Growth and Intracellular Development of a New Respiratory Virus

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The multiplication of a new, ether-sensitive, ribonucleic acid virus, 229E, isolated from the human respiratory tract, has been studied in cultures of WI-38 human diploid cells. In thin sections of these cells examined with the electron microscope, particles appeared in vesicles in the cytoplasm of cells at a time corresponding to the initial increase in infectious virus. Antigen was also detected in the cytoplasm of cells by the immunofluorescent technique. Extracellular particles of similar morphology were prominent soon after. These events preceded a detectable cytopathic effect. Later, an electron-dense particle appeared within vacuoles in the cytoplasm but was never found extracellularly. Its role in virus development is not known. Complement-fixing antigen developed along with the increase in infectious virus.

A new virus (229E) isolated from an acute specimen obtained during an upper respiratory infection in a medical student at the University of Chicago has recently been described (4). Its properties are as follows: ether sensitive; is a ribonucleic acid (RNA) virus (no inhibition by 5-fluorodeoxyuridine or 5-iododeoxyuridine); size is 89 mμ (gradocol filtration); grows in human kidney or human diploid cells, with production of cytopathic effect (CPE); does not grow in Rhesus monkey kidney or HEp-2 cells; does not grow in amnion or allantois of chick embryo; does not hemagglutinate chicken, guinea pig, or human O red blood cells at pH 7.2 in phosphate-buffered saline; not neutralized by myxovirus antisera—influenza A, B, C, parainfluenza 1, 2, 3, 4, respiratory syncytial virus, measles, mumps, and rubella; and complement-fixing (CF) antigen does not react with antisera for myxoviruses.

The present report deals with the multiplication of this virus in tissue cultures of human diploid fibroblasts as studied by plaque assay, CF antigen, immunofluorescence microscopy, and thin-section electron microscopy.

MATERIALS AND METHODS

Tissue cultures. Stock cultures of human diploid cell strain WI-38 were carried according to the method of Hayflick and Moorhead (5). Liquid maintenance medium was Eagle's minimal essential medium (MEM) with 1% fetal calf serum. Agar overlay for plaque assays consisted of MEM with 5% fetal calf serum, 400 μg of diethylaminoethyl dextran, 110 mg of sodium bicarbonate per 100 ml, and 0.9% Difco Special Agar (Noble).

Virus assays. Three petri dishes containing WI-38 cells were used for each virus dilution. Absorption was carried out for 1 hr at 33 C in a CO_2 atmosphere. The inoculum was not removed. After addition of the overlay medium, the petri dishes were incubated for 5 days at 33 C in 5% CO_2. Another overlay containing 0.01% neutral red was added, and the plaques were counted the next day.

Growth curve. Replicate cultures of WI-38 cells were prepared in 120-ml prescription bottles and in Leighton tubes containing sterile cover slips. Just before infection of these cultures with 229E virus, a count was made of the cells in one bottle and in one Leighton tube to calculate the virus input per cell. The schedule of sampling for the growth curve is given in Table 1. At 4 hr after inoculation, the medium on all bottles and Leighton tubes was replaced with fresh maintenance medium. The cultures were then harvested for the various tests.

Virus assay. For each virus assay, one bottle infected with 229E was harvested by transferring 9.5 ml of the maintenance medium from this bottle to a centrifuge tube containing 0.5 ml of inactivated chicken serum. This mixture was spun and the supernatant fluid was frozen at −85 C. The cells remaining on the glass surface of the bottle were washed twice with Hanks basal salt solution; then 9.5 ml of fresh maintenance medium plus 0.5 ml of inactivated chicken serum were added, and the bottle was frozen at −85 C. For assay of cell-associated virus, these bottles were thawed and refrozen three times, the fluids were centrifuged to remove debris, and the supernatant fluid was refrozen in several vials for assay by plaque count. To determine how much inactivation of 229E virus might occur during these manipulations, a sample of the standard pool used for inoculum in the growth curve experiments was frozen and thawed four times and was then assayed by plaque count. A reduction to one-third of the original in virus content occurred.

