JOURNAL OF VIROLOGY, Aug. 1970, p. 237-242
Vol. 6, No. 2

Morphogenesis of Respiratory Syncytial Virus in a Green Monkey Kidney Cell Line (Vero)

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Received for publication 4 May 1970

The structure and morphogenesis of respiratory syncytial (RS) virus particles in a green monkey kidney cell line (Vero) were examined. Infected cells contained dense intracytoplasmic inclusions composed of filamentous structures. In places where inclusion material was associated with membranes, structural modifications were induced. There was a thickening of the membrane and an addition of projections 12 to 15 nm in length. The same changes were most frequently observed after association of isolated filamentous structures with the cytoplasmic membrane. The budding-off process was clearly visualized. The diameter of mature virus particles varied between 90 and 130 nm and that of the internal component varied between 11 and 15 nm. The similarities between ultrastructural features of cells infected with RS virus and pneumonia virus of mice are pointed out. It is proposed that these two viruses should be classified together in a third subgroup of myxoviruses.

Respiratory syncytial (RS) virus particles contain ribonucleic acid associated with a helical nucleocapsid surrounded by an envelope. The fine structure of virion components has been determined in the electron microscope by use of the negative-contrast technique. In general, similar findings have been made, although the diameter of the relatively fragile nucleocapsid structure has been estimated to vary between 12 and 18 nm (3, 4, 8, 12, 16).

To obtain additional information on the structure of RS virus particles and also on their morphogenesis in cells, electron microscopic examination of infected cells was undertaken. The results obtained were considered to have some implications on the classification of RS virus.

MATERIALS AND METHODS

Virus and cell culture. The Long strain of RS virus was used. Both stock virus material used in earlier studies (3, 4) and fresh seed material obtained from the American Type Culture Collection, Rockville, Md., were used. The serological specificity of the strains was controlled in neutralization tests employing a reference horse hyperimmune serum against the virus (lot no. 830008, Flow Laboratories, Irvine, Scotland).

Vero cells, originally supplied to this laboratory by H. Liebhaber, Yale University School of Medicine, New Haven, Conn., were maintained on Eagle's minimal essential medium plus 3% inactivated calf serum. The cultures were seeded with virus at input multiplicities of 0.01 to 0.1. Samples for examination of morphological changes of infected cells were taken at different times after the appearance of distinct cytopathic degeneration.

Light microscopy. Cell monolayers for examination by light microscopy were grown on cover slips in Leighton tubes. After fixation in methanol, cells were stained with hematoxylin-eosin by using conventional techniques. Photographs were taken on Kodak Plus-X ASA 125 black and white film.

Electron microscopy. The virus was propagated in bottles containing about 10^7 cells. When a clear-cut cytopathic degeneration had developed, the bottles were harvested. The medium was discarded and the monolayer was washed once in fresh medium followed by the addition of a 3.0% glutaraldehyde solution in phosphate buffer (0.2 M, *p*H 7.2). The bottles were fixed for 10 min at room temperature, and the cells were removed with a "rubber policeman" and centrifuged at 150 × *g* for 10 min. Cell pellets were post-fixed in 0.5% osmium tetroxide and dehydrated in ethanol. They were then embedded in Araldite and sectioned with an LKB Ultratome. The sections were stained with 6% uranyl acetate in methanol and lead citrate by the method of Reynolds (15). The stained sections were examined in a Philips EM200 electron microscope.

RESULTS

Light microscopy. The cytopathic changes caused by RS virus multiplication in Vero cells can be seen in the hematoxylin-eosin stained preparations in Fig. 1. Small syncytia containing, at most, 20 to 25 nuclei and isolated cells displaying cytopathic degeneration were found. The relative

number of cells participating in the formation of syncytia varied from one preparation to the other. Usually they did not dominate the picture. The general outline of infected cells became irregular. Intensively stained eosinophilic inclusions were seen in the cytoplasm. The periphery of the inclusions either had a distinct or a more diffuse appearance. The latter kind of inclusion frequently occurred in the center of small syncytia surrounded by nuclei. There were no marked changes of nuclear structures until the cells started to become pycnotic.

