were found to have a shape suggestive of hexagonal symmetry (Fig. 1D, E).

Initially, the larger or normal virions were found to be the predominating species of virus in the infected cell supernatant. At 5 days after infection, however, the small and medium size particles were found collectively to exceed the normal particles in number by about 5 to 1. This ratio of smalls and mediums to normals did not change significantly in the supernatants of serially passaged virus infected cells.

The medium and small virions found in the concentrated progeny virus were apparently more labile than the normal size virions. The number of morphologically complete medium and small virions were found to decrease rapidly on standing at 4 C relative to the number of normals. Because of this instability of the smaller particles it has not been possible to confidently determine the relative numbers of small and medium size virions. We suspect that they decompose at different rates relative to the normal size virions and that decomposition may begin soon after release from the host cell.

Ultrathin-sectioned virions produced by Sindbis-infected Aedes albopictus cells. The three size classes of virions seen in the negatively stained concentrated supernatants described above could also be found in ultrathin sections of cell-associated virus (Fig. 2). The virions were generally difficult to find in thin sections possibly due to the relatively slow rate of virus production in tissue-cultured mosquito cells (16, 21). The thin-sectioned virions possess typical morphology and, as in the case of the negatively stained virus, differed from each other only in size. The particles were found to consist of an electron-dense central core sur-

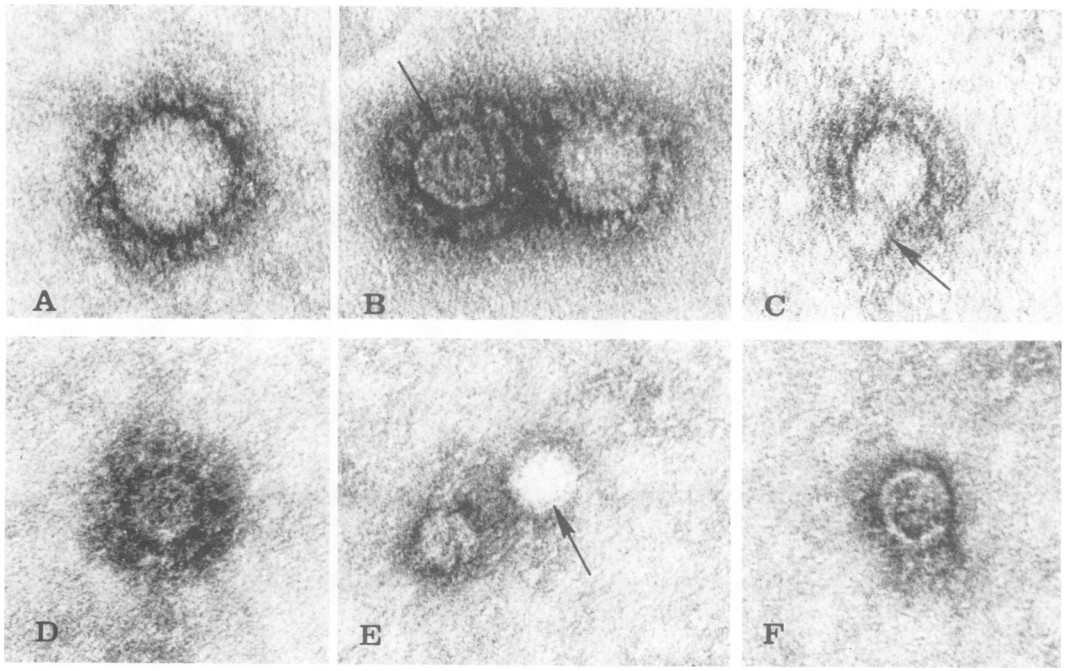

FIG. 1. *Electron micrographs of virus produced from Sindbis-infected A. albopictus cells at 5 days after infection. A, Normal size virion with spikes or fuzz; B, two medium sized virions with spikes. The particle on the left is more completely penetrated by stain revealing an inner layer of the envelope (arrow) possibly the unit membrane; C, a medium size particle with spikes which is apparently decomposing at one point (arrow); D, a small size virion with spikes; E, a spikeless small particle penetrated by stain to reveal the surrounding membrane (arrow); F, a partly degraded small revealing the surrounding membrane. Magnification ×300,000 for all.*

TABLE 1. *Mean diameters of Sindbis morphological variants*

Measurement	Negative stain			Ultrathin section		
	Avg diam (nm)	SD[a]	Normal size (%)	Avg diam (nm)	SD	Normal size (%)
Normal particles excluding spikes	50	±0.08	100	45	±0.10	100
Normal particles with spikes	66	±0.10	100			
Medium particles excluding spikes	37	±0.11	74	35	±0.13	78
Medium particles with spikes	52	±0.18	80			
Small particles excluding spikes	26	±0.15	52	24	±0.20	53
Small particles with spikes	39	±0.34	59			

[a] SD, standard deviation. Values were calculated on the basis of measurements obtained from 40 to 60 particles with the exception of the small particles with spikes where only 19 particles were measured.

rounded by a unit membrane about 7.6 nm thick. When the diameters of the virions were measured from the outer edge of the bilayer to the opposite outer edge of the membrane, the virions fell into three size classes closely corresponding to those seen in negatively stained preparations: normal virions (45 nm in diameter), medium (35 nm in diameter or 78% of normal size), and small (about 24 nm in diameter or 53% of normal size). Attempts were made to measure the size of viral nucleocapsids free in the cytoplasm of the infected mosquito cells. Although virus nucleocapsids of normal size could be found, the expected medium and small nucleocapsids could not be detected. The inability to detect and accurately measure these structures was probably a result of their small size, causing confusion in distinguishing them from ribosomes.

