Morphogenesis of Type 2 Parainfluenza Virus Examined by Light and Electron Microscopy

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The development of type 2 parainfluenza virus in HeLa and stable human amnion cells was examined by use of antisera labeled with fluorescein and ferritin. Serum containing antibody predominantly to soluble viral antigen gave specific fluorescence which was first detectable in small cytoplasmic foci 8 to 10 hr after initiation of infection. By 20 to 24 hr, when the production of infective virus and hemagglutinin was maximal, large perinuclear aggregates of fluorescence were observed which corresponded in distribution and time of appearance to the eosinophilic inclusions seen in similar preparations stained with azure eosin. The inclusions, examined by electron microscopy, were composed of fibrils, presumably viral ribonucleoprotein, which specifically bound the antibody labeled with ferritin. With antiserum to concentrated virus, on the other hand, specific fluorescence was most marked at the surface of infected cells. Foci of fluorescence at the surface represented segments of membrane which had become differentiated morphologically and antigenically to resemble the viral envelope. These were the sites where mature virions appeared. The latter exhibited marked pleomorphism; in some instances, particles were formed which lacked recognizable internal fibrils but which possessed an enclosing membrane bearing viral antigen. Filamentous forms showing an organized internal structure were also observed at the cell surface, but were never encountered in negatively stained preparations. No clear relationship between these filaments and the spherical or oval forms could be established. In negatively stained preparations, nucleocapsid released by rupture of viral particles was similar in appearance to that reported for other paramyxoviruses. It seems probable that this component has a helical configuration.

The paramyxoviruses are distinguished from the influenza virus group by their larger size, and by their ability to cause the formation of syncytia and eosinophilic inclusions in cultured cells. With the paramyxoviruses, moreover, synthesis of internal antigen is initiated in the cytoplasm, in contradistinction to influenza virus, the internal or "soluble" antigen of which is first recognized in the nucleus. Both groups, however, mature at the cell surface. In previous investigations, the morphological and antigenic changes which occur during the formation of influenza virus particles in cells of the chicken chorioallantoic membrane were elucidated by electron microscopy, with the aid of antibody conjugated with ferritin. The development of parainfluenza virus (type 2) has now been examined in relation to its growth cycle in human amnion and HeLa cells. Antisera, previously described in conjunction with immunochemical studies of this virus (10), were conjugated with fluorescein and with ferritin for use in this investigation.

MATERIALS AND METHODS

Stable amnion cells (FL) and HeLa cells were propagated as previously described (10). For light and electron microscopy, monolayers were grown on cover slips in Leighton tubes and were inoculated with virus at high input multiplicity. Antisera were prepared to ultracentrifuge-concentrated virus, to amnion cells lyophilized 4 days after infection, and to lyophilized normal amnion cells, as reported in detail in a previous communication (antigens 2, 3, and 4 in Table 1 of reference 10). Globulins were precipitated at 40% of saturation with sodium sulfate, and were conjugated with either fluorescein or ferritin (1). Before use, the conjugated antisera to viral antigens were absorbed with lyophilized and with freshly harvested viable amnion or HeLa cells. Absorbed fluorescein and ferritin conjugates gave no significant reaction with normal, uninfected amnion or HeLa cells.

For light microscopy, cover slips were fixed in Bouin's solution at appropriate intervals after infection and were stained with azure eosin. For fluorescence microscopy, similar preparations were fixed in cold acetone, dried in air, and treated with labeled

