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Squirrel Monkey Retrovirus: Electron Microscopy of a Virus From New World Monkeys and Comparison With Mason-Pfizer Monkey Virus

By

M. A. Gonda, D. L. Fine, and M. Gregg

Viral Oncology Program, NCI Frederick Cancer Research Center, Frederick, Maryland, U.S.A.

With 5 Figures

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Summary

The ultrastructural morphogenesis of squirrel monkey retrovirus (SMRV) and Mason-Pfizer monkey virus (MPMV) grown in cell culture were compared. Both viruses develop by a process that begins with the formation of intracytoplasmic A particles which are then enveloped at the plasma membrane during budding. SMRV also develops as a crescent-shaped nucleoid beneath a bulging plasma membrane, a development characteristic of type C oncornaviruses. Free extracellular mature SMRV was generally round with a centrally located electron-dense nucleoid enclosed by the viral envelope. In contrast, mature MPMV had a tubular-shaped nucleoid. Negative stained preparations of both viruses yielded head-tail forms with surface projections. By uranyl acetate/critical point drying, SMRV particles were usually round with an eccentric electron-dense nucleoid enclosed by the viral envelope, whereas MPMV particles were round and contained an electron-dense bar-shaped nucleoid. These morphological observations indicate that SMRV more closely resembles MPMV, presently the only member of genus oncornavirus type D, than other retroviruses species. However, since SMRV can be morphologically, biochemically, and immunologically distinguished from MPMV, it represents a new species within genus oncornavirus type D.

Introduction

Retroviruses (8, 10) have been found to be etiologic factors or associated with tumors of a number of avian and mammalian species, including primates (15, 18, 19, 20, 24, 25). However, several other primate retroviruses have not yet been shown to have an etiologic role in cancer. These include the endogenous type C viruses of baboons (26), the type D Mason-Pfizer monkey virus (MPMV) isolated from rhesus monkeys (1, 3, 4, 5), and squirrel monkey retrovirus (SMRV), a new isolate from squirrel monkeys (16).

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In a recent report by HEBERLING *et al.* (16), SMRV was described to be morphologically similar to but antigenically distinct from MPMV. Subsequent studies conducted in our laboratories (11) confirmed the observations of HEBERLING and coworkers and also showed these two viruses to have different structural protein profiles. Since these viruses can be distinguished biochemically and immunologically, we wanted to determine whether they could be distinguished morphologically when propagated in the same cell line. In this paper we wish to communicate observations on the ultrastructure and morphogenesis of SMRV and MPMV grown in human cells.

Materials and Methods

Cells

Human rhabdomyosarcoma cells (A 204) (13) were used for propagation of SMRV (22) and MPMV (12). These cells were tested and found to be free of mycoplasma contaminants [R. Del Giudice (Frederick Cancer Research Center)]. SMRV was also propagated in fetal canine thymus (FCf 2 Th) cells originally obtained from Dr. Richard Heberling (Southwest Foundation for Research and Education, San Antonio, Texas).

Virus

SMRV and MPMV propagated in A 204 cells were obtained from Charles Benton (Frederick Cancer Research Center, Frederick, Maryland) and purified according to methods previously published (17).

Tissue

Two healthy, 2-year-old, male squirrel monkeys *(Saimiri sciureus)* were obtained from the Institute of Marine Biomaterials (Wilmington, North Carolina) and biopsied for the presence of viral particles. Tissue was taken from the lung, spleen, liver, heart, kidney, medulla, lymph node, thymus, testes, bone marrow, cerebrum, and cerebellum of each monkey.

Electron Microscopy

Biopsied material was immediately fixed for 1 hour in 2.5 per cent glutaraldehyde in 0.5 X Dulbecco's phosphate buffered saline (PBS) and 0.1 M cacodylate buffer, pH 7.2. During fixation the tissue was minced into 1 mm square pieces to allow adequate penetration of the fixative.