TABLE 1. *Sampling schedule for cultures infected with 229E virus and observation of cytopathic effects*

Time after infection (hr)	Samples taken for[a]				CPE
	EM	VA	FA	CF	
2.7	x				None
6	x	x	x		None
12	x	x	x		None
24	x	x	x	x	None
36	x	x	x	x	Few foci
48	x	x		x	++
60		x		x	+++
72		x		x	++++

[a] EM = electron microscopy; VA = virus assay; FA = fluorescent antibody; and CF = complement-fixing antigen assay.

CF tests. For the assay of CF antigen, one bottle, with medium left on the cells, was frozen at each sampling time noted in Table 1. At the termination of the experiment, these bottles were thawed and refrozen three times, the suspension was centrifuged, and the supernatant fluid was tested for the presence of CF antigen with a known acute and convalescent human serum by use of the microtiter test with two exact units of complement and after fixation overnight at 4 C.

Fluorescent antibody specimens. For examination by the fluorescent antibody technique, the cover slips from two infected and two uninfected WI-38 cultures were fixed in acetone at each interval shown in Table 1. The indirect staining technique was employed with known acute and convalescent human antiserum (1).

Electron microscopy. Duplicate control and infected cultures were sampled for electron microscopic examination at the times shown in Table 1. The cells were removed from the glass surface by gentle rolling of glass beads over the cell monolayer (6) and were centrifuged at low speed to form a pellet. The pellet was fixed for 45 min with either cold 1.33% osmium tetroxide in s-collidine buffer at pH 7.2 or with 1% glutaraldehyde in phosphate buffer and processed routinely for examination of thin sections (7).

RESULTS

Growth curve. The results of the experiment shown in Fig. 1 were obtained after inoculation of virus averaging 1.8 plaque-forming units (PFU) per cell. Cell-associated virus increased rapidly to a maximum at 24 hr after infection (Fig. 1), although at this time no CPE could be detected (Table 1). The maximal amount of virus in the culture fluids was reached at 36 hr, when only minimal CPE was detectable. The titers of both cell-associated and released virus decreased slowly as the CPE became more extensive (Fig. 1 and Table 1).

By the fluorescent antibody technique, specific antigen was detected by bright green fluorescence

in the cytoplasm of a few infected cells as soon as 6 hr after infection (Fig. 2). By 12 hr after infection, the number of fluorescent cells had increased, and, at 24 hr, almost all of the cells showed specific fluorescence in the cytoplasm. Both infected and noninfected WI-38 cells stained with acute phase human antiserum as controls showed a faint green fluorescence which could not be completely removed by a single cycle of absorption of the antiserum on acetone-fixed monolayers of WI-38 cells. Control cultures treated with phosphate-buffered saline instead of human antiserum did not show any fluorescence.

The development of CF antigen paralleled the quantity of virus present in the cell cultures. The highest titer of antigen was obtained between 24 and 36 hr after inoculation. No attempt was made to determine whether this antigen was associated all or in part with the infectious particle.

Electron microscopy. All samples of noninfected WI-38 cultures examined at different times appeared the same ultrastructurally. The usual cytological organelles were present, and no abnormal or unusual structures were observed. In the infected cells, however, the following series of changes occurred. At 3 hr postinfection, an increase in the number of free ribosomes was observed, and, by 6 to 12 hr, there were large accumulations of single ribosomes in many cells (Fig. 3). These accumulations often lie between
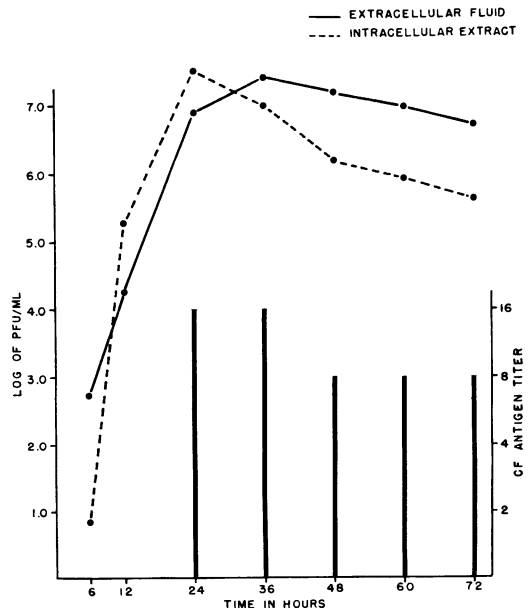