Electron microscopy. Ultrastructural changes appearing during multiplication of RS virus can most readily be presented by reference to different compartments of cells and also to different stages of virus maturation.

Cell nuclei. No major changes of nuclear structures were seen even in cultures displaying extensive cytopathic changes (Fig. 2a). In a few cells, a slight margination of the chromatin was noticed.

Cell cytoplasm. Compact inclusions appeared in large numbers during late stages of virus multiplication (Fig. 2a to c). At higher magnification, the inclusions could be seen to have a granular or threadlike appearance. Only occasionally could free-lying filamentous structures with dimensions corresponding to those of structures associated with inclusions be seen. Additional changes of cytoplasmic structures were of a more general degenerative character, including a marked accumulation of glycogen granulae.

Maturation of virions. The maturation of virus particles occurred at the cytoplasmic membrane or at the membrane of vesicles present within the cytoplasm. The possibility that the latter structures represent invaginations of the cytoplasmic membrane cannot be excluded. Distinct changes were seen at points where dense cytoplasmic inclusions came into direct contact with membranes of vesicles (Fig. 2b, c). There was a thickening of the membrane as well as an addition of short fringelike structures. The corresponding morphological modifications of membrane structures were seen at the surface of cells (Fig. 3 and 4a). In this case, the transformation of the triple-layered membrane occurred without any visible association with inclusion structures. Instead, a submembranous occurrence of distinct dots (interpreted to represent cross sections of filamentous components) with a diameter of 11 to 15 nm was frequently seem. Four to five dots appeared beneath the membrane of individual particles half way through their budding-off process (Fig. 3a). On a few occasions, it was observed that the dots seemed to be associated with cytoplasmic filamentous structures. In thin sections (Fig. 3a), the length of the membrane projections was estimated to be 12 to 15 nm. Some tendency for clustering of projections was observed. The budding-off process was readily visualized (Fig. 3a to c). The final particles displayed a certain pleomorphism (Fig. 4b). However, many of them exhibited a diameter falling within the range of 90 to 130 nm. The internal component of many virions was represented by dots (maximum number, 10 to 12 per particle) with a symmetrical circular arrangement.

Elongated forms budding off from the cytoplasmic membrane were frequently encountered (Fig. 4a). They displayed a relatively uniform diameter of about 100 to 130 nm. Their length, in some cases, exceeded 2 μm. In their center, they

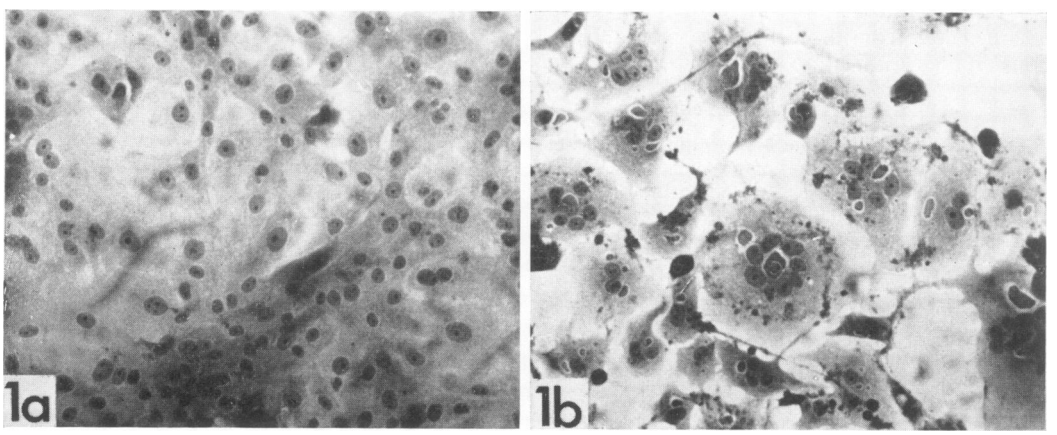

FIG. 1. *Cytopathology of RS virus infection in Vero cells. (a) Uninfected and (b) virus-infected culture. Note the appearance of intracytoplasmic inclusions and small size syncytia in (b). Stained with hematoxylin-eosin.*