For preparation of cell pellets, monolayers of SMRV-infected A 204 and FCf 2 Th cells and MPMV-infected A 204 cells were fixed in situ with 2.5 per cent glutaraldehyde in 0.5 X Dulbecco's PBS and 0.1 M cacodylate buffer, scraped, and sedimented immediately by low-speed centrifugation. The cell pellets and minced tissue were post-fixed in 1 per cent osmium in 0.2 M cacodylate buffer for 1 hour and en bloc stained in 0.25 per cent uranyl acetate (UA) in 4.5 per cent sucrose buffer. Specimens were dehydrated in graded ethanols, infiltrated with and embedded in Epon 812, and polymerized at 50° C for 24 hours followed by 70° C for 48 hours. Thin sections were cut on an LKB Ultrotome III equipped with a diamond knife, mounted naked on 300 mesh copper grids, and double-stained with a saturated UA solution (14) and Reynold's lead citrate (21). The mounted sections were stabilized by evaporating a thin layer of carbon onto the grids.

Purified virus was evaluated by negative stain (23) and critical point drying (CPD) techniques (9). Negative staining was performed by diluting (at a ratio of 1:4) one drop of virus suspension with 2 per cent phosphotungstic acid (PTA), pH 4.2. A drop of virus was allowed to adsorb to a glow-discharged carbon-coated Formvar 200 mesh grid and excess liquid was drawn off by absorption with filter paper. The grids were allowed to air dry.

UA/CPD was performed according to the methods of DE HARVEN *et al.* (9) with modifications noted. A drop of unfixed whole virus was allowed to adsorb to a carbon-

coated Formvar 300 mesh copper grid for 10 minutes with care to avoid air drying. The grids were placed in a Bomar CPD grid holder (Bomar Co., Tacoma, Washington), washed in 0.5X Dulbecco's PBS, pH 6.0, and fixed for 10 minutes in 1 per cent glutaraldehyde in the same buffer, followed by a second wash in buffer. The virus was dehydrated for 3 minutes in each of the graded ethanols containing 1 per cent UA followed by three changes of 100 per cent ethanol. The virus was then infiltrated and critical point dried with Freon (6).

Observations and micrographs of all thin sections and negative stains were made with an Hitachi HU-12A or HU-12 operated at 75 kV. Observations and micrographs of UA/CPD-prepared samples were made at 125 kV.

Results

Comparison of Morphogenesis of SMRV and MPMV

SMRV

SMRV-infected A204 cells demonstrated intracytoplasmic, membrane-associated, and free extracellular particles. The SMRV intracytoplasmic particles (Fig. 1 a—d), analogous to type A particles of MPMV, were most frequently found within enclosures of rough endoplasmic reticulum (RER). These particles were electron-dense and doughnut-shaped with a more electron-lucent center and an average diameter of 88 nm. In these RER areas containing virus the RER membrane was devoid of associated ribosomes. Often an electron-dense coating could be discerned surrounding the intracytoplasmic particles (Fig. 1 b and Fig. 1 c). As shown in Figure 1 c, it was not unusual to find crescent-shaped intracytoplasmic particles. In some instances the dense coating was absent and small projections were seen on the outer surface (Fig. 1 d).

Two distinct budding virus types were found in SMRV-infected A204 cells. The most prevalent form consisted of an intracytoplasmic A particle incorporated into a bulging plasma membrane (Fig. 2 a and 2 b). The transitional form of this could be found with the membrane further enveloping the nucleoid until a stalked particle resulted (Fig. 2 c). The inner membrane of the envelope was thicker and more electron-dense than the outer. A more electron-lucent region separated the viral core from the envelope. Although many of these stalked particles had doughnut-shaped nucleoids similar to the virus form seen in the cytoplasm, an equal number of budding viruses had round electron-dense cores, although they were still attached to the plasma membrane (Fig. 2 d). A second budding virus form was found in which a crescent-shaped nucleoid, similar to that seen in the cytoplasm, formed under the plasma membrane, resembling the morphogenesis of type C oncornaviruses (7) (Fig. 2 e and 2 f). It is presumed that the crescent-shaped nucleoid continues to develop into the completed doughnut form described above since no mature type C virions were encountered in extracellular spaces.

Extracellular mature virus believed to be derived from those particles seen budding at the plasma membrane were found in great numbers in the extracellular spaces (Fig. 3 a). These virions had a centrally located electron-dense core surrounded by the viral envelope. Extracellular particles averaged 140 nm in diameter. The viral nucleoid itself was surrounded by a double membrane tightly bound to the core (Fig. 3 a, inset). This membrane could also be discerned surrounding the core in budding virions (not shown).