The appearance of the medium and small size virions in the supernatant and cell-associated virus of Sindbis-infected *A. albopictus* cells

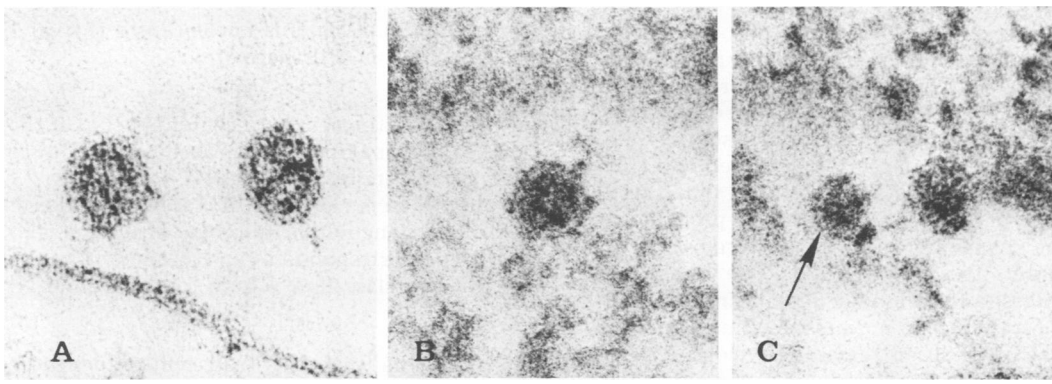

FIG. 2. *Ultrathin sections of Sindbis-infected A. albopictus cell-associated virus. A, Two normal virions; B, a medium virion; C, a small virion (arrow). All show electron-dense cores and surrounding membranes. Magnification ×257,200.*

raised the possibility that these cultured cells were carrying a contaminating virus or viruses, or that they were harboring some latent virus or viruses, the active production of which was induced by Sindbis infection. These two possibilities seem unlikely as no virion of any size could be seen in uninfected cell cultures or the supernatants of these cultures. Attempts to produce virions in noninfected cells by various treatments with ultraviolet light, 5 fluorodeoxyuridine, or actinomycin D were not successful. When purified normal virions were incubated in growth media for 5 days at 26 C, the number of intact particles in the viral suspension was greatly reduced. Electron microscope studies of preparations treated in this manner did not show an accumulation of particles resembling the smalls and mediums described above, suggesting that these smaller particles were not breakdown products of the normal size virions.

Because of the relative instability of the medium and small particles, attempts to purify them from each other and the normals have met with mixed success. Sucrose gradient analysis has shown the medium particles to have a sedimentation constant of 180 to 190*S* relative to normal virions which sediment at 273*S* (11). The medium size virions purified in this manner are antigenically similar to normal virions as determined by their ability to inactivate specific anti-Sindbis serum (serum-blocking power). Anti-Sindbis antiserum (obtained from Elmer Pfefferkorn) which was capable of reducing the plaque forming capability of normal sucrose gradient purified Sindbis virus from a titer of 5×10^9 PFU/ml to 4×10^4 PFU/ml after 5 min at 37 C, was reduced in its virus inactivating ability such that it would reduce the plaque forming capability of the same purified virus stock only to 7×10^7 (same conditions of temperature and time) after incubation with the medium particles for 15 min at 37 C. Attempts to obtain plaques on CEF cells with the medium particles were unsuccessful, suggesting that the sucrose-purified medium sized virions are not infective. Electron microscopy of cells and supernatants of cells (CEF or *A. albopictus*) infected with large numbers of medium sized particles (as determined by count in the electron microscope) revealed no intact virions or subviral particles. No observations in the physical or antigenic properties of the small size virions have yet been possible.

DISCUSSION

General observations on the production of Sindbis virus morphological variants. Sindbis virions produced under normal circumstances from chicken embryo fibroblasts cells have been shown by a variety of procedures to be composed of a 35-nm nucleocapsid surrounded by a tightly fitting unit membrane which is covered on its outermost surface by the viral glycoprotein, (1, 4, 7, 9, 10, 11, 15, 19). The overall diameter of the virion as determined by negative staining (19) and freeze etching (4) is about 65 nm.

