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In Vitro Morphology and Maturation of Lymphocystis Virus[1]

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The temporal sequence of development of lymphocystis disease virus (LDV) was studied by electron microscopy of thin sections of infected tissue-culture monolayers. Neither the typical cytoplasmic inclusion nor virus was detected at 4 days postinfection (PI). Inclusions, but no viruses, were detected at 8 days PI. Inclusions and associated virions were detected at 15 days PI, and by 28 days PI the undisrupted cells were filled with the typical virions. No release mechanism was detected, and severe clumping of particles was noted. Negatively stained preparations revealed particles 200 nm in diameter with no capsomere structure and apparent spikes associated with the particle. The relationship of LDV to the well-defined deoxyribonucleic acid virus groups is discussed.

Lymphocystis disease virus (LDV) is the inciting agent of a benign tumor in numerous species of fish (12). These tumors are composed of masses of cells (lymphocystis cells) that are greatly enlarged, unable to divide, contain massive Feulgen-positive cytoplasmic inclusions, and have a cell membrane modified into a thickened capsular structure (4). Recent cultivation of the virus in cell culture (15) allows study of the virus in infected cells in vitro, and of negatively stained particulate preparations. Virus particles in tumor sections have been reported as being 200 nm in diameter with a hexagonal profile and, occasionally, in crystalline array (9–11).

MATERIALS AND METHODS

Virus was grown in a fibroblastic cell line (BF-2) derived from the common bluegill (*Lepomis macrochirus*). The line was initiated at the Eastern Fish Disease Laboratory (15). Infected cells were harvested at appropriate intervals by use of Viokase-ethylenediaminetetraacetate. Suspended cells were sedimented in a clinical centrifuge at 1,200 × *g* for 30 min and washed with Hanks' balanced salt solution (BSS) before fixation. Fixation was accomplished with 5% glutaraldehyde in 0.1 M phosphate buffer at *p*H 7.0 for 2 hr at 4 C. After four washes, for 30 min each, with phosphate buffer, the cells were stained in 2.5% osmium tetroxide under vacuum. Cells were dehydrated in acetone and embedded in Epon 812 for sectioning.

Particulate preparations were obtained from 28-day harvests of infected cells and overlying medium containing approximately 10^6 ID_{50} per ml. Cells and medium were centrifuged at 1,200 × *g* for 30 min and the pellet was suspended in 0.1 volume of Hanks' BSS. The supernatant fluid was discharged. Most of the infectivity is known to be associated with the cells (15). The cell suspension, while immersed in an ice bath, was sonically treated for 2 min at 40 w output (macrotip) with a model W-185-C Sonifier (Branson Instruments, Inc., Stamford, Conn.). The sonically treated suspension was centrifuged for 30 min at 1,500 × *g* and the pellet was again suspended in 0.1 volume of distilled water (100-fold concentration in terms of the original harvest). This stock virus preparation was mixed with an equal volume of 2% phosphotungstic acid and applied, by the surface-spreading technique (8), to grids coated with Formvar-carbon.

RESULTS

Thin sections of infected cells were prepared at 0, 4, 8, 15, and 28 days PI. Unadsorbed clumps of virus were detectable in the interstices between packed cells in all sections prepared through 15 days (Fig. 1). The number and size of the aggregates decreased during this period. Walker also noted clumps of virus on disruption of infected cells (4). The clumping phenomenon probably accounts for the relatively low titer (10^6 ID_{50} per ml) obtained at 28 days even after sonic treatment of more than 10^6 cells, each of which must contain approximately 10^5 mature virus particles.

A count of the virions present in a section of a

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28-day cell (30 μ in diameter), the major portion of which is shown in Fig. 5, revealed more than 10^3 particles. Mature 28-day lymphocystis cells vary from 30 μ to slightly more than 100 μ in diameter. The average diameter of 100 infected cells at one week PI was 30 μ when measured as spheres in a hemocytometer. Therefore, 30 μ is a conservative estimate of the diameter of a mature lymphocystis cell. We assume the thickness of the section to be 0.2 μ, i.e., the diameter of a virus particle, although the sections averaged 0.15 μ as determined by interference coloration. Further assuming that the section in Fig. 5 is a cylinder through the equator of the cell, and that virions are uniformly distributed, as found in all sections of 28 day cells, it can be calculated that a minimum of 10^5 virions are present. Larger cells would contain larger numbers of particles.