antibody for 20 min at room temperature. After being washed in three changes of phosphate-buffered saline, the cover slips were mounted in buffered glycerine. For electron microscopy, unfixed cover slip preparations were stained with ferritin-conjugated antibody for 20 min at room temperature. The cells were scraped from the cover slips into phosphate-saline, washed twice in the same diluent, and centrifuged. The pellets were fixed for 1 hr in 1% glutaraldehyde, washed thoroughly, postfixed in 1% osmium tetroxide for 30 min, and, after dehydration, were embedded in epoxy resin 812. Thin sections, stained with uranyl acetate and lead, were examined in an RCA electron microscope. To facilitate the penetration of labeled antibody to intracellular constituents, some monolayers were first fixed in 4% Formalin. After being washed in saline and dipped in dimethyl sulfoxide, the cover slips were placed in Leighton tubes which were then immersed in carbon dioxide-ethyl alcohol to freeze the attached cells. After thawing, the conjugate was placed directly on the cover slips; alternatively, the cells were removed from the glass and suspended in the conjugate. The cells were then washed, fixed, and embedded in the manner described above. Virus concentrated from cultures 7 days after infection was used for negative staining. Cells remaining attached to the glass surface were scraped into the maintenance fluid, which was then centrifuged at $1,000 \times g$. The pellet was frozen and thawed three times, and was recombined with culture supernatant fluid. The latter was clarified and then ultracentrifuged at $80,000 \times g$ for 1 hr. The pellet was resuspended in balanced salt solution, and drops were placed on Formvar-coated grids. Phosphotungstic acid, 1%, buffered at pH 6.8, was added to the drop; the grid was then dried by blotting with filter paper.

RESULTS

Light microscopic observations related to the growth cycle. The results of investigation into the growth cycle of parainfluenza 2, to be reported in detail in a subsequent communication, may be briefly summarized. Under conditions of viral input in which over 90% of the cells could be shown to be simultaneously infected, an increase in infective cell-associated virus first appeared 8 to 10 hr after inoculation. Hemadsorption and hemagglutination appeared together at approximately this time. Production of infective virus and hemagglutinin reached a maximum by the 24th hr, when cell-associated neuraminidase activity first became detectable. Production of virus continued at least through the subsequent 48 hr, after which infectivity titers tended to fall owing to thermal inactivation of released virus. The single-cycle growth curve in HeLa cells was similar to that in amnion cells.

Replicate monolayers of HeLa cells were simultaneously infected, and were then examined at intervals during the first 48 hr after infection. Cover slips were fixed and stained with azure eosin

or with fluorescent antibody. The earliest changes were detected 8 hr after infection by specific immunofluorescence, with the use of antibody to infected cells. Many small foci appeared at this time in the cytoplasm, and were well defined by the 10th hr (Fig. 1). During the ensuing 20 hr, these cytoplasmic foci increased in size, coalescing into large aggregates which frequently filled the perinuclear area (Fig. 2). With antibody to concentrated virus, specific fluorescence was most marked at the cellular surface, where aggregates of viral antigen were revealed (Fig. 3) at 20 hr after initiation of infection. Some of these aggregates could be seen to overlie the nucleus. In addition, there was diffuse specific cytoplasmic fluorescence with this antibody, in contrast to the localized aggregates in the perinuclear area seen with antibody to infected cells.

The appearance of specific immunofluorescence preceded by about 20 hr the appearance of changes in similar preparations of HeLa cells stained with azure eosin. Eosinophilic inclusions were detected in the cytoplasm 24 hr after infection, accompanied by the development of cytoplasmic vacuoles and gradual loss of cell structure. The inclusions corresponded in their distribution to the areas of specific cytoplasmic fluorescence revealed with antiserum to infected cells (Fig. 4). No specific nuclear fluorescence or any striking tinctorial changes in the nucleus were noted. Although cells with several nuclei were frequently encountered, few true syncytia were formed in either cell line during the course of infection.

FIG. 1. HeLa cell 10 hr after infection, stained with fluorescent antibody to lyophilized infected amnion cells. Small cytoplasmic foci of specific fluorescence are seen.

FIG. 2. *HeLa cells 24 hr after infection, stained with the aforementioned antiserum. Large perinuclear aggregates of fluorescence are seen, along with some smaller more peripheral foci. Some of the latter may be at the cell surface. In the cell at the top, a large cytoplasmic focus of fluorescence overlies the nucleus, which itself shows no specific fluorescence.*

