JOURNAL OF VIROLOGY, Nov. 1973, p. 1173-1180

Vol. 12, No. 5
Printed in U.S.A.

Morphogenesis and Ultrastructure of Respiratory Syncytial Virus

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Received for publication 25 July 1973

Respiratory syncytial (RS) virus was grown in Vero cells and fixed for electron microscopy at various stages of maturation. Both filamentous and round or kidney-shaped particles, either with (complete) or without (incomplete) internal structure, were observed. All four morphological forms were identical with respect to their reactivity with ferritin-labeled antibody to RS virus. Freeze-etching revealed a structural feature apparently unique for RS virus, namely helical striations around the core on the internal aspect of the envelope. This specific configuration was already detectable during the early stages of viral differentiation of the host cell membrane. Concentration of free virus by zonal ultracentrifugation of culture fluids onto sucrose cushions yielded predominantly filamentous forms up to 10 μm in length.

Ultrastructural characteristics of respiratory syncytial (RS) virus have been used as the basis for tentative classification of this agent together with pneumonia virus of mice (PVM) and bovine RS virus as a separate subgroup of myxovirus (6, 8, 11, and 15). Studies on the morphogenesis of these viruses have revealed round or filamentous particles which mature by a budding process at the surface of infected cells. Attempts to discern structural features of RS virus and its components by the negative-staining technique have been handicapped by the extreme fragility of the virion (7, 11). The experiments reported here represent an effort at further characterization of RS virus with immunoferritin techniques and a comparison of its appearance in negatively stained, thin-sectioned, and freeze-etched preparations.

MATERIALS AND METHODS

Cells. Vero and KB cells were grown in Eagle minimal essential medium (MEM) supplemented with 10% inactivated fetal calf serum (FCS). Before infection, the monolayers were washed with Earle balanced salt solution (EBSS) at 37 C. After inoculation with virus, cells were maintained in MEM containing 2% FCS with 0.1% lactalbumin hydrolysate (LH).

Virus. RS virus (obtained from the American Type Culture Collection) was maintained by passage in Vero or KB cells. Washed, confluent monolayer cultures were infected with passage fluid at a dilution of 10^{-1} to 10^{-3} and maintained for 1 to 6 days in LH medium containing 2% FCS. Stock virus was usually harvested from infected cells 3 days later by scraping off the cells from the plastic surface and centrifuging for 10 min at 2,000 $\times$ g at 4 C. The supernatant fluid was then used for reinoculation of fresh cultures or was frozen and stored at −70 C. Infectivity titrations were carried out in replicate monolayers of Vero cells. Cultures were exposed to decimal dilutions of virus on a rocker at 37 C for 2 h. After washing with EBSS, infected cultures were maintained in LH medium for 6 days, and then were stained with Lillie azur-eosin to establish end points for calculation of mean tissue culture infective dose ($TCID_{50}$). In order to concentrate free virus, 500-ml volumes of culture fluid from infected Vero cells were centrifuged in a Ti-14 zonal rotor for 60 min at 4 C at 120,400 $\times$ g onto a cushion of 55% sucrose. Sucrose was removed by overnight dialysis of concentrated virus against phosphate-buffered saline (PBS), pH 7.2.

Antisera. Antiserum was prepared to RS virus grown in KB cells. After low-speed centrifugation of infected cells, virus was pelleted from the supernatant fluid by centrifugation for 120 min at 50,000 $\times$ g onto a cushion of 75% sucrose. After dialysis of the virus-containing fraction against PBS, rabbits were immunized by four weekly footpad injections of virus in complete Freund adjuvant. The animals were bled out 10 days after the last injection. The sera were inactivated (56 C, 30 min) and exhaustively absorbed with lyophilized Vero cells. Absorbed antisera neutralized 10 to 100 $TCID_{50}$ of virus to high titers. Globulin fractions were conjugated with ferritin or fluorescein by methods previously published (9).

Electron microscopy. Vero cell monolayers were processed for electron microscopy before, or at various stages after, infection with RS virus. Cells to be fixed for thin sectioning were washed with cold EBSS while still attached to the plastic surfaces. A mixture of 3%

glutaraldehyde and 3% acrolein in 0.05 M cacodylate buffer, pH 7.2, was added at 4 C. After 10 min the cells were scraped from the plastic surface, centrifuged in the same fixation mixture, and left for 30 min at 4 C. The cells were then washed in two changes of 0.1 M cacodylate buffer containing 0.18 M sucrose and postfixed for 60 min with 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, at 4 C. The fixed material was left overnight in a 2% solution of uranyl acetate before dehydration and embedding in Epon-Araldite. As indicated subsequently, washed monolayers were incubated with ferritin-labeled antibody for 30 min at 20 C and washed free of unreacted antibody prior to fixation. All thin sections were stained with lead citrate.

