An Electron Microscopic Study of Rabbit Syncytium Virus* (34523)

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To reduce the possibility of exposing human beings to animal viruses with unknown disease potential, elimination, or at least inactivation, of viruses that contaminate cell cultures used for growth of vaccine virus is essential. Since it is currently proposed (1) to produce rubella vaccine in cultures of rabbit kidney cells, characterization of viruses indigenous to the rabbit is important, especially of those that might be present in cultures of cells prepared from rabbit kidney.

Recently, a previously undescribed virus, tentatively designated rabbit syncytium virus, was isolated from tissues of an apparently healthy cottontail rabbit (2). Since then, a strain of the same virus has been found as a contaminant in a cell culture prepared from kidneys of a laboratory-bred New Zealand rabbit (3). The new virus, on the basis of chemical, physical, and biological properties, was provisionally classified as a myxovirus. To facilitate recognition of rabbit syncytium virus in contaminated cell cultures and to gather additional information that might be useful in classifying definitively the new agent, we studied its ultrastructure and virus-cell relations by electron microscopy. This paper is an interpretative report of our findings.

Materials and Methods. The BHK-21 continuous line of baby hamster kidney cells and primary cultures of dog kidney cells were selected in preference to rabbit kidney cells for use in this study as substrates for propagation of rabbit syncytium virus for two reasons: (1) rabbit syncytium virus grows to higher titers in these selected cell cultures than in rabbit kidney cells; and (2) cell cultures prepared from the kidneys of rabbits available to us frequently were contaminated with other viruses (3). The BHK-21 line of baby hamster kidney cells was propagated in No. 2 Falcon flasks in Eagle's basal medium with Earle's base and 3% fetal bovine serum; primary dog kidney cells were grown in glass bottles in medium 199 with 5% calf serum. The cells were inoculated with 10^7 50% tissue culture infectious doses of rabbit syncytium virus that had been passed 12 times in embryonated chicken eggs, 1 time in WI-38 cells, and twice in BHK-21 cells. The virus assay procedure employed has been described (2). Three days [this interval was selected because of the growth characteristics of rabbit syncytium virus (2)] after inoculation, when practically all cells were exhibiting virus-related cytopathic changes, the cultures were fixed by replacing the nutrient fluid with cold isotonic gluteraldehyde solution (2.25% gluteraldehyde in 0.06 *M* *s*-collidine buffer, pH 7.3). The fixed cells were scraped from the bottom of the flask and pelleted by low-speed (500*g*) centrifugation. The pellet was cut into 1-mm-diameter pieces; these were postfixed in isotonic osmium tetroxide (2% osmium tetroxide in 0.22 *M* *s*- collidine buffer, pH 7.3) for 30 min, dehydrated in graded ethanol solutions and propylene oxide, and infiltrated and embedded in Epon 812. Thin sections were cut with a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, and observed with an Hitachi HU 11-C electron microsope. Adjacent "thick sections" (1 μ thick) were prepared and stained