Electron microscopic observations of viral development at the cellular surface. In Fig. 5, the surface of an infected cell traverses the field vertically, with the cytoplasm to the left. Two distorted, vacuolated mitochondria are present in the left upper half of the field. Ferritin-conjugated antibody, marking the sites of viral antigen on the surface, has tagged rodlike forms (filaments) as well as irregularly shaped particles of differing density. The protrusion in the lower half of the field is illustrated at higher magnification in Fig. 6. The long filament tagged with ferritin appears to contain oval granules lining portions of the inner side of the coat. Fibrils of low density are also visible within the rod, as well as at the base and in the adjoining cytoplasm. Just to the right are two smaller protrusions coated with ferritin. Figure 7 shows the upper portion of Fig. 5 at higher magnification. Part of the surface of the cell has been altered so that it contains viral antigen. In this area, oval granules line the inner aspect and fibrils are present in the adjacent cytoplasm. Some of these fibrils are continuous with

the granules, suggesting that the latter are, in fact, fibrils seen in cross section. The surface of the cell to the left of the altered segment contains little or no viral antigen. In the three adjacent filamentous forms, which pass obliquely through the section, the fibrils appear in those portions where the upper and lower surface of the rods pass through the section, i.e., at the top and bottom. In Fig. 8, the altered portion of the cell surface is sharply demarcated by the presence of ferritin; in this area, the surface is composed of two layers separated by 40 to 50 Å. Immediately beneath, there is a collection of fibrils, the most peripheral of which are arranged regularly under the altered cell membrane.

Electron microscopy of viral morphology in thin section. In Fig. 9, two filamentous forms, which are fused distally, have been cut obliquely. Where the surfaces of the fused portion and the proximal portion of each filament are nearly parallel to the plane of section, a complex pattern of fibrils is seen. Proximally, there is a gradual transition to granular cytoplasmic matrix. In Fig. 10 (lower

FIG. 3. Amnion cells 20 hr after infection stained with fluorescent antibody to concentrated virus. Fluorescence is seen predominantly in foci at the cell surface; in some cells, these foci overlie the nucleus, which does not show any specific fluorescence. Diffuse cytoplasmic fluorescence is evident.

right), an obliquely cut filamentous form is seen in which the arrangement of internal component appears to be helical. At the thicker, proximal end of this filament, successive turns of the fibrils are seen in parallel transverse array, up to the central portion. Here the plane of section bisects the filament, and along either edge the cut ends of fibrils are seen as symmetrically arranged dots of electron-dense material alternately joined by transverse fibrillar segments. Distally, the plane of section approaches the surface, until at the very tip internal structure is no longer discernible. This distal portion of the filament is heavily tagged with conjugate throughout its entire breadth. A similar filament, which has become detached from the cell membrane, and in which the internal component also seems to be in helical arrangement, appears in Fig. 11 (center left).

At the top of Fig. 10, and in Fig. 11, there are large, irregularly contoured particles showing

FIG. 4. HeLa cells 3 days after infection, showing large perinuclear eosinophilic inclusions, cytoplasmic vacuolization, and characteristic "sponge"-like appearance. Bouin's fixation, azure eosin.

specific labeling with antibody around part or all of their perimeter. In the lowermost particle in Fig. 11, only a portion of the surface is labeled, and, in this area, there are several buds in process of formation. In all of these larger, irregularly shaped configurations, the specific surface antigenic change demarcated by ferritin is accompanied by the accumulation of a narrow zone of amorphous, electron-dense material subjacent to the closely spaced double membrane previously mentioned. This same change is also seen under portions of the intact cell membrane which are budding as if to form viral particles. Two such areas of budding are seen in Fig. 11 toward the top of the cell border, which is elsewhere free from ferritin. There is a notable lack of recognizable internal fibrillar component in the cytoplasm under these budding areas of cell membrane as well as in the several irregularly contoured particles just described.

By far the most frequently encountered form

FIG. 5. Part of an infected amnion cell treated with ferritin-conjugated antibody to concentrated virus. The free surface of the cell is at the right. $\times 45,000$.

FIG. 6. Lower right portion of the preceding micrograph at higher magnification. The surface of the cell traverses the field horizontally with the cytoplasm below. A viral filament containing fibrils extends from the surface. To the right, filaments have been sectioned obliquely. $\times 100,000$.

FIG. 7. Part of Fig. 5 at higher magnification. Fibrils are lined up just beneath a segment of the cell wall, which contains viral antigen. Above are several filaments cut obliquely. $\times 100,000$.

8

9

FIG. 8. Portion of the cell surface apparently in process of protrusion, and containing viral antigen. Fibrils and ribosomes are evident in the adjacent cytoplasm. $\times 110,000$.

FIG. 9. Fused filaments illustrating a complex array of fibrils in regions where their surface passes through the section. $\times 110,000$.