For negative staining, a droplet of concentrated virus was put on a carbon-coated grid and mixed with the same amount of 3% glutaraldehyde in PBS, pH 7.2. After 5 min of fixation at room temperature, the grid was washed with PBS and then was stained with 2% phosphotungstic acid pH 7.2.

Cells to be subjected to freeze-etching were held for 20 min at 4 C in EBSS containing 25% glycerol without prior fixation, and were then frozen and replicated according to standard procedures (14).

RESULTS

Standard passage of the RS virus in Vero cells at a dilution of 10^{-2} to 10^{-3} resulted in a cytopathic effect first detectable by light microscopy at 48 h. The formation of characteristic syncytia became more pronounced at 72 to 96 h, when 80% of the monolayer was found to consist of polykaryons containing up to 30 nuclei. The infectivity of virus harvested at this time usually was 10^4 to 10^5 $TCID_{50}$ per ml. Budding of virus particles was occasionally detectable by electron microscopy of thin-sectioned material as early as 15 h after infection, well before syncytia and intracytoplasmic inclusions were detectable by light microscopy.

The particles formed at the surface of the plasma membranes of infected Vero cells displayed great pleomorphism. All structures which were readily identified by their morphology as viral particles also reacted with specific ferritin antibody to RS virus (Fig. 1–6). Invariably, virus-associated bulging of the surface membrane occurred at sites which displayed an electron-dense structure measuring 4 nm in thickness apposed to the inner leaflet of the unit membrane constituting the cell surface. On the outside of the plasma membrane, budding areas were distinguished by the presence of a fuzzy coat of variable electron density. Ferritin antibody to RS virus also reacted specifically with these discrete segments of membrane altered by nascent virus.

Besides these commonly observed features of developing RS virus, many viral particles were observed which varied widely in the appearance of internal structure and overall shape. Round or kidney-shaped particles (Fig. 1–3) with a diameter of 150 to 250 nm frequently, but not invariably, contained electron-dense dots with a diameter of 15 nm (Fig. 3). The same differences were reflected among budding filamentous particles with a uniform diameter of 90 to 100 nm. Thus, the filaments shown in Fig. 4 do not contain any well-defined internal structures. On the other hand, three to five parallel strands with a diameter of 15 nm were detected in the core of some of these elongated virions (Fig. 5). Cross-sections of these filaments (Fig. 6) showed the same internal components appearing as dots with a diameter of 15 nm.

The different forms of RS virus described above (round and filamentous particles, either empty or containing electron-dense internal component) usually were observed to occur simultaneously in infected cultures. The filamentous forms always outnumbered the round particles, and coreless filaments, in turn, seemed to be more frequent than those with distinct internal components. The duration of infection prior to fixation (15 to 96 h) did not influence the relative frequency with which any of the four different forms occurred; nor was it possible to control the pleomorphism of progeny virus by varying the input multiplicity of infecting virus.

Freeze-etching of infected cultures revealed predominantly filamentous forms of a caliber similar to those measured in the thin-sectioned material. Figure 7 shows a plasma membrane from which several filamentous virus particles appear to be budding. Within the plasma membrane itself, there are typical membrane particles in irregular, clustered distribution. The viral filaments appear as tube-like structures which have been fractured at two different levels. Either the major part of the virus has been broken away, leaving behind a concave shell, or the fracture plane has occurred along the top of the filament, resulting in removal of the envelope only and exposure of the convex structure of the core. The latter reveals distinct structures consisting of oblique striations with a pitch of 6 nm. The striations seem to form a helical structure with an angle of 60° around the core exposed by the removal of the envelope. The latter is visible as a rim measuring 15 nm at the outside of the core (Fig. 7, 9, and 10). The inner surface of the envelope, exposed by removal of the core, usually had a granular aspect. Budding particles were detectable at relatively early stages by differentiation of the cytoplasmic membrane. At sites at which maturation of virus was taking place, striations were