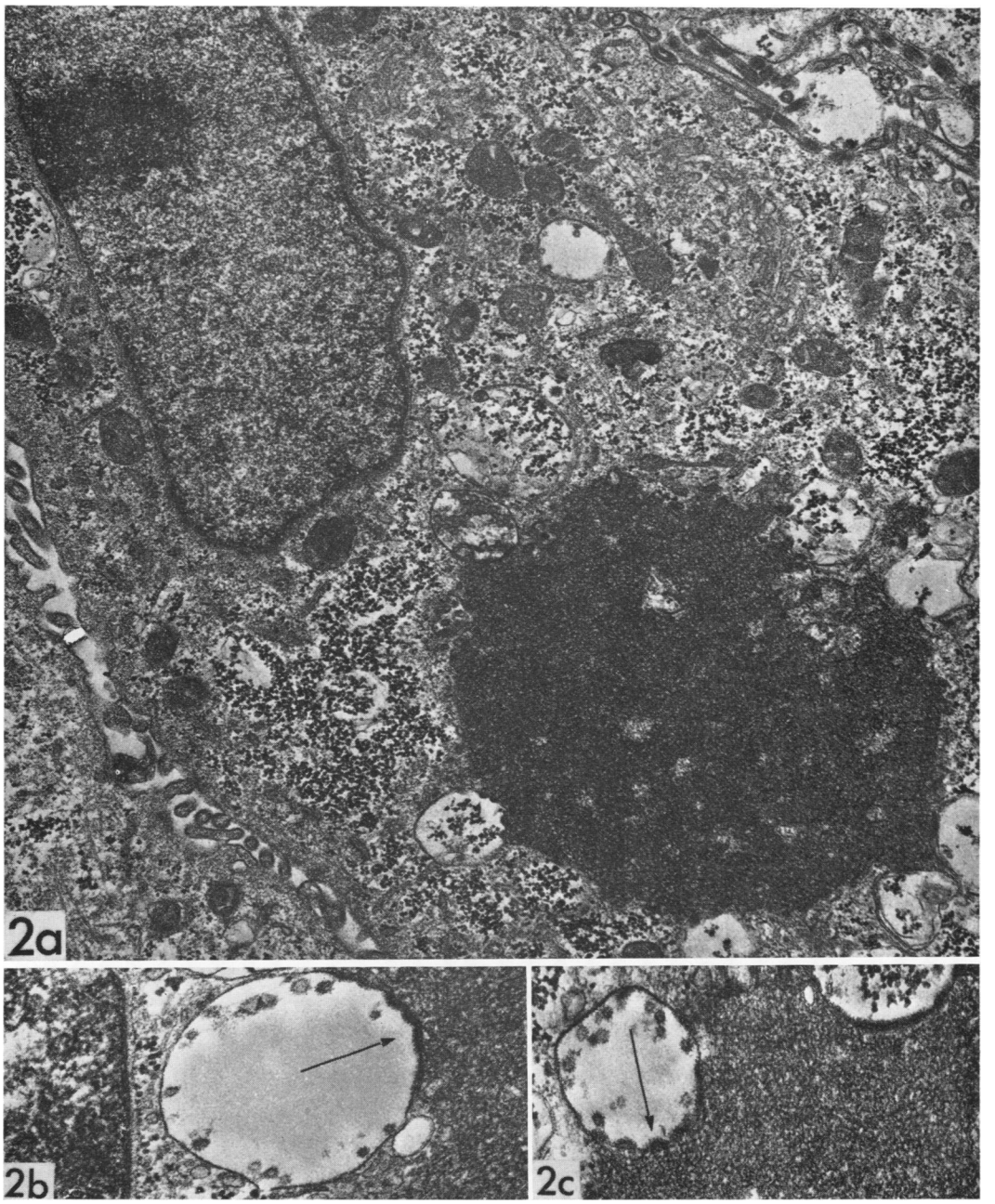

FIG. 2. *Fine structure of dense intracytoplasmic inclusions in RS virus-infected Vero cells. The cell in (a) displays a large inclusion as well as intracytoplasmic accumulation of glycogen granulae and budding of virus particles at the cell surface. At the point of contact (arrows) between intracytoplasmic inclusions and vacuoles in (b) and (c), a morphological transformation of the membrane can be seen. (a) × 24,000; (b) and (c) × 34,000.*

contained filamentous structures seen either in longitudinal or cross sections. The tips of filaments often had the appearance of a budding-off virus particle.

DISCUSSION

The cytopathology of RS virus multiplication in Vero cells does not include the formation of extensive syncytia. This is in agreement with pre-