FIG. 10. Filament containing fibrils which may be in helical array. The irregular structure at the upper left contains viral antigen on its surface. $\times 130,000$. Inset: spherical forms of the virus in a very thin section. The internal filaments are visible. Particles central to the plane of section exhibit a diffuse peripheral coat of low density. $\times 130,000$.

FIG. 11. Spherical viral particles of the type most commonly observed. This section is considerably thicker than the one illustrated in the inset to Fig. 10; hence, there is superimposition of internal components. $\times 70,000$.

of the virus was spherical or oval, with double-contoured membrane and a less-distinct outer zone, as shown in Fig. 12. This micrograph shows the high degree of specificity with which the ferritin conjugate is bound to viral particles, leaving the microvilli and detached cytoplasmic fragments free from ferritin. The arrangement of internal viral component is obscured because of superimposition. However, in exceptionally thin sections, such as that shown in the inset to Fig. 10, fibrils are recognizable within the viral particles.

Figures 13 and 14 illustrate preparations stained with antibody specific for normal uninfected amnion cells. In Fig. 13, three viral particles with visible internal structure are seen, free from ferritin and adjacent to an area of unaltered cell surface. In Fig. 14, virus in the process of budding is likewise untagged, and what appear to be regularly spaced structures are arranged around the periphery of the partially formed virion. In contrast, the unaltered cell surface shown in Fig. 13 and 14, as well as the free, nonviral particles shown in Fig. 13, have bound antibody specifically. In Fig. 14, several phagocytic vacuoles deep within the cytoplasm are seen to contain ferritin.

In contrast to the helical arrangement of internal component suggested by the appearance of the filaments in Fig. 10 and 11, the configuration shown at higher magnification in Fig. 15 is quite different. At its base, and along one margin, a filament is still attached to the cell, the zone of fusion being indistinct because of the oblique plane of section; however, it contains ferritin granules throughout its length, indicating the presence of viral antigen.

Electron microscopy of viral morphology by negative staining. Figures 16 and 17 illustrate particles negatively stained with phosphotungstic acid. The internal component, which has been released by rupture of the particle as shown in Fig. 17, exhibits a characteristic structure. At higher magnification, as in Fig. 18, fragments of internal component of varying lengths are seen, along with hollow circular structures of the same diameter. These latter appear to be segments which have become disengaged from the fibrils and lie on the substrate. In Fig. 16 and 17, surface projections on the viral envelope are discernible. These are more clearly shown in the inset to Fig. 18, in which only the surface of a negatively stained particle appears, without visible internal component.

Cytoplasmic viral antigen. Figure 19 shows the cytoplasm of an amnion cell which had become detached from the glass 3 days after infection. The cellular surface is on the left. Part of the nucleus

is on the right, showing the chromatin displaced to the nuclear periphery. The mitochondria contain dense material and, along with the ribosomes and other cytoplasmic components, have been displaced peripherally by large irregularly shaped aggregates of fibrils. Figure 20 illustrates a portion of one of these aggregates in which the individual elements are so tortuous and interwoven that their length is difficult to measure. These aggregates appear to be densely packed in cells such as that illustrated in Fig. 21; ferritin conjugate has tagged the surface, but has failed to penetrate to the interior of the mass. Figure 22 shows, at lower magnification, the aggregate illustrated in Fig. 21. This preparation was not frozen prior to treatment with conjugate, but antibody was able to reach the intracytoplasmic inclusion because of the marked degenerative changes in cell structure which are readily discernible. Earlier stages in the formation of these aggregates are shown in Fig. 23 and 24. In these preparations, taken at 38 and 24 hr after infection, respectively, the cells were still attached to the glass, and penetration of antibody into the cell was facilitated by freezing and thawing before exposure to antibody. In the upper half of Fig. 23, a significant concentration of ferritin is seen in the interior of an aggregate, whereas in Fig. 24 specific staining is more pronounced toward the periphery of a similar mass of fibrils. In the lower part of Fig. 23, there are several regions of staining which are not associated with fibrillar structures, but which are nonetheless specific for infection with parainfluenza 2 virus. This antigenic material differs from the larger aggregates in being relatively amorphous, and in being found on occasion at the cell surface, where it is equally well identified by its binding of specific conjugate. In Fig. 25, this amorphous antigen is shown apparently in the process of being released from the cell. Toward the top of the figure, there appears to be a narrow channel connecting the perinuclear with the extracellular space.