FIG. 1. *Multiplication of 229E virus in WI-38 cell cultures and titration of CF antigen. Symbols: solid line, extracellular fluids, dashed line, intracellular extract.*
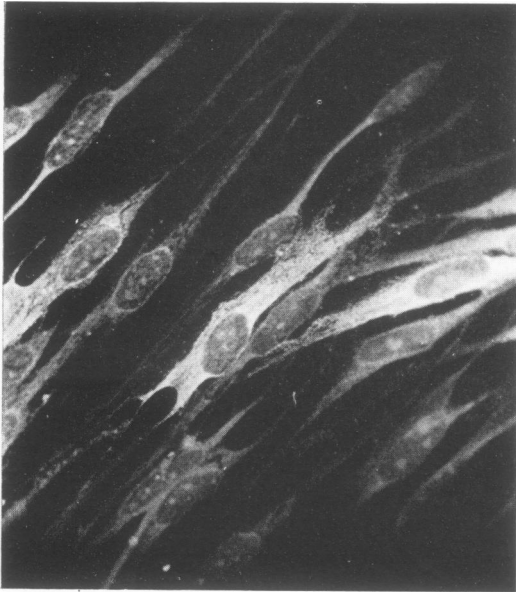
areas of endoplasmic reticulum, but membranous structures were not observed within the areas of free ribosomes.

At 6 hr postinfection, one type of virus-like particle was noticed within dilated vesicles of endoplasmic reticulum. The particles were spherical, 80 to 100 mμ in diameter, and con-

tained a "hollow" or electron-transparent central area 35 to 50 mμ in diameter (Fig. 4). Beneath the outer surface and delineating the hollow core was an electron-dense ring. This ring was not always the same thickness at all points of its circumference, but varied from 5 to 10 mμ to virtually nothing (Fig. 4 and 5). None of these particles contained an electron-dense nucleoid.

The particles were often located in a vesicle of rough endoplasmic reticulum that was in continuity with the space between the inner and outer layers of the nuclear envelope (Fig. 5). By 12 hr postinfection, the particles were seen in large quantities extracellularly, often lining the cell membrane (Fig. 6). Presumably, the particles were liberated into the extracellular space by rupture of the intracytoplasmic vesicles containing them. At later times postinfection, no further changes were observed in the amount or location of these particles.

In addition, after 12 hr, intracytoplasmic, smooth, spherical vacuoles were observed which contained many of these particles along with what appeared to be fragments of them (Fig. 7). These vacuoles were clearly distinguishable from the vesicles of endoplasmic reticulum by their shape and by the absence of ribosomes from the membrane. No other types of particles or membranous components were present within these vacuoles.

At 24 hr and later, a different morphological type of particle was observed in addition to the one previously described. These particles appeared within spherical vacuoles in the cytoplasm. They measured from 70 to 80 mμ in total diameter
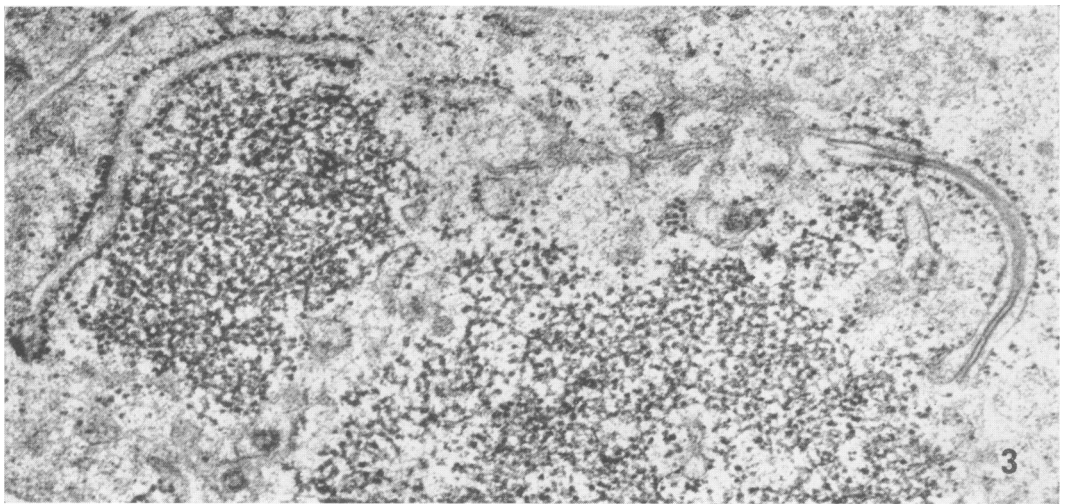