At 8 days PI, no virus particles were seen in either nucleus or cytoplasm although the typical inclusion bodies formed during infection are visible (Fig. 2). The inclusion appears to be an amorphous substance and is not bounded by a membrane.

By 15 days PI, virus particles are visible either immediately adjacent to the inclusion body or contained within the fenestrations of the inclusion (Fig. 3, 4). From serial sections, it is apparent that the fenestrations are not wholly contained within the inclusion but, at some point, are contiguous with the cytoplasm. We interpret Walkers' electron micrograph (13) as a section through a fenestration, and he has referred to folded in "new surface" (10). The fenestrations evidently provide a larger inclusion surface area at or near which assembly of the virus particles takes place.

By 28 days after infection, almost the entire cytoplasm not occupied by cellular organelles is packed with virus particles (Fig. 5–7). We have not observed regular crystal arrays. The inclusions have moved from the juxtanuclear position to the periphery of the cell as described by Dunbar and Wolf (4). No particles are found in the nucleus and no accessory membrane is present either in sections of cells or in particulate preparations. There is no evidence of liberation of mature particles; the cell remains essentially intact.

Measurement of 25 particles, chosen at random from three photographic plates, gave a mean particle size at the greatest diameter of 196 nm and a range of 192 to 215 nm. These measurements are in excellent agreement with those of Walker, who found a mean of 200 nm and a range of 180 to 220 nm in tumors from *Stizostedion vitreum* (9), and a mean of 200 nm in tumors from *Lepomis macrochirus* (9–11).

In negatively stained preparations, undistorted particles are uniformly hexagonal in outline. There is no evidence of an acessory membrane and there are no distinct capsomeres. Occasionally, there are indications of surface projections (Fig. 10, 11). The mean size of 25 negatively stained particles was 200 nm.

DISCUSSION

Our results in vitro confirm those of Walker on the tumor cell in vivo. The virus is hexagonal in outline, and may well be an icosahedron with an average particle size near 200 nm and no accessory membrane or visible capsomeres. Walker also noted the initial appearance of mature virus particles in or immediately around the area of the inclusion (10, 13). The results of an autoradiographic study (R. G. Malsberger, *unpublished data*) confirm that the inclusion body is the site of deoxyribonucleic acid (DNA) synthesis. The electron micrographs are consistent with the interpretation that the viral capsid is added to the DNA on, or near, the inclusion body. The sequence of appearance of mature particles correlates reasonably well with the in vivo multiplication curves presented by Wolf et al. (4, 14), who detected an increase over the residual level at 9 days PI.

Our micrographs indicate a large increase in the number of virions present during the late stages of infection, i.e., 15 to 28 days. This rise is inconsistent with the plateau of infectivity detected by these authors. We agree that the plateau may be accounted for by the clumping effect mentioned by Dunbar and Wolf (4) and confirmed by our results. The failure of Wolf et al. (4, 14) to detect the increase can be attributed to the clarification procedures employed, which may have removed the aggregates and thus depressed the virus titer.

Although it has been suggested that LDV is a member of the pox virus group (13) on the basis of the formation of Feulgen-positive cytoplasmic inclusions, the morphology of the particle is clearly unlike the "mulberry" forms of the pox group, and is not round or oval in cross section. Multiplication in the cytoplasm and lack of an accessory membrane exclude it from the herpesvirus group. The particle size is too large for inclusion in the other DNA animal-virus categories.

The hexagonal profile of the LDV particle, which lacks apparent capsomeres, closely resembles the profile of several amphibian viruses both in thin sections (3, 6) and in negatively stained preparations (7). This profile is evident also in insect iridescent viruses such as Tipula and Sericesthis (1, 2, 5). All of these viruses develop in the cytoplasm in association with