DISCUSSION

Since the original isolation of parainfluenza virus type 2 (croup-associated or CA virus) by Chanock (5) and, independently, by Beale (2), observations have been made on the effect of this agent in various cell systems. The formation of eosinophilic cytoplasmic inclusions has been described in human amnion cells (13), in KB cells (22, 32), in HEP-2 cells (4), and in primary cultures of human (13, 14), canine (4), and monkey (4, 22) kidney. The inclusions, containing sulfhydrated ribonucleic acid (22) in high concentration, are a relatively late manifestation of infection. Observations with other paramyxovi-

FIG. 12. *Extracellular virus showing the extreme degree of pleomorphism frequently encountered. Two particles in process of budding from the host cell are visible in the upper third; there are no fibrils in the subjacent cytoplasm. $\times 90,000$.*

FIG. 13. Surface of an infected cell treated with ferritin-conjugated antibody to normal amnion cells. The virus is not tagged. $\times 70,000$.

FIG. 14. Budding forms showing absence of tagging with ferritin-conjugated antibody to normal amnion cells. $\times 140,000$.

FIG. 15. Viral particle at the surface of a cell. The arrangement of fibrils is clearly seen. The cell wall to the right is cut obliquely. $\times 200,000$.

FIG. 16. Viral particle (about $3,600 \text{ \AA}$ in diameter) negatively stained with phosphotungstic acid. The internal fibrils (nucleoprotein) are evident. The external spikes are not well preserved. $\times 180,000$.

FIG. 17. Disrupted viral particle. The fibrils in process of release exhibit a structure which is not visible in the thin-section micrographs. Note also the striking difference in diameter of the fibrils compared with those in Fig. 15. $\times 200,000$.

FIG. 18. Nucleoprotein component released from a viral particle, the edge of which appears at the right border of the field. The diameter of the fibrils averages 160 $\text{\AA}$. The central zone of lesser density is about 70 $\text{\AA}$ in diameter. There are numerous fragments, some of which are in the form of rings. $\times 250,000$. Inset: a small viral particle 1,000 $\text{\AA}$ in diameter. The surface spikes (140 $\text{\AA}$ in length) are visible in profile at the periphery; toward the center, their random orientation is evident. No internal component is visible. $\times 340,000$.

FIG. 19. Part of the cytoplasm of an amnion cell containing a large aggregate of fibrils. The free surface of the cell is on the left; a portion of the nucleus, on the right. $\times 37,000$.

FIG. 20. *Aggregate of fibrils at higher magnification. $\times 120,000$.*

FIG. 21. *Part of an aggregate of fibrils in the cytoplasm of an amnion cell treated with ferritin-conjugated antibody to infected cells. $\times 90,000$.*

FIG. 22. Same aggregate as that illustrated in the preceding micrograph. Since the cell had become detached from the glass and necrosis was advanced, the ferritin conjugate could penetrate to intracellular sites without prior freezing and thawing of the preparation.

FIG. 23. *Infected HeLa cell which, after being frozen and thawed, was treated with ferritin-conjugated antibody to infected amnion cells. In the upper half, an aggregate of filaments is tagged. In the lower half, collections of amorphous infection-specific antigen are heavily stained with ferritin. $\times 55,000$.*

FIG. 24. Fibrils tagged with ferritin. The surrounding cytoplasmic structures are devoid of ferritin. $\times 130,000$.

FIG. 25. Cytoplasm of a cell at an advanced stage of infection. The nucleus is on the right. Amorphous antigen heavily labeled with ferritin lies within the perinuclear space. The continuity of this space with the surface of the cell is evident at the upper left. $\times 40,000$.

ruses, notably parainfluenza 1 (Sendai), have shown cytopathology essentially similar to that of parainfluenza 2 virus (8, 27). The bovine strain of parainfluenza 3 (SF-4), however, produces intranuclear eosinophilic inclusions (7, 20, 25, 29), a feature not generally characteristic of human parainfluenza viruses. Studies with fluorescent antibody (23, 25, 31) have substantiated the fact that the initial sites of antigen synthesis are cytoplasmic. Newcastle disease virus (NDV) is generally similar to the parainfluenza viruses in that the soluble antigen develops primarily in the cytoplasm (28), although the transient occurrence of viral antigen in the nuclei of HEP-2 cells infected with this virus has been reported (19). The significance of nucleolar involvement in the synthesis of parainfluenza 1 viral antigen (35) and of NDV (36) remains to be determined. A recently isolated avian myxovirus (11), serologically distinct from NDV, mumps, and parainfluenza 1, 2, or 3, has cytopathic characteristics similar to those of other paramyxoviruses.