Fig. 3. *Electron monograph of WI-38 cells 6 hr after infection with 229E. Large areas of free ribosomes are present.* × 47,200.
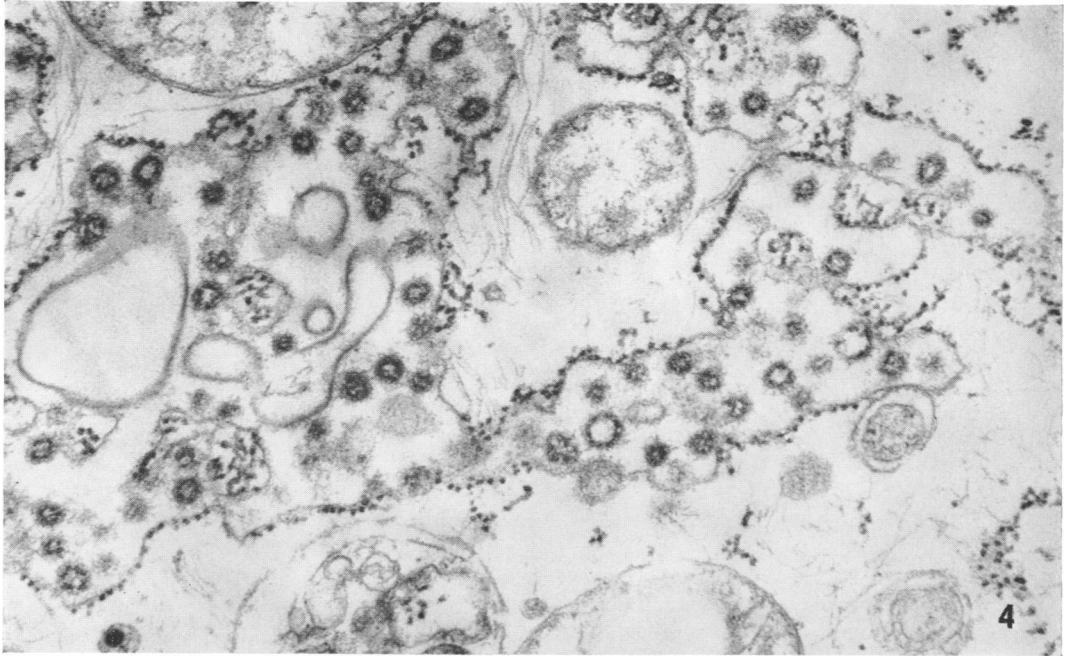
FIG. 4. *Another cell 6 hr postinfection. The virus particles are localized within vacuoles of rough endoplasmic reticulum. × 61,600.*
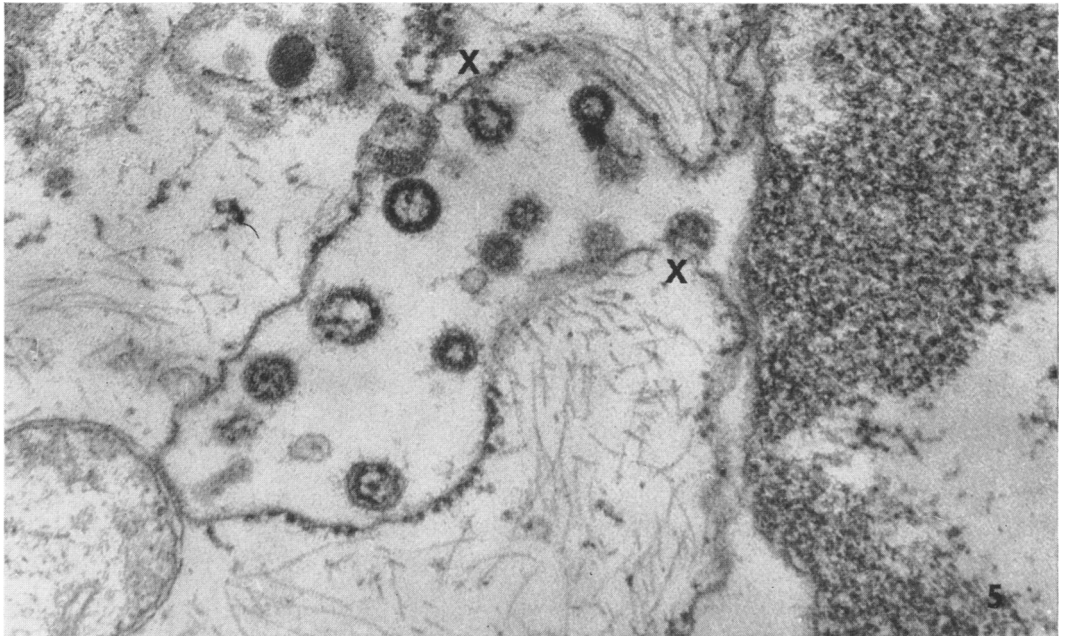