We have found that when tissue-cultured *A. albopictus* cells are infected with morphologically homogeneous Sindbis virus, three sizes of membrane-bound particles were produced. These particles were classified as normals, mediums, and smalls. On well-preserved normal size particles the spike layer was found to have a thickness of about 8 nm. Spikeless virions were 50 nm in diameter, roughly the expected value if only the outer spikes or fuzz have been lost. We presume then that the particles measured by excluding the spikes (listed in Table 1) consist of the protein nucleocapsid surrounded by an

intact membrane bilayer. That this is not an unreasonable assumption can be demonstrated by subtracting the size of the normal nucleocapsid (4, 11, 19) from the diameter of the spikeless virion. The result allows 7.5 nm on either side of the nucleocapsid to accommodate the bilayer of the envelope. Negatively stained and thin-sectioned particles demonstrated that the difference in size of the virions was probably due to the change in size of the centermost part of the virion, the nucleocapsid. The outer layer of spikes and the unit membrane were of one constant size in normals, mediums, and smalls. The observation that the envelope components of the three size classes are of constant thickness suggests that normal and variant nucleocapsids are assembled together in the cytoplasm of the infected cell and are matured through a common modified membrane.

Electron microscopy of concentrated mosquito cell culture supernatants collected at various times after infection with Sindbis virus suggests that the production of the small and medium size particles initially lags behind that of the normal size virions. At 5 days after infection the medium and small virions represent the majority of the observed progeny virus.

Several previously published studies have suggested the progressively increasing production of defective Sindbis virus components when plaque-purified virus is serially passaged at high multiplicity in vertebrate cells (18, 20, 22). These conclusions have been based on the observation of a small (12S) double-stranded RNA component produced in cells infected with high-passage level virus, and upon the observation of specific interference with normal Sindbis virus production by components in the high-passage virus stocks. We are presently attempting to determine if the morphological variants described herein represent the interfering agent described in these other studies.

Possible morphology and triangulation numbers of Sindbis virus nucleocapsids. Brown et al. (4) have suggested that the 35-nm nucleocapsid is composed of subunits having a center to center spacing of 7.0 nm. In some instances these capsomeres were seen organized in a fivefold array strongly suggestive of icosahedral symmetry. Applying the principles of virus construction developed by Caspar and Klug (5, 6), it is possible to calculate a triangulation number (T) for an icosahedron of given diameter composed of subunits of given dimensions. The length of an edge E of one of the 20 equilateral triangular facets of the icosahedron can be determined by the equation:

$$E = 0.618\ D \quad (1)$$

where D is the diameter of the icosahedron (8). For the normal Sindbis nucleocapsid the value of E is 21.6 nm. Furthermore,

$$E = dT^{1/2} \quad (2)$$

where (d) is the center to center spacing of the capsomeres making up the icosahedron (6). For the normal Sindbis nucleocapsid, T = 9.5.

This value is closest to T = 9 in the series of possible triangulation numbers of Caspar and Klug and corresponds to an icosahedron of the nonskewed class P = 1 with total subunits N:

$$N = 10T + 2 = 92 \quad (3)$$

and suggests that the true center to center spacing of the subunit is 7.2 nm which is within measuring error of the value 7.0 published by Brown et al. (4).

If one were to construct class P = 1 icosahedra smaller than the normal Sindbis nucleocapsid using the same capsomeres or morphological units (size = 7.2 nm), the possible triangulation numbers of these smaller morphological variants would be T = 4, and T = 1 (5). Using equation (3) above, these capsids would possess 42, and 12 capsomeres, respectively. Their diameters may be calcuated from equations (1) and (2) above, and would be 23.3 and 11.7 nm in diameter, respectively.

Using a thickness of 7.6 nm for the unit membrane surrounding the Sindbis nucleocapsid (1, 4, 15, and this study) and the observation by electron diffraction that the unit membrane is tightly associated with the nucleocapsid (10), it is possible to predict the sizes of the spikeless particles which would be produced by maturation of the medium and small nucleocapsids. The predicted sizes are summarized in Table 2 and are compared to the measured values obtained in negatively strained and thin-sectioned particles (Table 1). Good agreement is obtained in the negatively stained preparations, whereas the thin-sectioned virions are consistently smaller than the predicted values by about 10%. The smaller diameters obtained in thin-sectioned particles probably result from shrinkage during dehydration and embedding (3).

We conclude that the nucleocapsid of the normal Sindbis virion is a member of the nonskewed class P = 1 of icosadeltahedra. Its triangulation number is 9 and it is composed of 92 subunits which have a center to center spacing of 7.2 nm. We can find no other architectural arrangement (even completely hypothetical) which would allow the construction of the normal viral nucleocapsid and the two morphological variants given the experimentally obtained dimensions.

TABLE 2. *Triangulation numbers and comparison of predicted diameters of Sindbis variants with diameters obtained by direct measurement*

Triangulation no. T	Predicted size of nucleocapsid (nm)	Predicted size of capsid and unit membrane (7.6 nm)	Measured size by negative staining and % of predicted size	Measured size by thin sectioning and % of predicted size
9	35	50.1	50 (99.8)	45 (89)
4	23.3	38.5	37 (96.1)	35 (90)
1	11.7	26.9	26 (96.2)	24 (89)

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