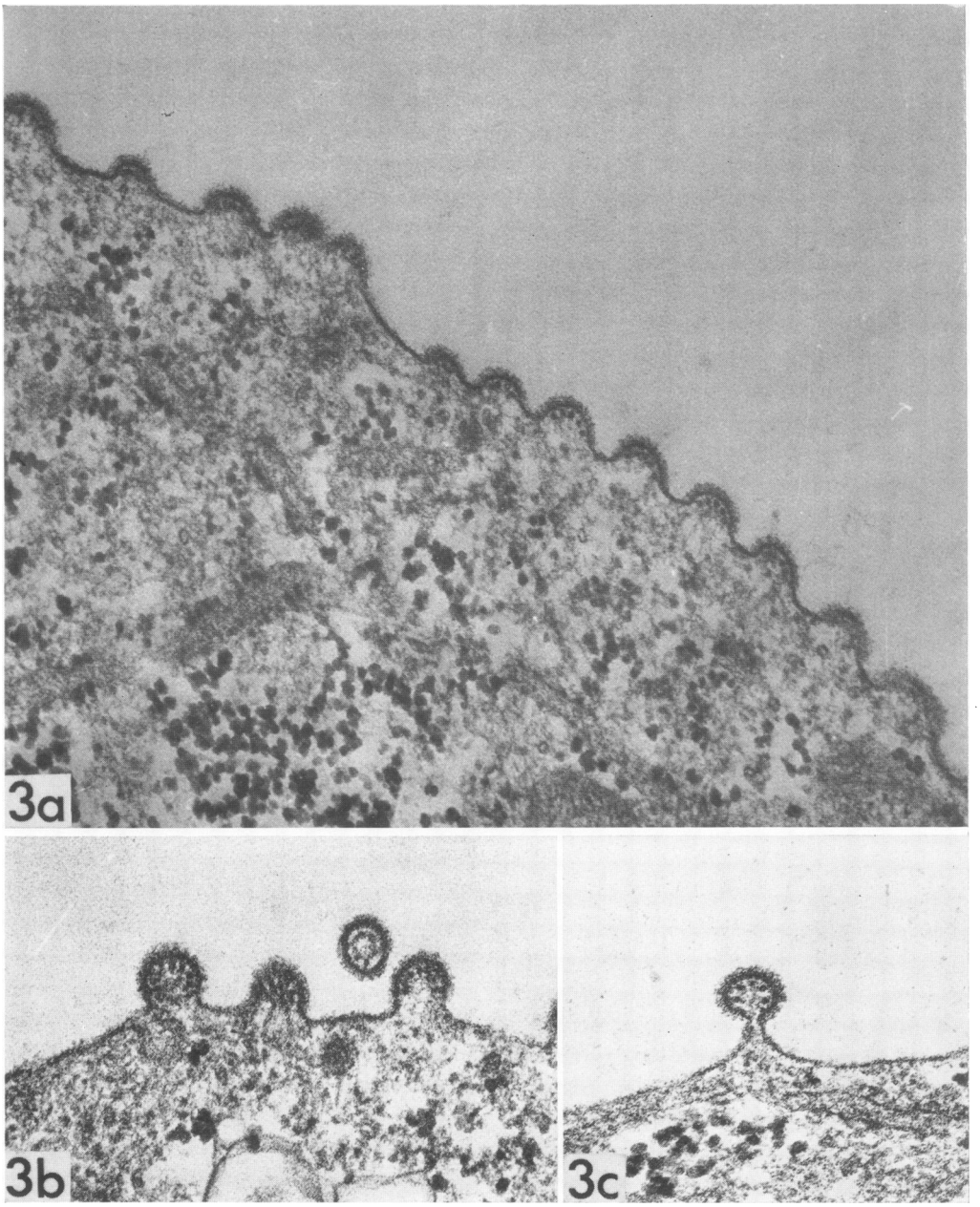

FIG. 3. *Different stages of the budding-off process of RS virus particles from the cytoplasmic membrane of Vero cells. Note the appearance of fringelike projections, thickening of membranes, and presence of submembranous structures (symmetrically arranged dots when cross-sectioned) at places where budding occurs. (a) × 75,000, (b) × 76,000, and (c) × 86,000.*

vious reports (13) that RS virus, in spite of its name, causes effects of this kind only in certain cell systems. In the present study of morphogenesis of RS virus, only cultures displaying a distinct cytopathic