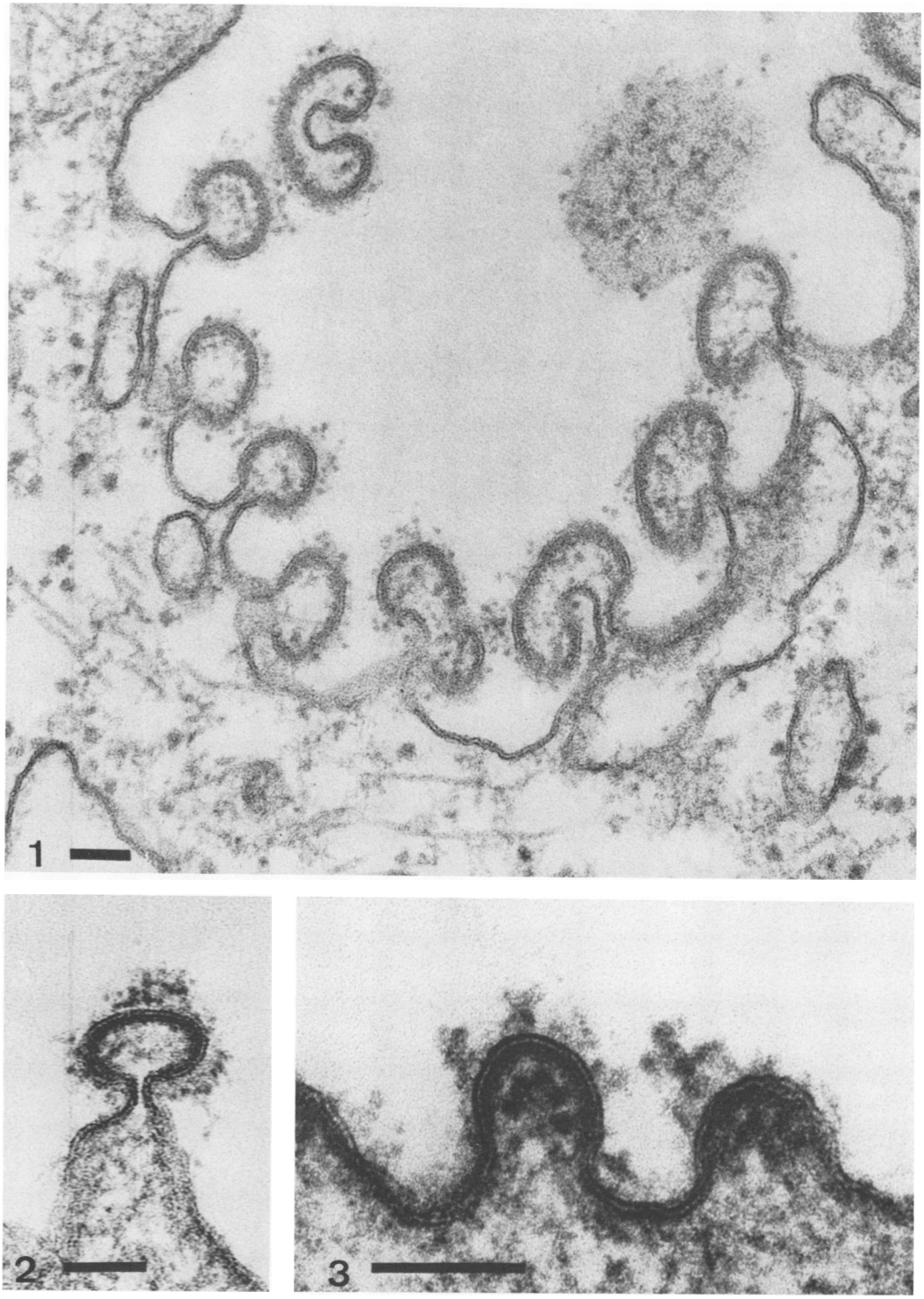
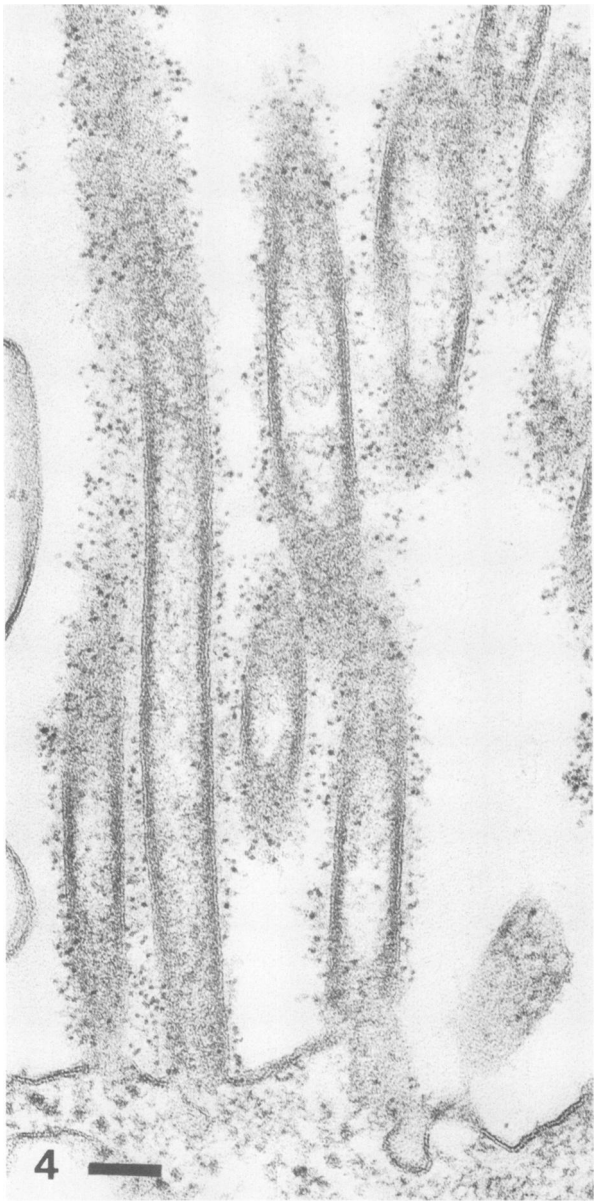