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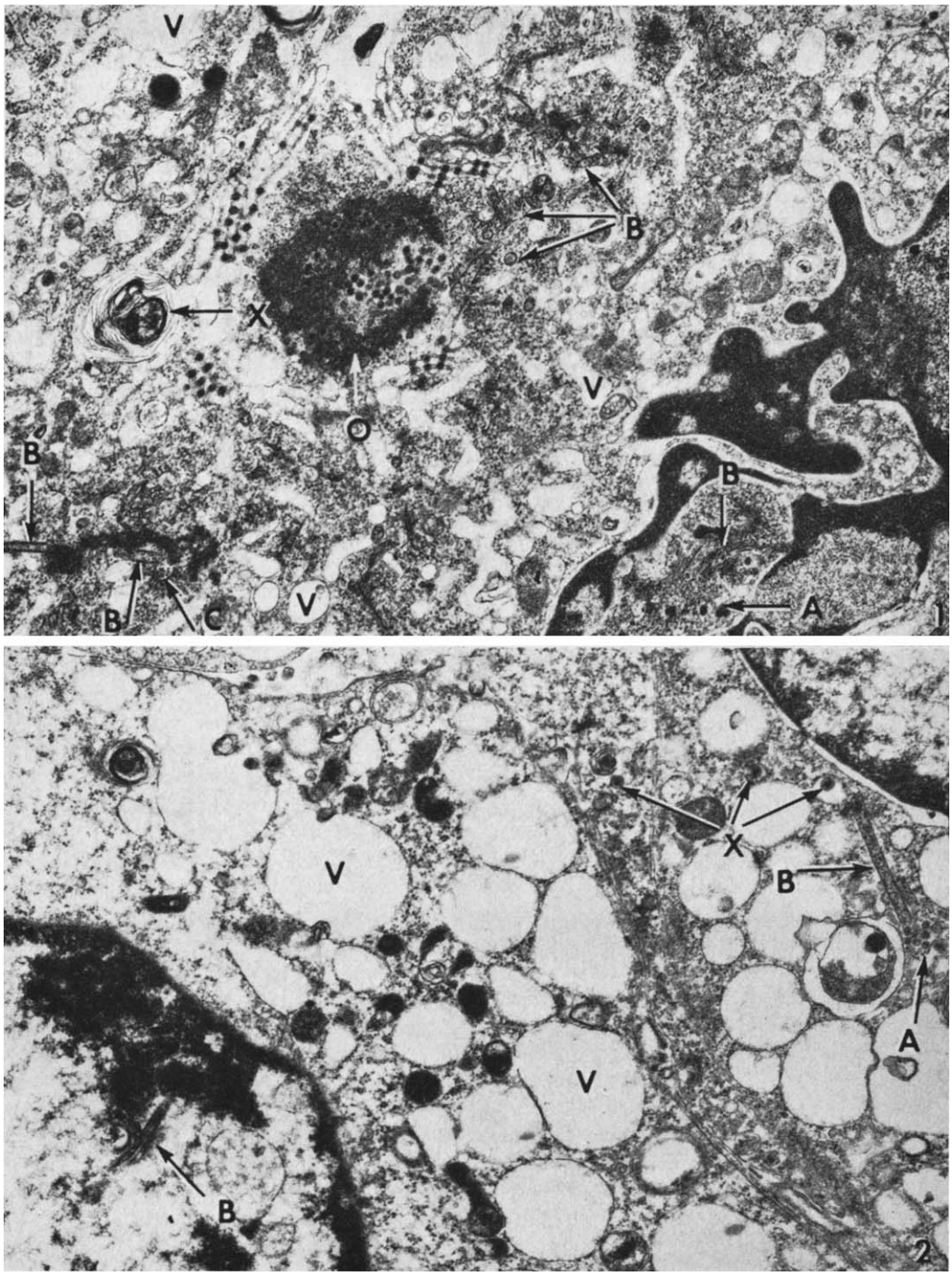

FIG. 1. Part of a degenerating cell with nuclear chromatin margination (lower, right), slight cytoplasmic vesiculations (V), and a single myelin figure (X→). Spherical virus is forming in a dense granular cytoplasmic inclusion (O→). Near the cytoplasmic inclusion, virus is linearly arranged in cytoplasm between smooth membranes. Spherical virus (A→) is also in the nucleus. Rods (B→) are in the cytoplasm and nucleus. In the lower left, the apparent end of a rod is associated with dense

granular material in which spherical virus appears to be forming. (Fig. 4 is an enlargement of a portion of this area.) C→ denotes an apparent dilated portion of rod showing thin tapered projections; ×14,700.

FIG. 2. Portions of several cells showing marked cytoplasmic vesiculation (V) and nuclear chromatin margination. Rods (B→) are in the nucleus (lower, left) and in cytoplasm (upper right), where spherical virus (A→) is associated with the apparent end of a rod. Spherical virus indigenous to the BHK-21 cell culture are also present (X→) within cytoplasmic vesicles; ×24,000.

with toluidine blue (4). Negative-stained infected materials were prepared by Chamber's method (5) from gluteraldehyde-fixed pellets and unfixed supernatant fluids and pellets. Uninfected BHK-21 and primary cultures of dog-kidney cells were propagated, processed, and examined by the same methods used in studying the infected cultures.

Results. In the virus-infected BHK-21 and primary dog-kidney cultures, practically all cells examined showed varying degrees of cell damage characterized by nuclear pyknosis and karyolysis, chromatin margination, and cytoplasmic fragmentation and vesiculation. Similar damage appeared in an occasional cell in uninfected control cultures. The cytoplasmic vesicles were lined by a unit-type membrane. Most were electron lucent, and a few (apparently phagosomes) contained granular, membranous, and amorphous material (Figs. 1 and 2). Mitochondria, ribosomes, and other organelles were morphologically similar in infected and uninfected cells when the degrees of cytopathic changes were correspondent. Rabbit syncytium virus did not induce formation of syncytia in fibroblastic BHK-21 and dog-kidney cells used. In this respect, the cell cultures respond differently from virus-inoculated epithelial-cell cultures, in which syncytia formation is characteristically induced (2).