degeneration were examined. No attempts were made to study separately early events of virus multiplication.

The general features of RS virus morphogene-

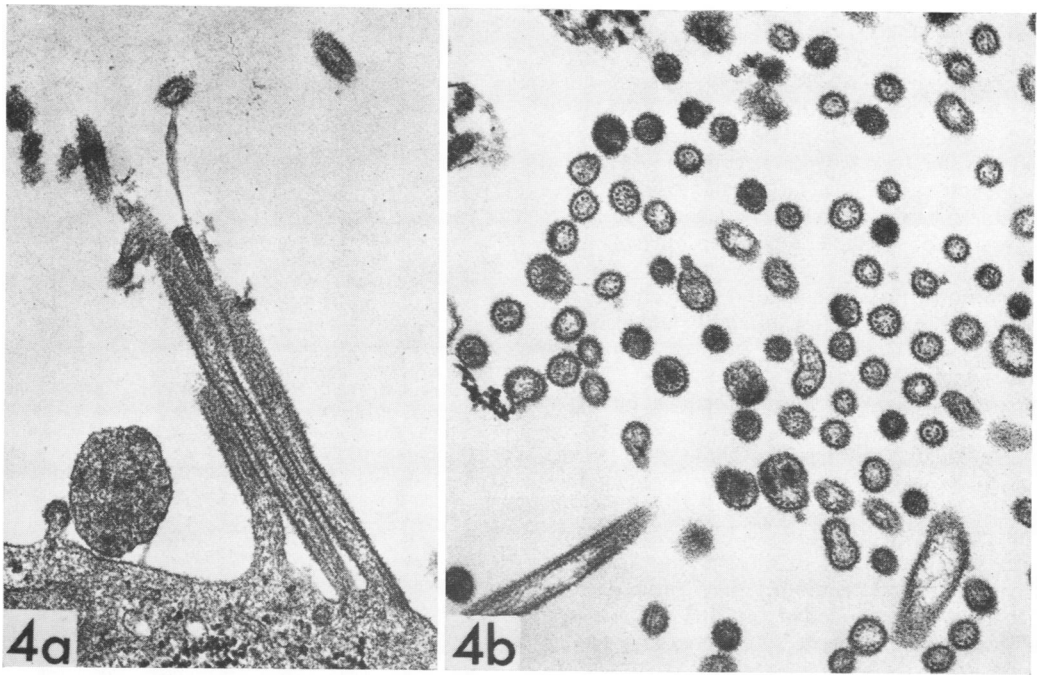

FIG. 4. *Examples of (a) elongated forms of virus particles extending from an infected cell and of (b) free-lying virus particles. Internal component(s) can partly be seen in longitudinal sections within the filamentous particles. In (b), cross sections of internal components of a number of particles have a symmetrical, circular arrangement, suggesting an organized packing of this component. (a) × 42,000 and (b) × 36,000.*