The present study of the development of type 2 parainfluenza virus was followed with the use of three antisera. The first of these, to whole virus concentrated by ultracentrifugation, contained predominantly antibody to viral envelope constituents (hemagglutinin and neuraminidase), and gave an appreciable neutralization titer. Accordingly, with the fluorescein conjugate (Fig. 3), surface aggregates of viral antigen were revealed which were doubtless identical with the sharply demarcated segments of altered cell membrane seen to react with the same antibody conjugated with ferritin. The second antiserum, made to amnion cells lyophilized 4 days after infection, contained antibody to soluble viral antigen or internal component, as shown by the reactivity of fluorescein and ferritin conjugates with intracytoplasmic nucleocapsid (Fig. 1, 2, 21-24). A third antiserum, made against normal amnion cells, contained antibody predominantly against surface antigens, as shown by agglutination and absorption studies (10). Accordingly, the ferritin conjugate of this antibody reacted only with portions of cell membrane remaining unaltered by the process of viral development (Fig. 13 and 14).

In the two cell systems used (FL amnion and HeLa), the rates of development of infective virus were virtually identical. As observed in HeLa cells, the first evidence of specific viral antigen was revealed by immunofluorescence 8 to 10 hr after infection, corresponding to the almost simultaneous development of hemadsorption and hemagglutination. With the progression of infection and the concomitant increase in released virus, large foci of cytoplasmic fluorescence were

encountered. From our observations, and from those of other investigators, there is little doubt that these fluorescent aggregates were the large inclusions seen in the later stages of infection. As shown subsequently, the inclusions also corresponded to the aggregates of fibrils seen by electron microscopy. With the death of the cell, and its disintegration, the large aggregates remaining were released, but, as already noted, appeared to be resistant to mechanical disruption as well as to the penetration of ferritin-conjugated antibody. The identity of the amorphous antigen detected in intracellular and extracellular sites (Fig. 23 and 25) by its specific binding of antiviral antibody remains uncertain. However, it is possible that this material, as well as cytoplasmic nucleocapsid, may be among the infection-specific precipitating antigens demonstrable by immunoelectrophoretic analysis of cells producing infective virus (10).

Our electron microscopic findings are in general agreement with, and extend, the reported observations of others on the paramyxoviruses. Thus, Reczko and Bögel (27), in examining calf kidney cells infected with parainfluenza 3 virus, noted filamentous forms which appeared to be in process of detachment from the surface of cells and which they supposed became segmented into smaller viral subunits. In addition, intracytoplasmic clusters of fibrils were seen which were thought to constitute the inclusions observed by light microscopy. These authors suggested that the fibrils were incorporated into viral particles budding from the cell surface, and they were able to show specific binding of ferritin conjugate by viral particles. A detailed consideration of specific surface changes was given by Berkloff (3) and by Colobert and Berkloff (9) in a study of parainfluenza virus 1 in KB cells. These investigators showed that localized segments of the cell surface became transformed morphologically to resemble the envelope of the virion, and that aggregates of fibrils, presumably nucleoprotein, were gathered under these altered areas and subsequently became incorporated into viral particles as the internal component. These observations are supported by the results of the present study of parainfluenza 2 virus, demonstrating that the localized areas of cellular surface which bind antiviral antibody are the sites at which virions emerge. Subjacent cytoplasmic nucleocapsid, however, was not invariably present at these sites (Fig. 11). This latter finding is not wholly consistent with the statement of Colobert and Berkloff (9) that the membrane differentiates only in the presence of capsid. The development of parainfluenza viruses in discrete foci is in contrast to the morphogenesis of influenza virus

during which the entire surface of the host cell becomes altered so as to contain viral antigen and to produce virions at myriads of loci (12, 24).