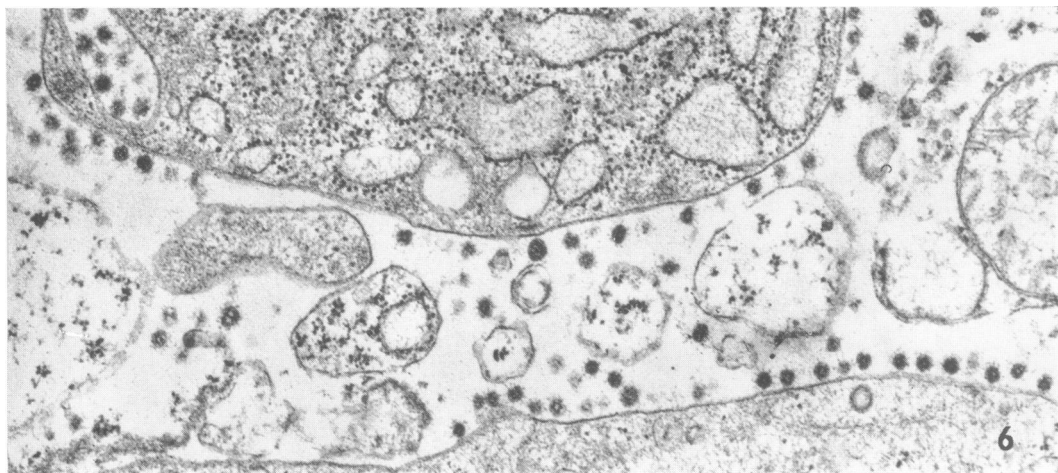FIG. 5. *At 12 hr postinfection. Two possible budding structures are visible (X). × 88,000.*

FIG. 6. *At 12 hr postinfection. Many virus particles are present extracellularly, often lining the plasma membrane* × *39,000.*
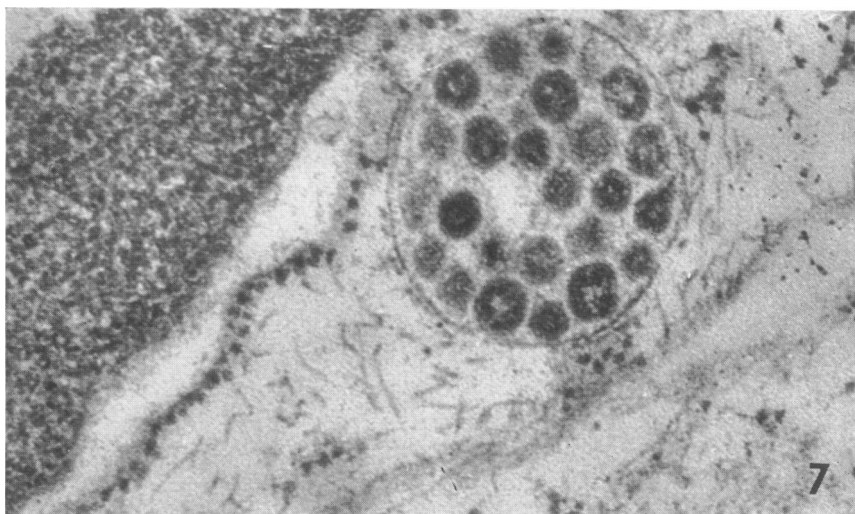