FIG. 1. *Respiratory syncytial (RS) virus budding at the surface of Vero cells 48 h after infection. Round and kidney-shaped particles react with specific ferritin rabbit antibody to RS virus. Invervening segments of cytoplasmic membrane remain unreactive with antibody. Viral envelopes consist of a trilaminar membrane continuous with host membrane. Viral component is distinguished by a fuzzy outer coat (spikes) and an electron-dense layer apposed to the inner side of the membrane. The viral particles are coreless (incomplete).* $\times$*90,000. In all figures, except Fig. 11, bars represent 100 nm.*

FIG. 2. *Same as Fig. 1. Incomplete respiratory syncytial virus particle in final budding stage. Virus is connected to the host cell membrane by a narrow stalk formed by undifferentiated host cell membrane unreactive with ferritin antibody.* $\times$*120,000.*

FIG. 3. *Respiratory syncytial virus budding from Vero cell membrane 48 h after infection. In addition to the structures recognized in Fig. 1 and 2, the core reveals up to five electron-dense structures corresponding to the nucleoproteins of the virion (complete virus).* $\times$*230,000.*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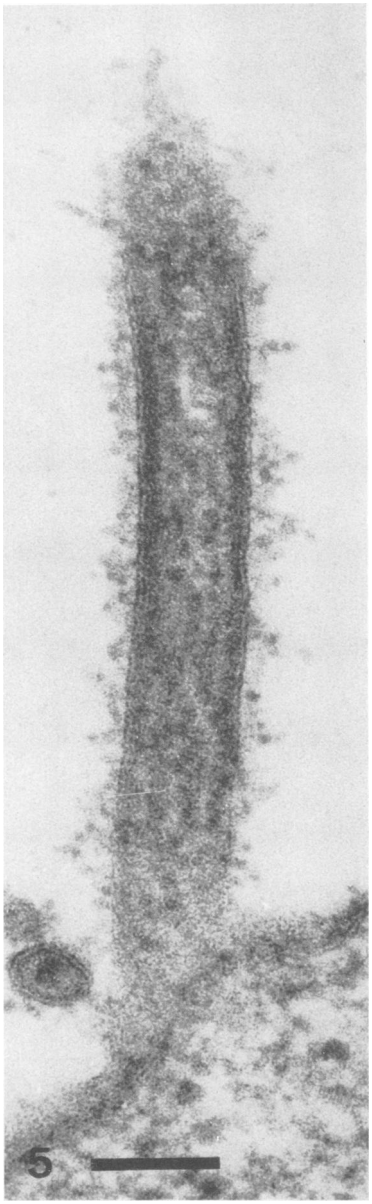
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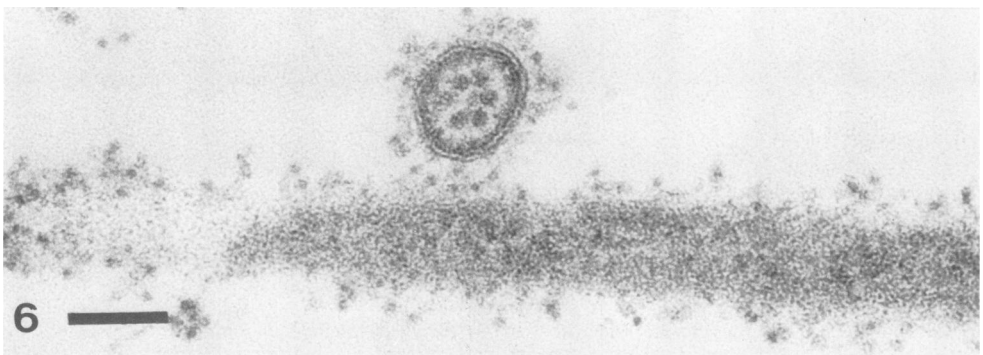
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observed which resembled those on the cores of filaments (Fig. 8). Freeze-etching of infected cells did not otherwise reveal internal components, although some differences in the striations of the core were noted. Although most virus particles seemed to bear the striations as a characteristic feature, other filaments, though undoubtedly of viral origin, lacked this configuration (Fig. 7). Round particles, corresponding to those observed in thin-sectioned preparations, were not readily identified in replicas of frozen-etched material.