Two ultrastructural forms were seen in thin sections of cells infected with rabbit syncytium virus, but not in those of uninfected control cells. One form consisted of a rounded, intracytoplasmic particle, 650 Å in diameter, with an indistinct, moderately electron-dense periphery 50-100 Å thick (Figs. 1, 2, and 3). Inside this periphery was a 50-Å thick membrane confining a center consisting of an exceedingly electron-dense 200-Å diameter central part, with no delineating membrane, and a less-electron-dense 100-Å thick outer part. Negative-stained preparations (Fig. 5) contained round particles 500-600 Å in diameter, with nodular or spiked peripheries suggestive of capsomers overlying a membrane that limited a center 375-475 Å in diameter. In negative-stained preparations, some of the particles showed the central part of the membrane-delineated center to be penetrated by stain, resulting in marked electron density, but the peripheral part of the center was only moderately electron dense (Fig. 5, left). Other particles showed moderate electron density of the entire center (Fig. 5, right). Helical strands were not seen in the particles in negative-stained preparations.

These round particles seemed to originate within dense, granular, cytoplasmic inclusions (Figs. 1 and 3), which were found easily in most cells and showed no particular fixed localization within the cell cytoplasm. Rarely, these particles seemed to form in similar dense, granular material in cell nuclei. In some nuclei, these particles were present singly or in groups of two or more, unassociated with dense granular inclusion material (Fig. 1). They were not found extracellularly, except in rare instances when they were seen inside a membrane-bound cytoplasmic vesicle (Fig. 6). When found in this location, the diameter of the particles was 100-150 Å larger than those found in the cytoplasm. In passing through the wall of the cytoplasmic vesicle, particles apparently acquired a coat derived from the vesicle membrane (Fig. 6), acquisition of which probably accounts for the increased size of the intravesicular spherical particles. Centers of the particles within vesicles were more electron dense than those in the cell cytoplasm.

The second structure observed in cells infected with rabbit syncytium virus was a nonsegmented rod (Figs. 1, 2, and 4) of 750-1100 Å diameter and of indeterminant length. The periphery of the rod was a unit-like membrane. Rarely, a rod with a curved

end, suggestive of a closed tube, was seen. Within the rods were structures 100-150 Å in diameter (Fig. 4), which were indistinguishable from ribosomes. The structures were usually distributed in a relatively electronlucent, finely granular substance. Infrequently, an apparently localized, dilated area in the rod was seen that had radiating, thin, tapered projections (Fig. 1) which were covered with a membrane continuous with that of the rod.