FIG. 7. *At 12 hr postinfection. Hollow type virus particles within a vacuole lined by a smooth membrane.* × *88,000.*

and were completely electron-dense with the exception of a faint peripheral membrane (Fig. 8). Other particles with different grades of density were observed. Often there were other membranous structures within the same vacuole. Most commonly, these particles were distributed around the periphery of the vacuole, and no fragments or incomplete particles were observed. This dense particle was never observed extracellularly in thin sections of cells in the 50-hr observation period.

DISCUSSION

The significance of the two kinds of virus-like structures is not completely clear. The parallel development of the hollow particles shown by electron microscopy with the increased biological activity of the supernatant fluids suggests that these structures are the infectious virus. The fact that the dense particles do not appear until 24 hr postinfection and that they are never seen extracellularly in thin sections of cells argues against their being the biologically active agent. However,
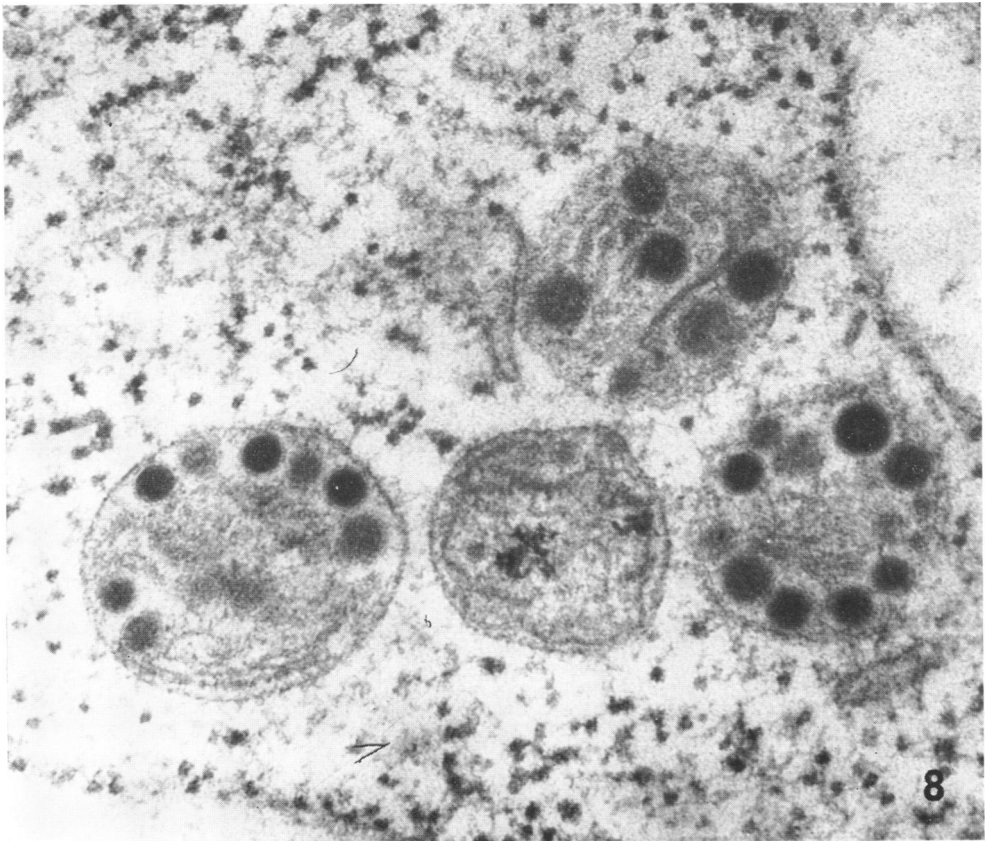
FIG. 8. *At 24 hr postinfection. Dense particles are seen inside vacuoles along with membranous fragments* × *96,000.*

centrifuged culture fluids were not examined for the presence of these structures. These particles could be degenerating virus, incomplete viral structures, or a reaction to the cell injury. Similarly, the nature of the hollow particles within spherical vacuoles is unclear, but this could represent either an alternate synthetic site or a degradation vacuole for adsorbed or phagocytosed particles.