Our attempts to visualize virus particles in unconcentrated supernatant fluids of infected cell cultures were never successful. Virions were therefore concentrated onto a cushion of sucrose by ultracentrifugation of large volumes of culture supernatant fluid in a zonal rotor. The virus was found mainly in an aggregated form and was localized in a fraction containing 43 to 50% sucrose. After dialysis at 4 C overnight against PBS, pH 7.2, the infectivity titer of this concentrate was 10^7; the infectivity titer of the original unconcentrated culture fluid was 10^5. Brief fixation was found to be necessary in order to guarantee the morphological integrity of the virus. The concentrated material, when negatively stained, was seen to consist mainly of filaments measuring between 1 and 4 μm (Fig. 11). Material submitted to negative staining without prior fixation revealed some filaments in which the viral envelope, with its fringe of spikes, seemed to be peeling away from the core (Fig. 14). Occasionally, round virus particles were seen with diameters varying between 150 to 500 nm (Fig. 11 and 12). All of these forms revealed peripheral spikes which measured 10 nm in length (Fig. 11, 12, and 15).

Nucleocapsid structures, released from ruptured particles, displayed a typical herring bone pattern (5). Single units appeared as doughnuts with an inner diameter of 10 nm and an outer diameter of 15 nm (Fig. 13). The same material, when examined in a dark-field microscope, showed numerous filaments up to 10 μm in length.

DISCUSSION

Our findings with respect to the morphology of RS virus, revealed by negative staining, are in general agreement with those of others, particularly as regards the appearance of released nucleocapsid and the spikes on both round and spherical viral particles or envelope fragments (5, 7, 10, 11, 15). In contrast to ortho- and paramyxoviruses, no biological functions, such as hemagglutinin and neuraminidase, have been found to be associated with RS virus (16).