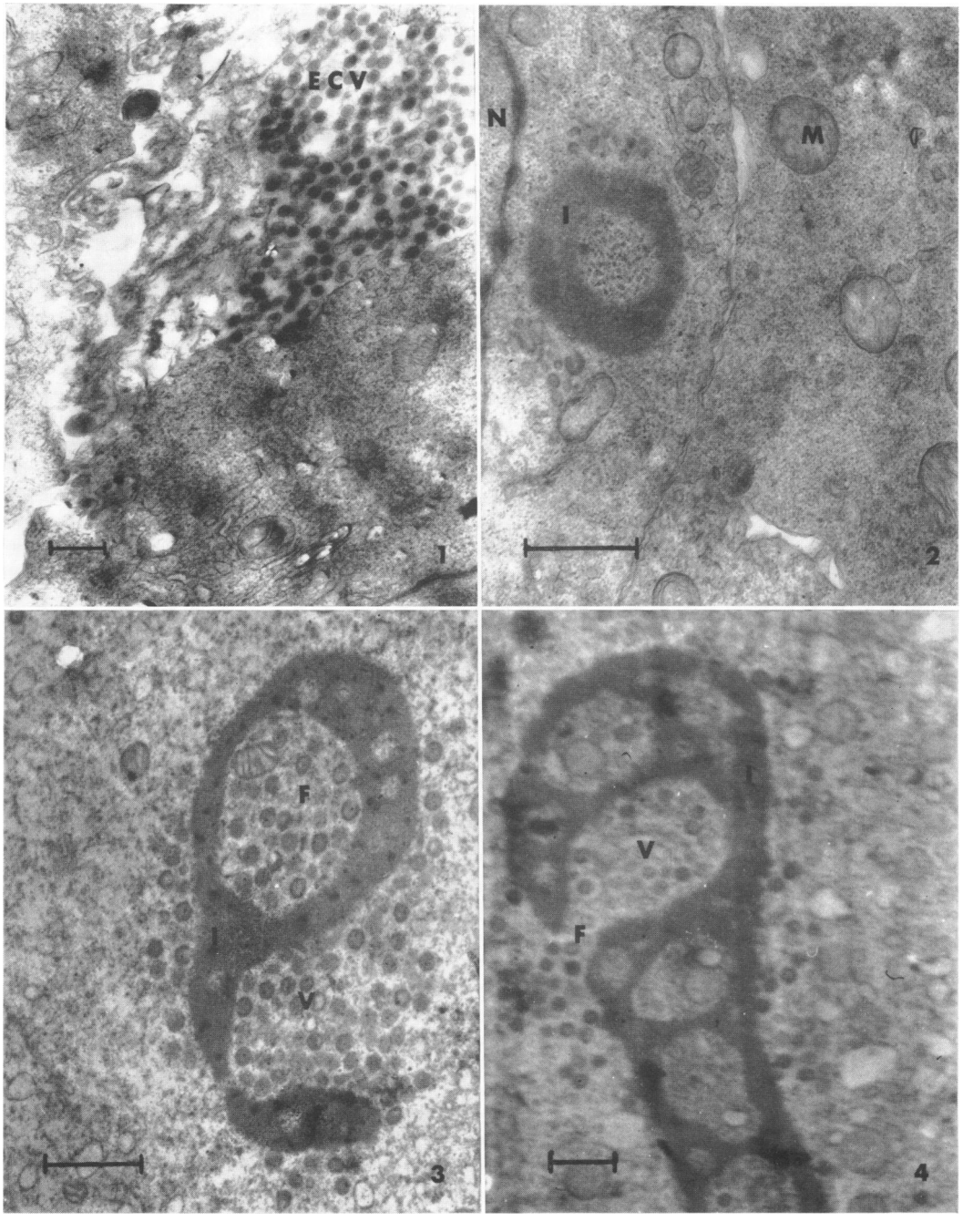

FIG. 1–4. *Electron micrographs of thin sections of infected BF-2 cells. The bar in each figure represents 1 μ.*

FIG. 1. *Section of cells harvested 4 days PI showing clumps of extracellular virus (ECV). Cells otherwise resemble normal cells except that the numerous cytoplasmic processes have withdrawn. × 9,000.*

FIG. 2. *Section of cells harvested 8 days PI showing the perinuclear inclusion body (I), nucleus (N), and numerous mitochondria (M). × 18,000.*

FIG. 3–4. *Section of cells harvested 15 days PI showing inclusion (I), fenestrations (F), and numerous virions (V) adjacent to the amorphous inclusion substance and within the fenestrations. Note continuity of fenestrations with the cytoplasmic matrix. FIG. 3, × 16,000. FIG. 4, × 11,000.*