Another morphologic type of extracellular virus had central doughnut-shaped cores with electron-lucent centers. These are believed to be either early forms of free extracellular virus or images arising from the plane of section of viruses still attached to some cell surface structure (e.g., microvilli) not visible in the plane of section (Fig. 3 b). Another frequently encountered form of mature extracellular virus had 2 to 3 viral nucleoids within one viral envelope (Fig. 3 c). Also found were budding forms of virus, primarily those that closely resembled type C virus,

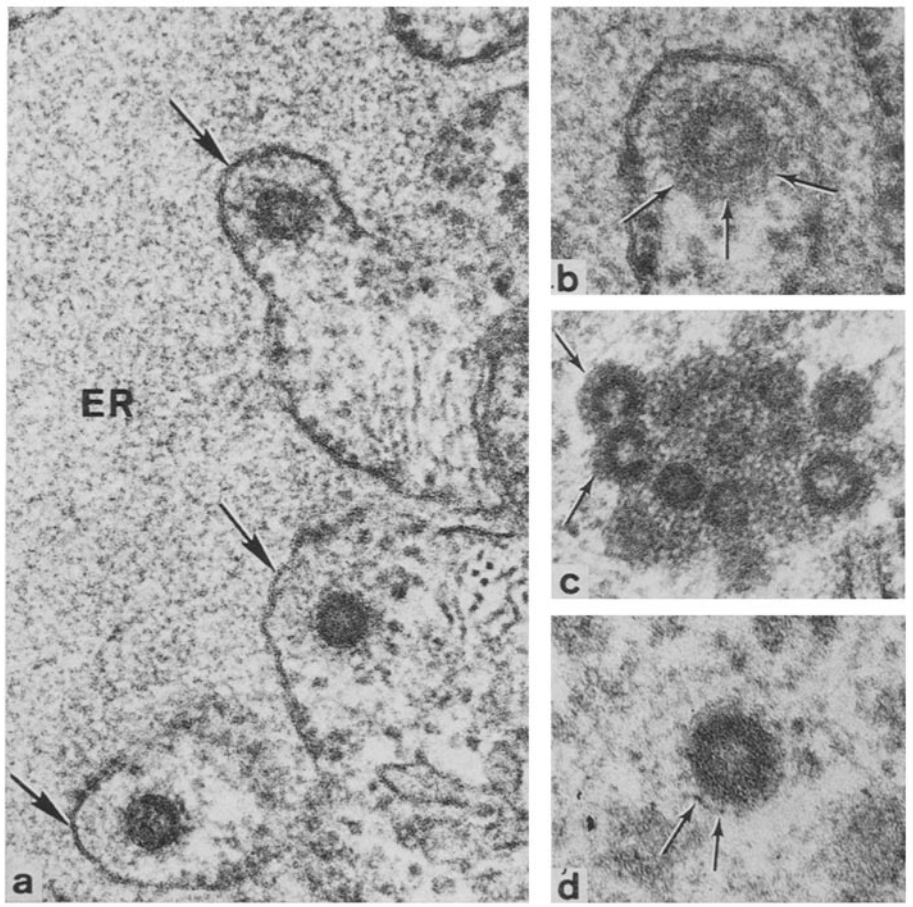

Fig. 1. SMRV intracytoplasmic A particles in infected A 204 cells

a. Electron-dense doughnut-shaped particles with more electron-lucent center enclosed in fold of expanded RER. Note that RER membrane surrounding the virions is devoid of associated ribosomes (→), × 81,000

b. Higher magnification of expanded RER with doughnut-shaped particle showing electron-dense coating surrounding nucleoid (→), × 112,700

c. Cluster of doughnut- and crescent-shaped (→) intracytoplasmic nucleoids with electron-dense coatings, × 77,000

d. Intracytoplasmic A particle with projections surrounding perimeter of nucleoid (→), × 140,000

in which 2 to 3 nucleoids were forming in one enlarged area of the cell membrane (Fig. 3 d). It is conceivable that the mature extracellular virion containing more than one nucleoid arose from these cell-associated forms. Although only results of SMRV grown in A 204 have been discussed in detail here, SMRV showed a similar morphogenesis in the FCf 2 Th canine cells originally obtained from Dr. Heberling.