Rods were often found in the cytoplasm

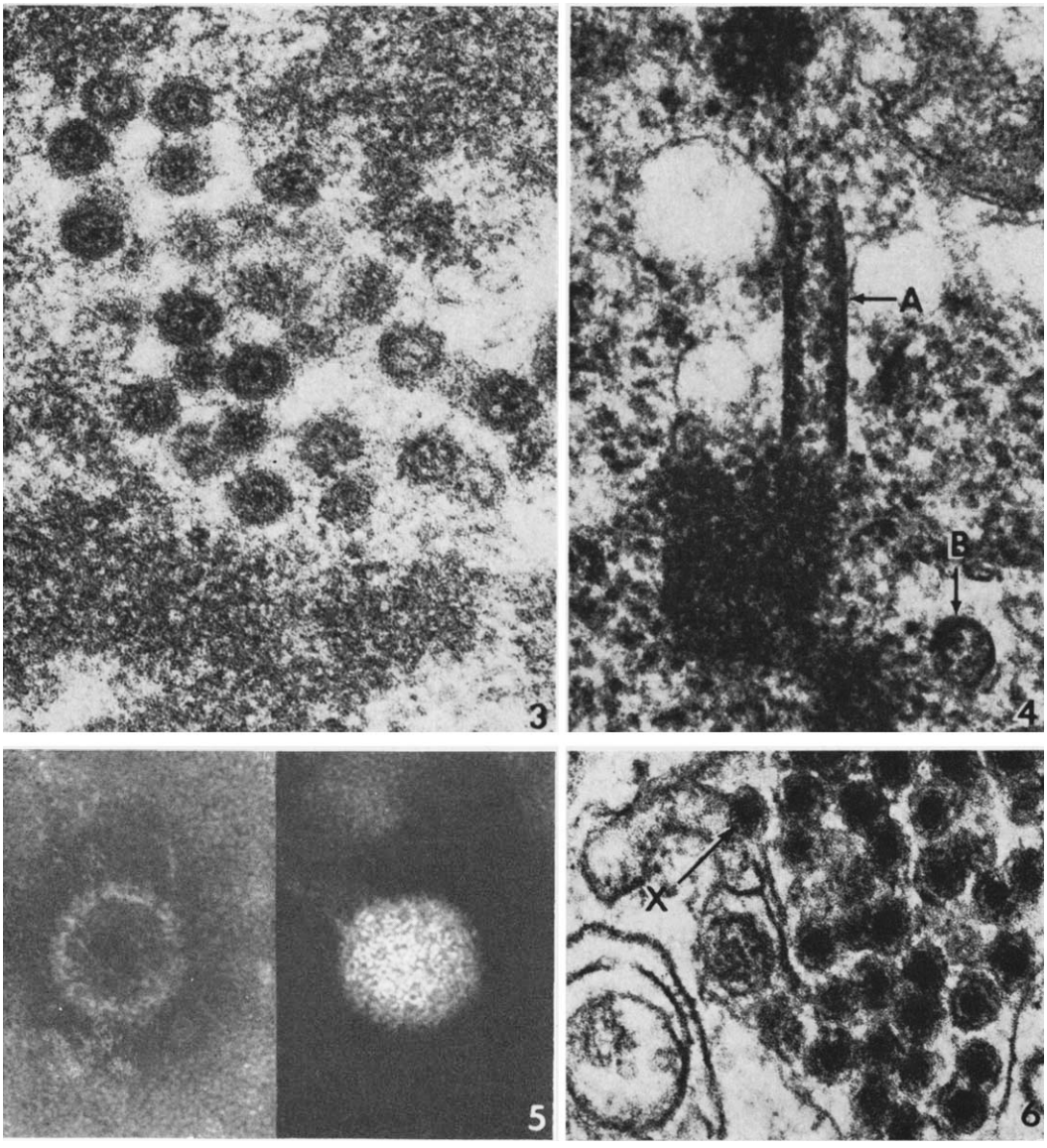

FIG. 3. Spherical virus in a dense cytoplasmic inclusion; ×123,000.

FIG. 4. Rods (A→ = longitudinal section, and B→ = cross section) showing dense granular material at the apparent end of a rod. (High magnification of part of a rod shown in Fig. 1.) ×100,000.

FIG. 5. Negative-stain of two spherical viruses from a virus-inoculated, primary dog kidney cell culture. The one on the left has a stain-penetrated central area; ×300,000.

FIG. 6. Membrane-coated spherical virus (750 Å-diam.) in a membrane-bound cytoplasmic vesicle. X→ indicates a virus apparently passing through the vesicle membrane obtaining a peripheral membrane coat; × 109,000.

haphazardly arranged in masses just beneath the cell membrane. Present in these masses were closely packed membranous structures that appeared to result from incomplete rod formation. Rods were usually found distributed singly or in clusters in the cytoplasm. But in rare instances, they were found in the nucleus (Figs. 1 and 2). When located intranuclearly, their presence was usually associated with nuclear chromatin margination or pyknosis (Figs. 1 and 2). Rods were not seen to bud from the cell surface, to enter a cell, to penetrate the nuclear membrane, or to occur extracellularly. This suggests that these structures were cellular lesions resulting from virus infection, rather than a body representing a stage in the multiplication cycle of rabbit syncytium virus. Nevertheless, occasionally, spherical particles were seen at the apparent end of a rod, where they were associated with dense granular material (Figs. 1, 2, and 4). In no instance were spherical particles found within a rod. It was not uncommon, however, to find rods associated with packed membranous structures that appeared to be "abortive rods"; these, in some instances, were adjacent to dense inclusions containing virus particles.

Infrequently seen in both infected and control BHK-21 cells was an intravesicular and extracellular virus particle with a diameter of 1000-1100 Å (Fig. 2). It had a 250-300 Å-thick, medium electron dense, segmented periphery that had an outer 50-75-Å-thick limiting membrane from which protruded small knobs. A similar membrane limited a central dense nucleoid. It appeared to pass from cytoplasm into cytoplasmic vesicles by budding, acquiring its outer membrane in the process. This particle appears to be morphologically identical to the particles of unknown significance reported by others to be present in the BHK-21 cell line (6).