The results of infectivity, complement fixation, electron microscopy, and fluorescent antibody studies are in agreement concerning the development of maximal viral activity. The appearance of extracellular virus particles at 12 hr by electron microscopy is consistent with the time course of the growth curve. The immunofluorescent localization of viral antigen exclusively in the cytoplasm is supported by the absence of viral structures in the nuclei of infected cells.

The mechanism of viral assembly has not been resolved by these experiments. Careful examina-

tion of many samples did not clearly demonstrate virus reproduction by budding at the cell membrane or into intracytoplasmic vesicles as is seen with influenza virus (9) and with the RNA tumor viruses (2). Rarely, however, structures were observed at the surfaces of virus-containing vacuoles that could be interpreted as virus in the process of formation by budding (Fig. 5). These structures were seen so infrequently that their relation to virus development is questionable.

The properties listed in the introduction are compatible with those of certain subgroups of the myxoviruses. Waterson and Almeida (10) have suggested the name pseudomyxovirus for myxoviruses which have Newcastle disease virus (NDV) morphology but do not interact with mucoprotein. However, 229E does not resemble NDV morphologically. A recent investigation by McIntosh et al. (8) has demonstrated the similarity in morphology between 229E and several new viruses which they isolated in organ cultures

inoculated with specimens from human respiratory illnesses. Negatively stained particles have club-shaped projections on the surface rather than the dense array of rods typical of NDV. They point out that a similar club-shaped surface has been found on the infectious bronchitis virus of chickens (3). The morphology of the exterior of 229E (8), the demonstration of intracytoplasmic particles in thin sections of infected cells presented in this study, and the other characistics summarized in the introduction may eventually lead to the definition of another group of viruses distinct from the myxoviruses.

LITERATURE CITED

1. BENNETT, C. R., JR., AND D. HAMRE. 1962. Growth and serological characteristics of respiratory syncytial virus. J. Infect. Diseases 110:8–16.

2. BERNHARD, W. 1960. The detection and study of tumor viruses with the electron microscope. Cancer Res. 20:712–727.

3. BERRY, D. M., J. C. CRUICKSHANK, H. P. CHU, AND R. J. H. WELLS. 1964. The structure of infectious bronchitis virus. Virology 23:403–407.

4. HAMRE, D., AND J. J. PROCKNOW, 1966. A new virus isolated from the human respiratory tract. Proc. Soc. Exptl. Biol. Med. 121:190–193.

5. HAYFLICK, L., AND P. S. MOORHEAD, 1961. The serial cultivation of human diploid cell strains. Exptl. Cell Res. 25:585–621.

6. KINDIG, D. A., AND W. H. KIRSTEN, 1967. Virus-like particles in established murine cell lines: electron microscopic observations. Science 155:1543–1545.

7. KIRSTEN, W. H., L. A. MAYER, AND C. W. WEDLANDER, 1966. Infectious and non-infectious viral murine leukemias. Natl. Cancer Inst. Monograph 22:369–377.

8. MCINTOSH, K., J. H. DEES, W. B. BECKER, A. Z. KAPIKIAN, AND R. M. CHANOCK, 1967. Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. Proc. Natl. Acad. Sci. U.S. 57:933–940.

9. MORGAN, C., H. M. ROSE, AND D. MOORE, 1956. Structure and development of viruses observed in the electron microscope. III. Influenza virus. J. Exptl. Med. 104:171–182.

10. WATERSON, A. P., AND J. D. ALMEIDA, 1966. Taxonomic implications of 'myxovirus.' Nature 210:1138–1140.