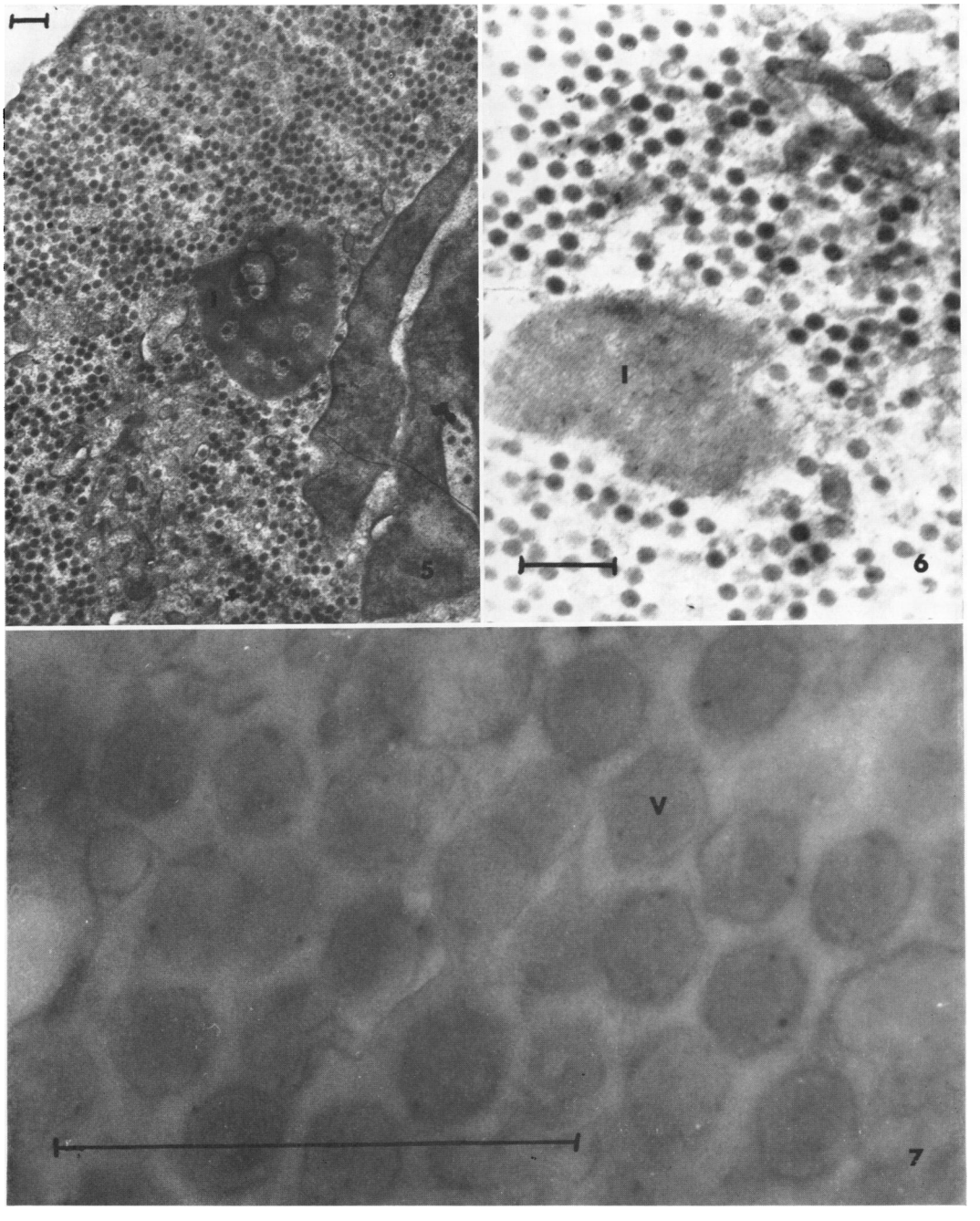

FIG. 5–7. *The bar in each figure represents 1 μ. Sections of cells harvested 28 days PI showing the complete disintegration of cellular structures, the persistence of the inclusion substance (I), and the massive accumulation of virus particles (V) within the cells. No release was noted in any sections examined.* FIG. *5,* × *6,000.* FIG. *6,* × *15,000.* FIG. *7,* × *80,000.*