Discussion. The intracellular distribution and morphologic properties of rabbit syncytium virus, consisting of intracytoplasmic and intranuclear spherical particles associated with intracytoplasmic and intranuclear rod-like elements are, as far as we are aware, distinctive and unlike those reported for previously described viruses. The distribution and morphology of the virus, however, closely resemble those of epizootic diarrhea of infant mice (EDIM) (7, 8). Both rabbit syncytium and EDIM viruses are spheres of similar size. Both originate in morphologically similar inclusions, and both acquire coats from vesicular membrane as they pass from the cytoplasm into cytoplasmic vesicles. ED IM virus, however, has not been seen in the nucleus of infected cells; in this respect, it differs from rabbit syncytium virus. But the most striking morphologic differences between the two types of viruses are concerned with their associated intracellular rods. The rod associated with rabbit syncytium virus possesses a single limiting membrane, contains ribosome-like particles, and is located most commonly in the cytoplasm in densely packed accumulations that frequently contain membranous structures interpreted to be rods aborted in their formative process. On the other hand, the rod associated with EDIM virus possesses a single or a double membrane, contains a continuous, dense, centrally located core, is found distributed singly or in accumulations of two or three in the cytoplasm and in cytoplasmic vesicles, and is not associated with abortive rod forms. Moreover, rabbit syncytium and EDIM viruses can be differentiated on the basis of their cultural, serologic, and physical properties. Rabbit syncytium virus propagates readily in embryonated chicken eggs and in a variety of cultured cells, whereas, EDIM virus has not been reported to propagate in any of these experimental hosts (9). Furthermore, rabbit syncytium and EDIM viruses do not cross-react in appropriate virus neutralization tests (Morris, J. A., unpublished). Also, rabbit syncytium virus is inactivated by exposure to heat (56° for 30 min) and to diethyl ether (2), but EDIM virus is not (8).

Rabbit syncytium virus in negative-stained preparations differs morphologically from described myxoviruses, paramyxoviruses, and respiratory syncytial virus. The rabbit syncytium virus particles seen in negative-stained preparations appeared to have cubic

symmetry. These probably represent intracytoplasmic particles that have not acquired a peripheral membrane by passing extracellularly, since they were of the same size as the intracytoplasmic spherical particles seen in thin sections. Myxoviruses, paramyxoviruses, and respiratory syncytial virus show helical symmetry and are not discernible until they begin to acquire, or have acquired, their coats by budding. Extracellular rod structures, considered to be virus forms, have been seen in electron micrographs of cell cultures infected with myxoviruses, paramyxoviruses, respiratory syncytial virus, and certain arboviruses. These viral rods reproduce by budding from cell or vesicle membranes and, in this respect, differ from the rods seen in cells infected with rabbit syncytium virus.

On the basis of its ultrastructure, intracellular distribution, and apparent mode of reproduction, and on the basis of previously described physical and biologic properties (2), rabbit syncytium virus seems to be distinct from viruses in established groups (10). Its identification, however, when encountered as a contaminant in cultures of rabbit kidney cells used in production of vaccines destined for use in man, should present little difficulty. In fact, a tentative identification of rabbit syncytium virus might be made by electron microscopy alone since, on the basis of the preliminary information in this report, the virus seems to induce distinctive changes in infected BHK-21 and dog kidney cells.

The presence of the spherical virus-like particle indigenous to the BHK-21 line of cells does not seem to interfere in the use of this cell line as a substrate for growth of rabbit syncytium virus. This indigenous particle can be differentiated readily from rabbit syncytium virus: the former structure is larger (1000 Å in diameter for the indigenous particle, against 650 Å for the intracytoplasmic form and 750-800 Å for the intravesicular form of rabbit syncytium virus) and possesses a "segmented" periphery with knobs that are absent in the rabbit syncytium virus particle.

Summary. The ultrastructure and virus-cell relation of rabbit syncytium virus was studied by electron microscopy. The virus was seen in infected cell cultures, frequently in the cytoplasm and rarely in the nucleus, as a 650 Å-diameter, nonmembrane-bound sphere. It was rarely found in intracytoplasmic vesicles as a 750-800 Å-diameter, membrane-bound particle, the latter particle having acquired, in passing intravesicularly, a membrane derived from the vesicle wall The virus appeared to originate within dense cytoplasmic inclusions, and rarely, within similar nuclear inclusions. The virus particles were frequently associated with membrane-bound intracytoplasmic and intranuclear rods whose diameter was 750–1100 Å. The biologic significance of the rod structures is not known. Our findings suggest that the ultrastructure and the intracellular behavior of rabbit syncytium are unlike those associated with any known virus, but approach most nearly those associated with the virus of epizootic diarrhea of infant mice. Since the pathogenic potential of rabbit syncytium virus for man is unknown, we suggest that its absence be demonstrated in rabbit kidney cell cultures that might be used in preparation of vaccines for human use.

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