Kuhn and Harford (21) examined the inclusion bodies produced in HeLa cells by parainfluenza 2, and they also suggested that the fibrillar structures of which the inclusion bodies were composed might be viral ribonucleoprotein. The specific reaction of these fibrillar aggregates with ferritin conjugate confirms the morphological identification of this material as viral ribonucleoprotein (Fig. 21-24). Convincing evidence for the presence of nucleocapsid in the cytoplasm of infected cells has recently been presented by Prose et al. (26), in an electron microscopic study of WB virus, a myxovirus antigenically distinct from the other human parainfluenza viruses but related to simian type 2 myxovirus. These authors also demonstrated the incorporation of internal component into viral filaments at the cell surface; moreover, some of their electron micrographs suggest that the internal component in detached filaments might be arranged helically. In many respects, the findings with WB virus parallel closely our own findings with parainfluenza 2 virus with regard to mode of filament formation and development of spherical and oval particles at the cell surface. However, budding from filamentous forms containing morphologically recognizable internal component was not observed with parainfluenza 2 virus. Also, in contrast to the findings with WB virus, some of the filaments observed with parainfluenza 2 virus showed an arrangement of internal component which was clearly not helical (Fig. 9 and 15).

The use of ferritin-conjugated antibody permitted the specific identification of released particles which contained viral antigen, but not all of which were morphologically complete virions. Several such forms (Fig. 10 and 11) are shown which lacked closely packed fibrils and whose surface was not uniformly tagged with ferritin. This suggests that portions of the cell membrane, some containing and others devoid of viral envelope antigens, may have become detached from the cell. Such particles would contribute to the total hemagglutinin produced, but not to the total infectious virus, since they apparently lacked the ribonucleoprotein core essential for infectivity. Although "incomplete" parainfluenza virus has not to our knowledge been described, recent unpublished evidence from this laboratory lends support to the belief that certain conditions of virus propagation favor a shift in the infectivity-hemagglutinin ratio consistent with this possibility.

It is noteworthy that filamentous forms are not mentioned in any of the published reports of

paramyxoviruses examined by the negative staining technique (6, 15-17, 30, 33, 34), and none was encountered in our negatively stained preparations of type 2 parainfluenza virus (Fig. 16-19). The failure of the filaments to survive the preparative procedure may be taken as an index of their greater fragility as compared with the spherical or oval forms, which perhaps represent the principal infectious units. These exhibit considerable variation in size. In the case of type 1 parainfluenza virus, recent studies have been reported (18) in which several classes of round or oval infective virions were separated by zone centrifugation, the largest particles apparently containing multiples of a smaller nucleocapsid unit. The pleomorphism of the virions was thought to be related to heterogeneity in the length and conformation of nucleocapsid and to flexibility of the envelope. As seen in the negatively stained preparations of type 2 parainfluenza virus (Fig. 18, inset), the envelope bears characteristic "spikes," and the internal component is apparently randomly arranged. With rupture of the viral particle, nucleocapsid is released and its fine structure is revealed (Fig. 17 and 18). The interpretation of such photomicrographs as showing a helical configuration of nucleocapsid is attended by some uncertainty, since the conditions of preparation, particularly pH, have an important bearing on the observed morphology of the fragments (30). Moreover, the rings or "doughnuts" seen with type 2 parainfluenza virus (Fig. 18) may be subunits of an arrangement other than helical. A further question may be raised concerning the width of the nucleoprotein fibrils, which in negatively stained preparations exhibit a diameter of 160 Å; in thin sections, their diameter is 100 Å. In sectioned filaments, such as those illustrated in Fig. 7-10, the fibrils exhibit regular and consistent spacing, which suggests that they are close-packed. Assuming that the irregular margin visible after negative staining is not sufficiently dense to be seen in sections, the center-to-center spacing should approximate 160 Å. In actuality, however, it is 200 Å.

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ADDENDUM IN PROOF

Since submission of this paper for publication, Compans et al. (Virology 30:411, 1966) described

the development of parainfluenza virus, type 5 (SV5), in primary monkey kidney cell cultures and in a stable line of baby hamster kidney cells. The morphological changes accompanying the maturation and release of SV5 at the cellular surface correspond closely to those we have observed with parainfluenza 2 virus in stable amnion and HeLa cells.

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