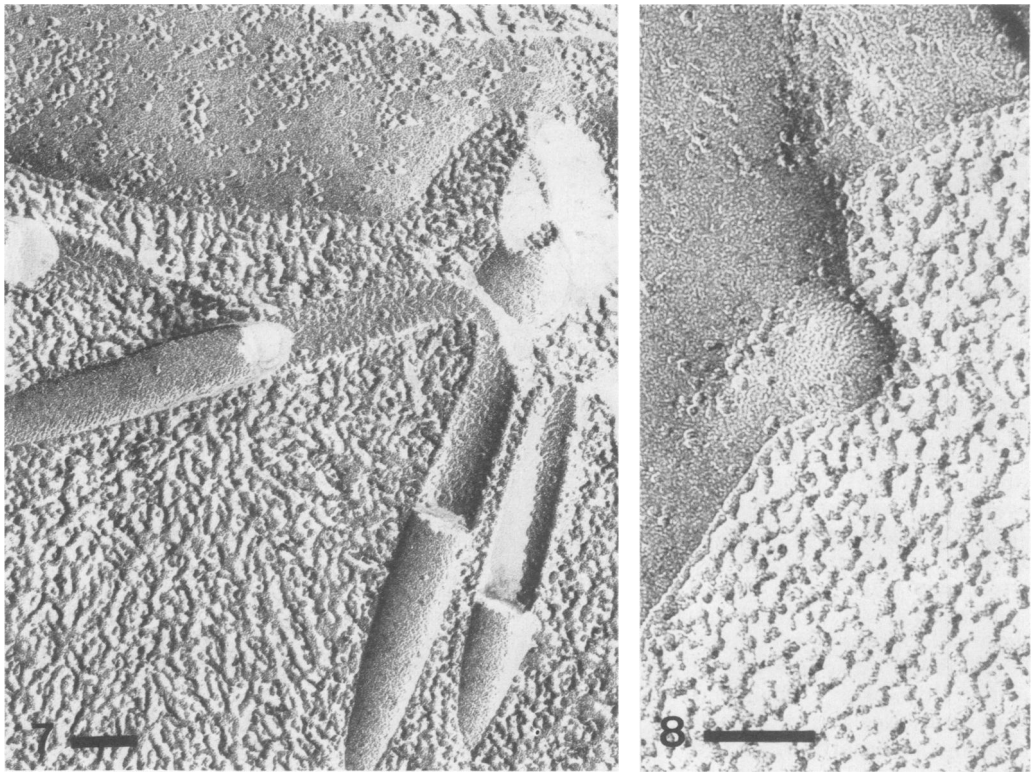
Not hitherto reported is the predominance of free filamentous forms obtained from culture fluids by procedures which were most likely to yield intact virions in sufficient concentration for negative staining. In most such preparations, as well as in thin sections of infected Vero cells, filamentous forms without inner structure (incomplete virions) outnumbered either filamentous or round forms containing inner components recognizable morphologically as nucleocapsid (complete virions) (15). Presumably only the latter are infectious. Thus, the consistently observed predominance of apparently incomplete virus might account for the generally low order of infectivity encountered with RS virus in any of several cell systems (10, 12, 13). Measurable infectivity may doubtless also be lost as a result of ultracentrifugation methods ordinarily used to concentrate RS virus from culture fluids. As shown herein, damage to fragile virions was avoided by zonal centrifugation onto sucrose cushions, a concentration procedure which yielded not only a 10- to 100-fold enhancement of infectivity, but also a superabundance of incomplete viral filaments. The factors governing the appearance of incomplete virus have not yet been elucidated.

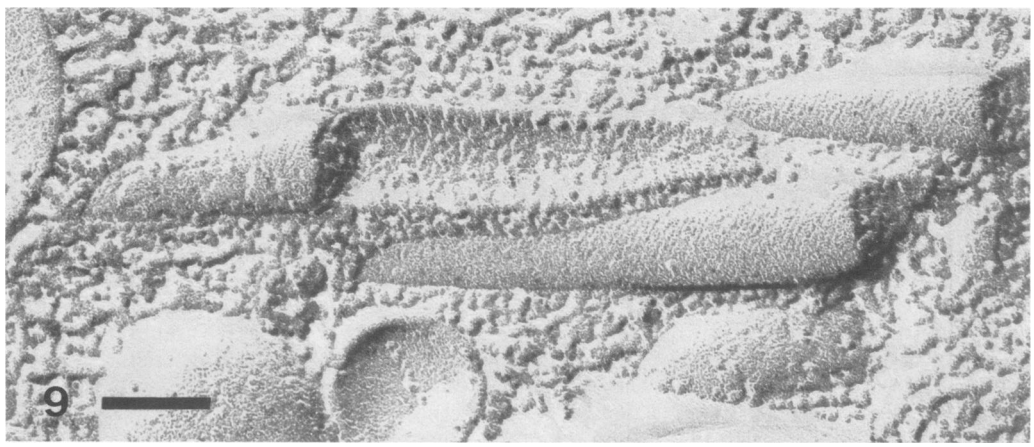
RS virus examined with freeze-etching techniques displayed a unique structural feature not discernable with any other preparative method. Virus particles, fractured so that the core was exposed, showed striations not observed thus far in other enveloped virus. Influenza virus, when replicated with the freeze-etching technique, showed a smooth core structure (1, 3), whereas Sendai virus seemed to have an obliquely arranged network of granules covering the core (4). With RS virus, the appearance of striations was first noted in conjunction with maturation of virus at the cell membrane (Fig. 8) and was accompanied by rearrangement of the host cell intramembranal particles (Fig. 7 and 8). The

FIG. 4. *Respiratory syncytial virus filaments budding from Vero cells 48 h after infection. Immunoreaction with ferritin-labeled antibody to virus is limited to the viral membranes. Filaments reveal no defined internal structures (incomplete).* ×*95,000.*

FIG. 5. *Same as Fig. 4. Longitudinal, stained strands in the filament suggest the presence of nucleoproteins in the virus (complete filament).* ×*170,000.*

FIG. 6. *Same as Fig. 5. Cross-section of filament reveals six electron-dense structures similar to those seen in the budding particles in Fig. 3. Note ferritin attached to the viral envelope. Lower part of micrograph shows longitudinally cut complete filament.* ×*130,000.*