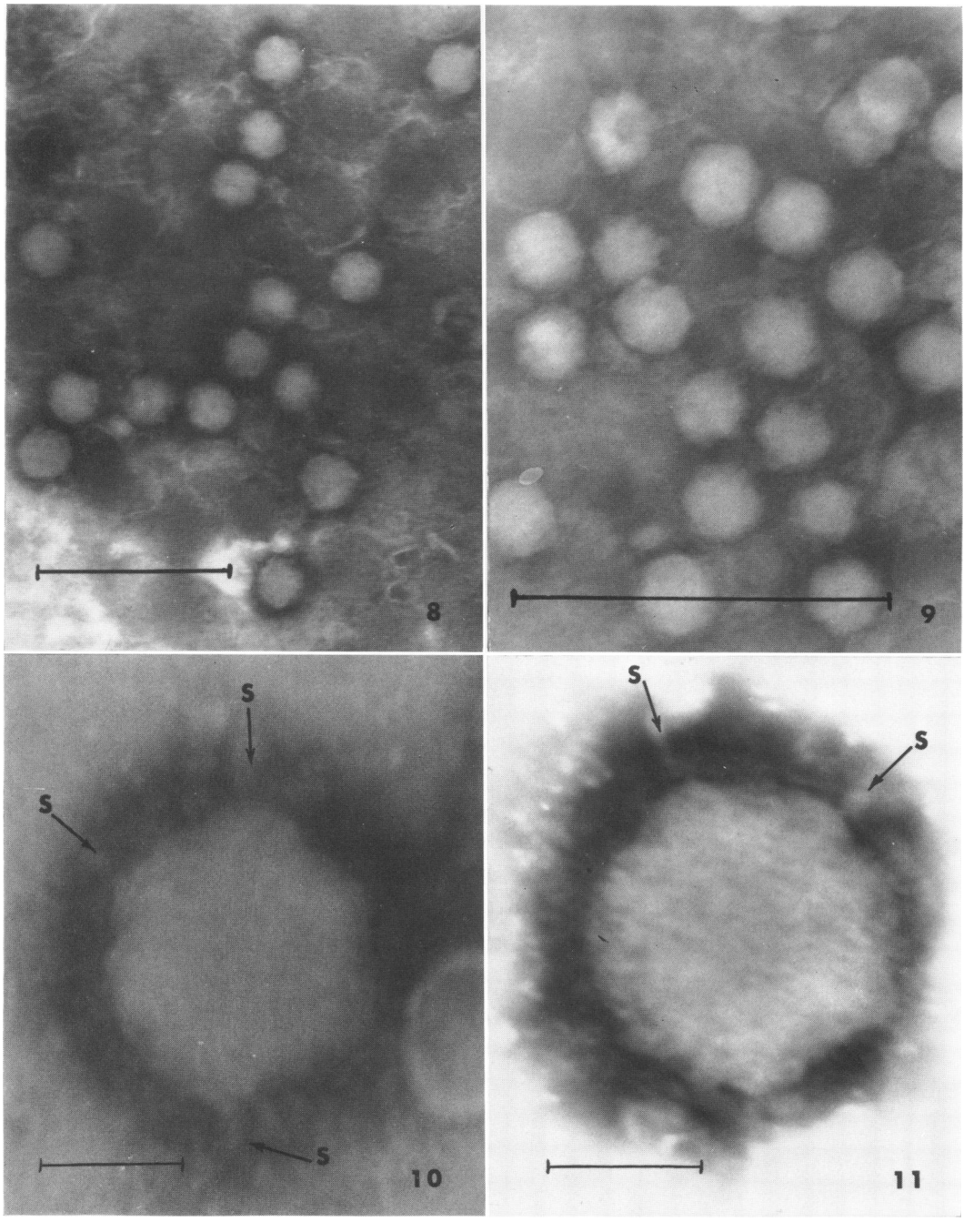

FIG. 8–11. *Electron micrographs of negatively stained lymphocystis virus particles.*

FIG. 8–9. *The bar in each figure represents 1 μ. Negatively stained LDV particles from BF-2 cells, 28 days PI, and associated cell debris. No capsomere structure was evident. Close inspection indicates a membranous capsid collapsed about the core. FIG. 8, × 31,000. FIG. 9, × 60,000.*

FIG. 10–11. *The bar in each figure represents 0.1 μ. Negatively stained LDV particles from BF-2 cells, 28 days PI, showing presumptive spikes (S) or filaments projecting into the surrounding phosphotungstic acid stain. FIG. 10, × 220,000. FIG. 11, × 240,000.*

inclusions that contain DNA as determined by Feulgen staining and acridine-orange ultraviolet fluorescence. The inclusions associated with the amphibian viruses appear to be of a more diffuse type than those of LDV and the insect viruses. Both these amphibian viruses and the insect iridescent viruses replicate in a more rapid temporal sequence than does LDV, and all except Tipula iridescent virus acquire an accessory membrane by a budding process at the cytoplasmic membrane during liberation from the cell. LDV differs from these viruses by virtue of its larger diameter and lack of an accessory membrane. LDV may be related to the amphibian and insect viruses mentioned above, although defective with regard to acquisition of an accessory membrane. It is obvious that LDV does not fit into any of the established groups of the present classification scheme.

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