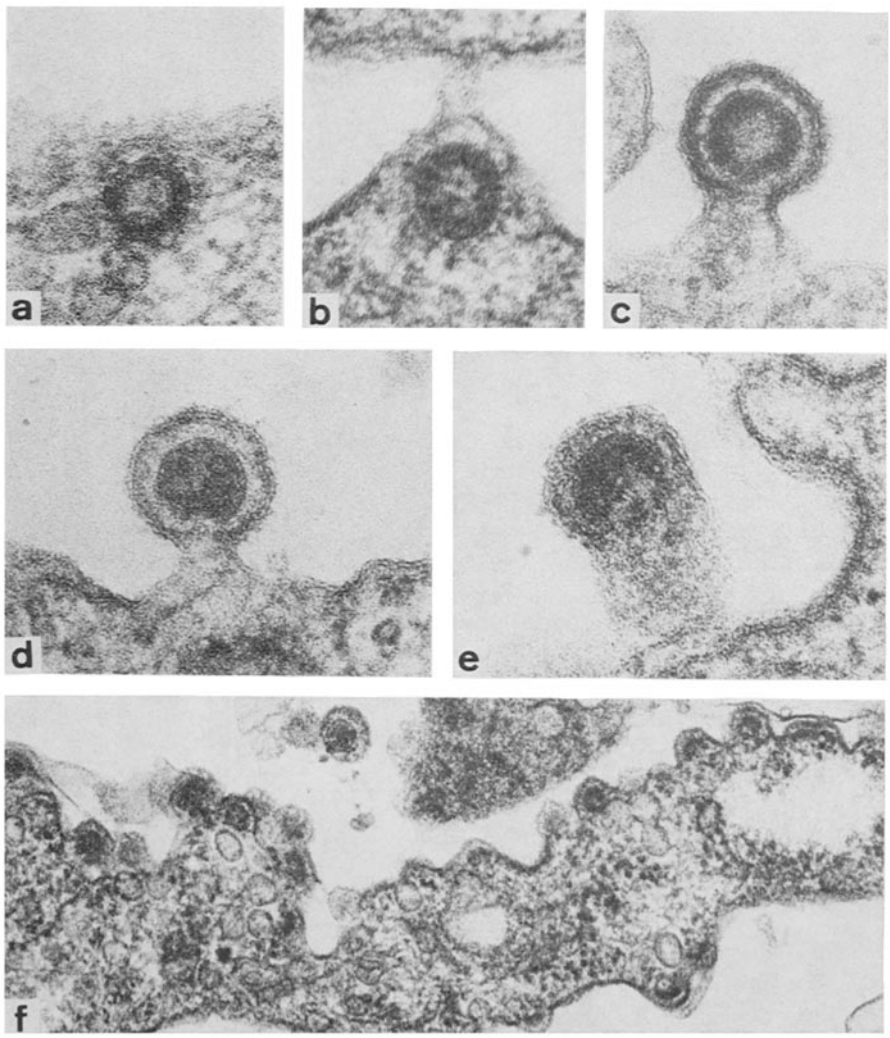

Fig. 2. Morphogenesis of budding SMRV in infected A 204 cells

a. Intracytoplasmic A particle directly underlying plasma membrane

b. Later stage of budding showing initial envelopment of nucleoid by plasma membrane

c. Stalked particle with doughnut-shaped nucleoid

d. Budding stalked particle with condensed electron-dense core

e. Budding SMRV with electron-dense crescent-shaped nucleoid resembling type C oncornavirus maturation, 2 A—E, × 140,000

f. Area of plasma membrane with many underlying crescent-shaped nucleoids, × 45,000