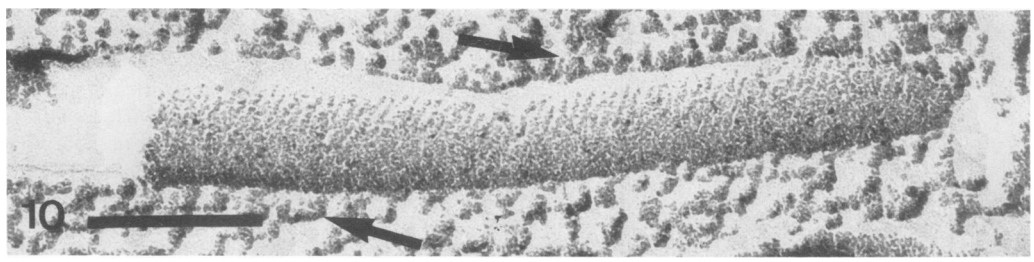
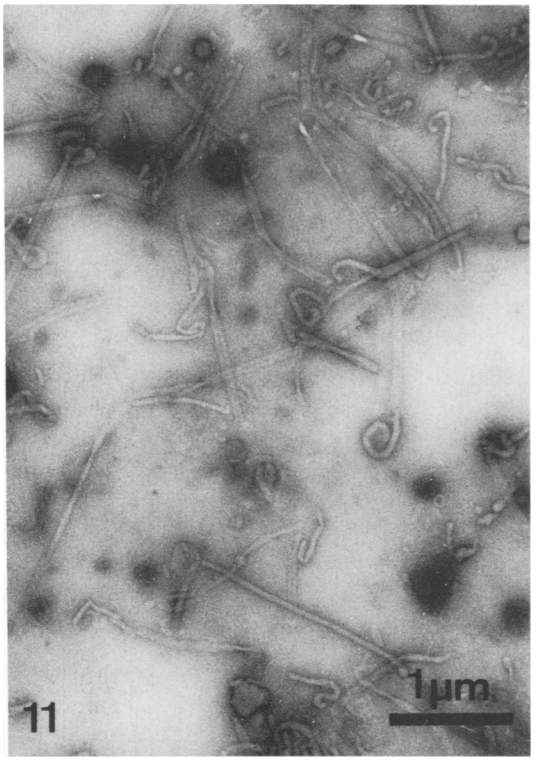


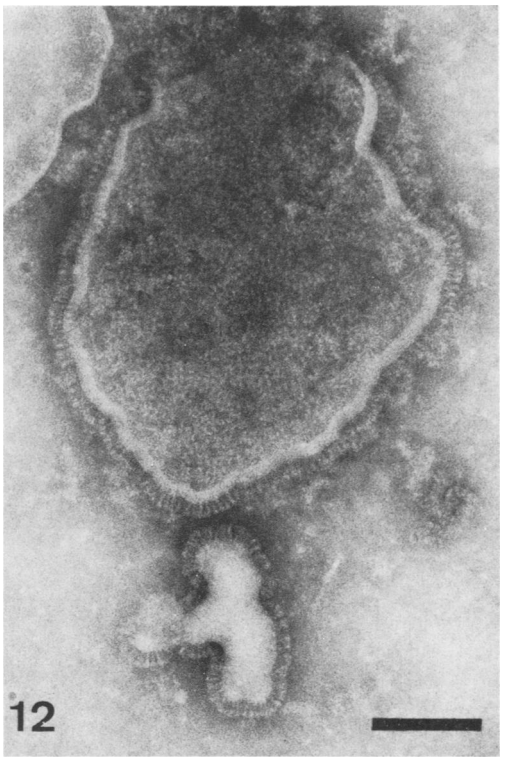
FIG. 7. *Respiratory syncytial virus 48 h after infection of Vero cells. Freeze-etching of virus filaments reveals a core with striated structures which are exposed by the removal of the envelope by the fracturing process. Removal of the core itself reveals the concave, inner surface of the viral envelope.* ×*85,000.*