sis, as presented above, resemble that of a myxovirus. This was also concluded in the only available publication on electron microscopy of RS virus-cell (HeLa) interactions (1). In this limited study, the particle diameter was estimated to be 65 nm, a value difficult to reconcile with findings in the present study. Furthermore, the intranuclear inclusions described to occur in HeLa cells were not seen in Vero cells.

Within the group of myxoviruses, two distinct subgroups can be distinguished. In addition, pneumonia virus of mice (PVM) has been proposed to represent a third subgroup (7). Earlier attempts to determine the subgroup membership of RS virus were based on ultrastructural examination of negatively contrasted virus components. The general experience in these studies was that both RS virions and nucleocapsids display a relatively high degree of lability. The diameter of the nucleocapsid has been estimated to be between 12 and 18 nm (3, 4, 8, 12, 16). In the most recent publication (12), the diameter was given as 12.5 to 15 nm, and it was pointed out that the nucleocapsid of RS virus resembled that of PVM. A comparative analysis of the ultrastructure of RS virus-infected Vero cells with that described for other myxoviruses seems to give further support to the hypothesis of a relationship between RS virus and PVM. The following features seem to be of particular importance.

Intracytoplasmic inclusions. The dense intracytoplasmic inclusions (presumably representing internal components) induced by RS virus resemble those found in PVM-infected cells (7). In contrast, intracytoplasmic inclusions observed in cells infected with paramyxoviruses are composed of loosely arranged nucleocapsids with a diameter of 17 to 18 nm (2, 6, 9, 11, 14).

Particle size. Although a certain variation in size occurred among RS virions seen in sections, a good number fell within the range of 90 to 130 nm. This range of diameter values tallies well with that given for PVM particles (7), whereas paramyxoviruses usually are described to have a diameter of 120 nm or more.

Nucleocapsid diameter. Nucleocapsid structures were readily identified in sectioned RS virus particles. In most cases, dots (cross sections) with a symmetrical distribution were seen, suggesting an orderly arrangement of the internal component. The diameter of cross-sectioned nucleocapsids was 11 to 15 nm. This value agrees with that previously described for PVM (7) and negatively contrasted RS virus components (12, 16), but is

higher and lower than that given for influenza viruses (9 nm) and paramyxoviruses (17 to 18 nm), respectively (10). If the value 11 to 15 nm is the "true" value for the diameter of RS virus nucleocapsids, it remains to be explained why, under some conditions, negatively contrasted components display a larger diameter (4). One possible explanation may be changes in the degree of twisting of the labile nucleocapsid structure of RS virus.

Additional properties shared between RS virus and PVM are their extreme fragility and also their tendency to cause the formation of filamentous particles. As regards the relationship of RS virus to paramyxoviruses, some differences in biological characteristics may be pointed out. Differences in their cytopathology have been reported (5). RS virus preparations of high antigen content have not been found to be capable of giving any demonstrable agglutination or lysis of red blood cells, activities carried by most paramyxoviruses. It can finally be mentioned that features of the syncytium-forming activity of RS virus appear to be somewhat different from those of paramyxoviruses (Norrby, *unpublished data*).

On the basis of the above-mentioned structural and biological characteristics of RS virus and PVM, as compared to those of other myxoviruses, it is suggested that the former two viruses should be classified together as a separate subgroup of myxoviruses.

ACKNOWLEDGMENTS

The technical assistance of Ylva Gollmar and Margareta Jurstrand is gratefully acknowledged.

This investigation was supported by grants from the Swedish Medical Research Council (project no. B70-16x-744-05).

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