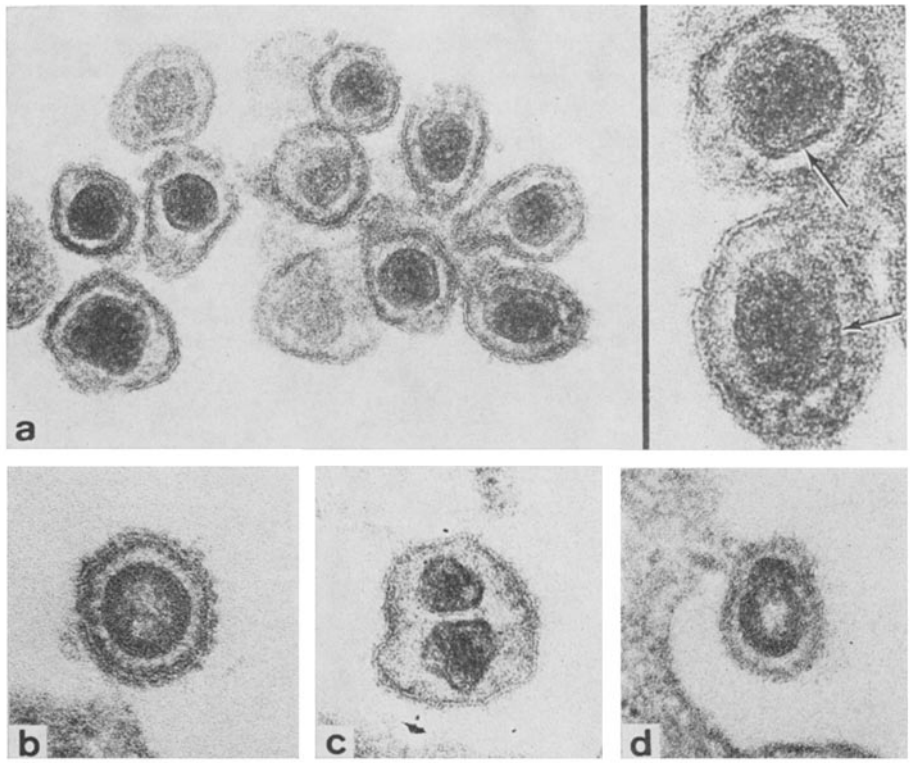

Fig. 3. Mature free extracellular SMRV virion types from infected A 204 cells

a. Aggregation of mature extracellular virion with centrally located electron-dense cores. Magnified inset shows double membrane coat bound to core (→), × 92,000; inset, × 178,500

b. Second form of free extracellular mature SMRV consisting of central electron-dense doughnut-shaped core with electron-lucent center, × 143,500

c. Mature SMRV particles containing two electron-dense nucleoids bounded by single viral envelope, × 104,000

d. Two nucleoids developing within one viral envelope while still attached to plasma membrane, × 108,500

MPMV

The intracytoplasmic A particles of MPMV from A 204 cells were electron-dense doughnut-shaped particles averaging 107 nm in diameter (Fig. 4 a). Projections were clearly visible along the outer perimeter of the nucleoid. Dimensions for the intracytoplasmic nucleoid include these projections.

MPMV nucleoids were enveloped at the plasma membrane (Fig. 4 b) of either vacuoles or the limiting cell membrane. More progressive forms of budding virus are depicted in Figures 4 c and 4 d, where the plasma membrane further surrounds the emerging nucleoid. A more electron-lucent region separates the viral core from the envelope.

Two types of free extracellular particles were found. The first were spherical particles with a doughnut-shaped electron-dense nucleoid with a more electron-

lucent center (Fig. 4e), resembling intracytoplasmic A particles. The more predominant form of extracellular particle was a round particle averaging 130 nm in diameter that resembled the Greek letter Θ (Fig. 4f). We believe this form to develop from the condensation of the spherical nucleoid resulting in the formation