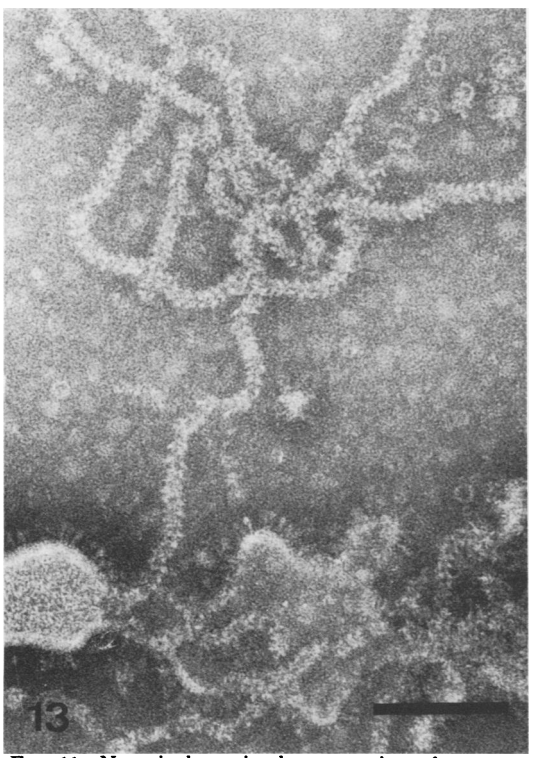
FIG. 8. *Same as Fig. 7. Budding particle is discernable by absence of membrane granules at budding site and virus-specific differentiation of the membrane expressed by striations similar to those seen on viral cores in Fig. 7, 9, and 10.* ×*145,000.*

FIG. 9. *Filaments of respiratory syncytial virus revealing striated helical structures with an angle of 60° on the core and a pitch of 6 mm.* ×*140,000.*

FIG. 10. *Same as Fig. 9. Fringe-like structure (arrows) surrounds the striated core and represents spikes seen in negatively stained preparations.* ×*230,000.*







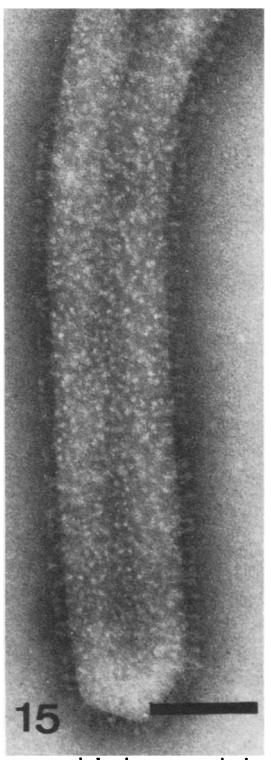


FIG. 11. *Negatively stained preparation of concentrated infective respiratory syncytial virus, consisting mainly of filamentous forms.* ×*16,000.*

FIG. 12. *Infective respiratory syncytial virus in concentrated preparation. The size of round particles is variable. Note spikes of the viral envelope.* ×*140,000.*

FIG. 13. *Nucleoprotein filaments released from ruptured respiratory syncytial virus particle.* ×*180,000.*

FIG. 14. *Respiratory syncytial virus filament in process of disintegration after negative staining of concentrated material without prior fixation. Envelope of filament is peeling off the core.* ×*60,000.*

FIG. 15. *Intact respiratory syncytial virus filament. Spikes are morphologically identical with those observed on round particles (Fig. 12).* ×*140,000.*

latter, in turn, were displaced from the sites of active viral budding and were found in intervening areas in irregular patterns or clusters. This process, which resembled the sequence of events occurring during maturation of myxoviruses (1, 3), may reflect the removal of host glycoproteins from the lipoprotein of the viral envelope. It could also represent a structural change in the membrane analogous to that seen during the course of erythrocyte fusion induced exogenously with Sendai virus (2, 4). The coincidence of syncytium formation with the appearance of viral antigen in the plasma membrane has been observed in cells infected with RS virus (12).

The exact location of the regular, striated structures in the RS viral envelope was not clearly definable. The fracture plane produced in the viral envelope with the freeze-fracturing method corresponds with the hydrophobic region of the lipid bilayer. Thus, pictures revealing the core of RS virus presented to view the inner leaflet of the viral envelope unpenetrated by the spikes. Alternatively, the hollow, concave envelopes, with cores removed, displayed granulated structures which might be taken as the bases of spikes. Some of the filaments appeared to lack striations (Fig. 7). This defect probably reflected poor local resolution of the replica rather than any real structural differences among the virus particles. In any event, the structural details revealed in cores of RS virus by freeze-etching techniques may represent biological characteristics peculiar to this agent and, if also eventually discovered in bovine RS virus and PVM, may strengthen the basis for considering these viruses together as a distinct taxonomic group.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10945 from the National Institute of Allergy and Infectious Diseases and AM-13200 from the National Institute of Arthritis and Metabolic Diseases.

The technical assistance of R. Keller and R. Leemann is gratefully acknowledged.

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