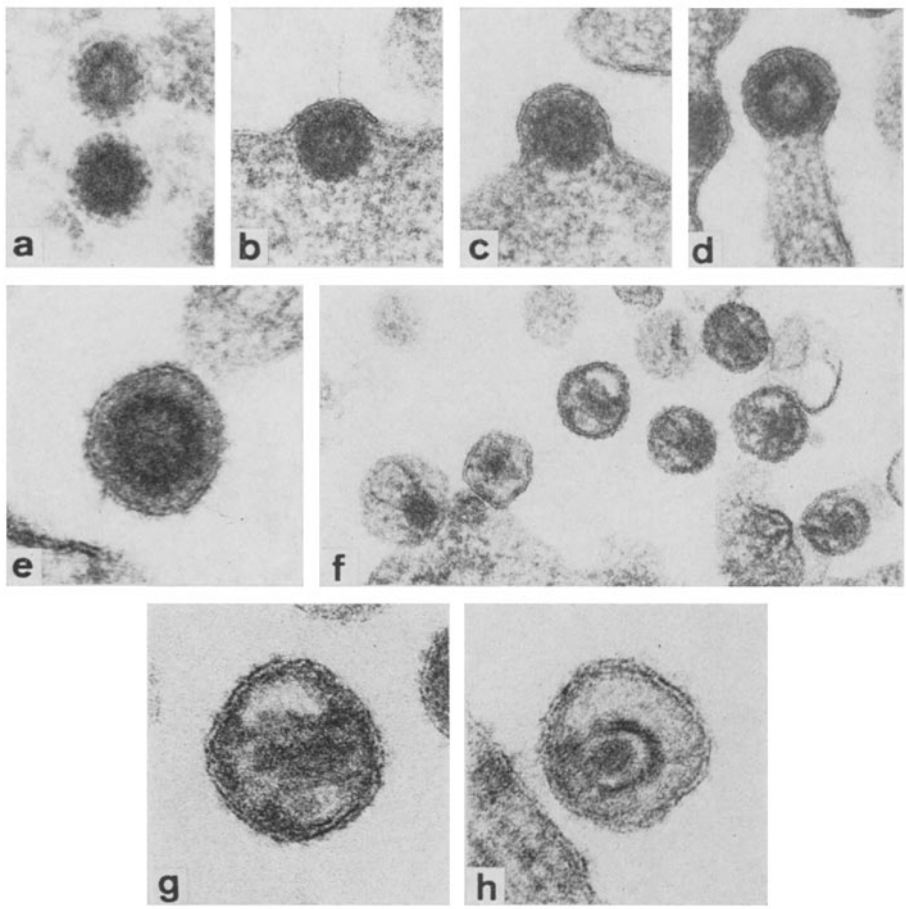

Fig. 4. Morphogenesis of MPMV in thin sections of infected A 204 cells

a. Intracytoplasmic A particles containing electron-dense doughnut-shaped virions with projections on outer perimeters

b, c. Early stages of budding in which intracytoplasmic A particle is enveloped by plasma membrane

d. Stalked late-budding particle with electron-dense doughnut-shaped nucleoid and electron-lucent center. 4a—d, × 93,000

e. Free extracellular MPMV particle containing electron-dense doughnut-shaped nucleoid with viral envelope. × 144,000

f. Free extracellular mature MPMV particles which are essentially round and contain tube-shaped nucleoids, × 63,600

g. Mature extracellular particle demonstrating tubular nucleoid at higher magnification, × 144,000

h. Mature MPMV particle resulting from plane of section being perpendicular to that in Figure 4g, × 143,500

of a bipolar tube-shaped nucleoid. The tube appears to be composed of a double membrane-like outer coat with a more electron-dense core (Fig. 4 g and 4 h).

EM of Density Gradient Purified Virus

Purified virus from tissue culture was examined by negative staining with 2 per cent PTA. Both SMRV (Fig. 5 a) and MPMV (Fig. 5 b) yielded primarily tailed particles (approximately 150 nm in diameter) with surface projections covering the perimeter of the virus. Spherical particles with surface projections were also encountered with both viruses (not shown).

When SMRV and MPMV were examined by UA/CPD, the following was observed. SMRV (Fig. 5 c) virions were essentially round to ellipsoidal, approximately 102 nm in diameter, with an eccentric electron-dense nucleoid. The envelope was a double unit membrane without surface projections, in contrast to the negative stained virus shown in Figure 5A. MPMV virions (Fig. 5 d) were also

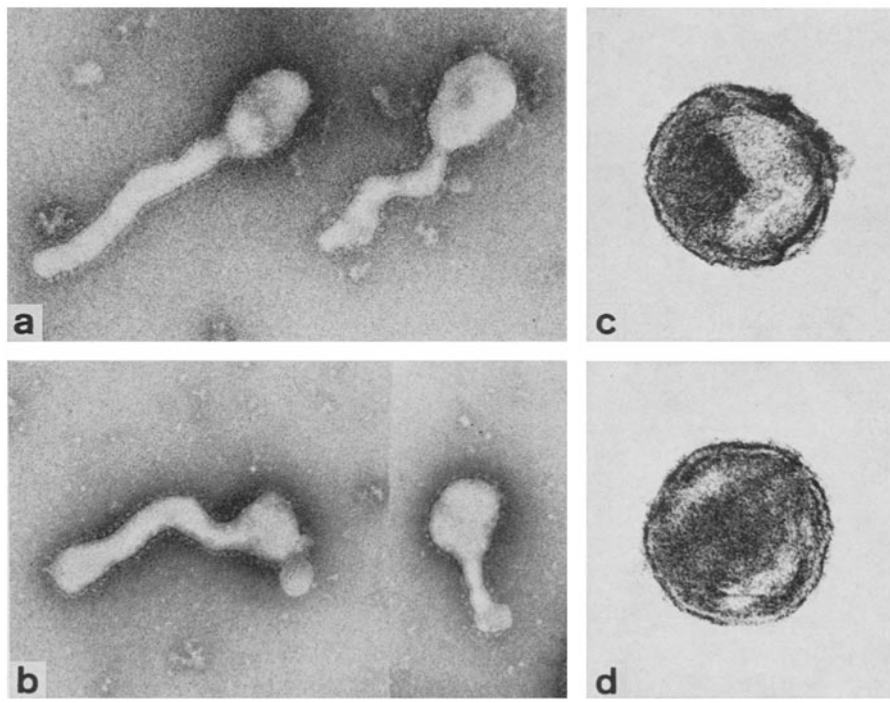

Fig. 5. SMRV and MPMV purified virus suspensions prepared by PTA negative staining and UA/CPD techniques

a. Negative stain of SMRV showing tailed particles with small surface projections, × 86,000

b. Negative stain of MPMV showing tailed particles with small surface projections, × 86,250

c. UA/CPD-prepared SMRV showing spherical particles with eccentric electron-dense nucleoids surrounded by double unit membrane, × 240,000

d. UA/CPD-prepared MPMV particles showing spherical particles containing bipolar condensed bar-shaped core surrounded by viral envelope, × 240,000

round to ellipsoidal, approximately 101 nm in diameter, with a double unit membrane surrounding a condensed bar-shaped core (analogous to the tubular structure seen in thin sections).

Thin sections of SMRV and MPMV viral pellets yielded results similar to those described above for extracellular particles in cell pellets (Figs. 3a—c and 4f—h) and are not shown here.

Discussion

Although SMRV and MPMV have similar morphogenetic development, they can be distinguished on the basis of the morphology of mature extracellular particles in thin section. The most discriminating characteristic of mature extracellular SMRV is the centrally located electron-dense nucleoid, as compared with the bipolar-positioned tubular nucleoids of MPMV (compare Fig. 3a and Fig. 4f and 4g). Mature SMRV having tubular nucleoids were not encountered in SMRV from A204 or SMRV-infected FCf2Th cells.

SMRV was also found to bud from the cell membrane in two forms: a) as preformed intracytoplasmic A particles enveloped by the plasma membrane, and b) as crescents under a bulging plasma membrane. The formation of a crescent-shaped nucleoid typical of type C oncornaviruses (7) is not unique to SMRV and has been reported recently for MPMV (2). Although we did not find budding MPMV developing as crescent-shaped nucleoids in the A204 cells, we have observed these forms in MPMV-infected rhesus monkey foreskin cells (unpublished data).

In an attempt to visualize SMRV *in vivo*, biopsied material from two healthy male squirrel monkeys was examined by thin section EM. None of these samples revealed SMRV or any other virus (data not shown). The absence of a morphologically recognizable virus in tissues from squirrel monkey and the ability to isolate SMRV when squirrel monkey lung tissue is cocultivated with mammalian cells other than squirrel monkey supports the contention that SMRV is a xenotropic endogenous virus of squirrel monkeys (16). This is supported by recent nucleic acid hybridization studies (Schlom, personal communication).

These observations and previously published results (11, 16, 22) support the candidacy of SMRV for genus oncornavirus type D. However, its antigenic, biochemical, and morphological properties distinguish it from MPMV, presently the only member of this genus. SMRV therefore represents a new species within genus oncornavirus type D.

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Authors' address: Dr. M. A. Gonda, Viral Oncology Program, National Cancer Institute, Frederick Cancer Research Center, P.O. Box B, Frederick, MD 21701, U.S.A.

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