Partial Purification and Electron Microscopy of Virus in the EB-3 Cell Line Derived from a Burkitt Lymphoma

Abstract. *The virus in the EB-3 cell line derived from a Burkitt lymphoma was partially purified by digestion with proteolytic enzyme and by density-gradient centrifugation on potassium tartrate. Electron microscope studies of both sectioned and negatively stained samples suggest that the virus belongs to the herpesvirus group, although the exact number of capsomeres was not unequivocally established.*

Epstein and others have reported on the continuous cultivation in vitro of a number of cell lines derived from biopsy specimens taken from patients afflicted with Burkitt lymphoma (*1*). Several of the cell lines carry a virus that morphologically resembles the herpes simplex and Lucké frog carcinoma viruses seen in electron microscope studies of thin-sectioned cells (*1, 2*). However, efforts to isolate the virus from lymphoma cultures by routine diagnostic procedures have failed (*2*). Interference studies with some of the cell lines have provided additional evidence for a viral-carrier state infection with an as yet unidentified agent (*3*). No relation of the virus to etiology of the disease has been demonstrated. This report is an account of (i) partial purification of virus from the EB-3 cell line by the density-gradient centrifugation technique and (ii) a study of the morphology of the virus by electron microscopy with the use of sectioning and negative-staining procedures.

Packs of wet EB-3 cells (*4*) (0.2 to 0.5 ml) were suspended in six to ten volumes of hypotonic buffer [0.015*M* NaCl, 0.001*M* ethylenediaminetetraacetate (EDTA), and 0.001*M* tris, *p*H 7.5] and disrupted by several cycles of freezing and thawing followed by homogenization with a motor-driven Teflon–glass homogenizer; or they were disrupted with high-frequency sound (M.S.E. ultrasonic disintegrator; 60 watts, 20 kc/sec) for 30 seconds in an ice bath, and further cooled for 15 seconds, the treatment being repeated five or six times. Disrupted cell suspensions were either layered directly on potassium tartrate gradients (*5*) or digested with a proteolytic enzyme before density-gradient centrifugation. Digestion was with 0.5 to 2 percent protease P-6 (*6*) or with 0.5 to 2 percent ficin (*7*) at 34° to 36°C for 30 minutes. Linear potassium tartrate gradients of 25 to 27 ml were prepared and ranged in density from 1.05 to 1.32. Cell suspensions were layered on top of the gradients and centrifuged at 25,000 rev/min for 90 minutes in the Spinco SW 25.1 rotor.

Generally, two distinct visible bands were obtained after density-gradient centrifugation of enzyme-digested suspensions of EB-3: a broad opalescent band at a density range of 1.13 to 1.16; and a distinctive, cottony, flocculent band at a range of 1.19 to 1.21. With the undigested suspensions of EB-3, the heavier band was much more intense and assumed a viscous, pellicle form. There was no distinct lighter band from these suspensions, but a strong opalescence extended from the flocculent band to the sample zone on top of the gradient. Densities of these various bands were relatively consistent between experiments, although centrifugation times were not sufficient to allow materials to reach equilibrium. The fractions of interest were removed from the gradient with a band-recovery apparatus (*8*), diluted to 12 ml with hypotonic buffer, and pelletized by centrifugation at 35,000 rev/min for 60 minutes in the Spinco 40 rotor.

For electron microscope examination by sectioning, the pellets were fixed with 2 percent glutaraldehyde in phosphate buffered saline, *p*H 6.8, fixed again in chrome-osmium, dehydrated in graded ethanol, and embedded in epoxy resin. The 50 percent ethanol bath contained 2 percent uranyl acetate. For negative staining, the pellets were suspended in 0.2 to 0.5 ml of buffer (0.15*M* NaCl, 0.001*M* EDTA, and 0.001*M* tris, *p*H 7.5), and the suspension was mixed with two volumes of potassium phosphotungstate (PTA), *p*H 4.5. Carbon-coated grids were applied to the surface of the mixture, drained onto filter paper, and dried.

The *A* to *G* series of Fig. 1 illustrates the main morphological entities by which the virus can be recognized in sections; the *H* to *L* series illustrates their appearance in negative stains. In preparations that were not treated with enzyme any of these forms could be found: (i) particles consisting of "naked" capsids either empty (Fig. 1, *A* and *H*) or with nucleoids at various stages of maturation (Fig. 1, *B, I,* and *C*); (ii) particles consisting of "coated" capsids again either empty (Fig. 1, *D* and *J*) or nucleated (Fig. 1, *E, K,* and *F*); and (iii) naked capsids either empty or nucleated (Fig 1, *G* and *L*), surrounded by an envelope of unit membrane structure. Coated capsids are never seen to be enveloped.

Many particles, regardless of their maturation stage, exhibited a hexagonal appearance in sections and with negative stains. PTA penetrates freely into the core of empty and nucleated, either naked or coated, particles, regardless of previous treatment, and prevents the formation of clear images of the capsid surface (compare *H, I, J,* and *K* in Fig. 1). However, in rare cases, points of five- and sixfold symmetry were seen. This suggests that the particles are built up according to icosahedral symmetry. Distinct "furrows" were seen on the capsid surface of favorably oriented, naked, negatively stained particles (Fig. 1*H*). When a particle was viewed along a probable axis of twofold symmetry, furrows could sometimes be seen arising from three parallel rows of capsomeres (Fig. 1*H*). This suggests that three capsomeres may be located on an edge between two points of fivefold symmetry, which in turn suggests, but does not prove, a possible total of 162 capsomeres (as in herpes simplex virus). This is compatible with a count of 23 to 25 capsomeres along the meridian of favorably oriented particles. The total number of capsomeres has not yet been unequivocally established because: (i) two neighboring fivefold vertices have not been seen, and (ii) the disposition of neighboring hexamers and pentamers could not be ascertained.

At some time during disintegration of the virus-producing cells, the surface of the latter, as well as the surface of the viral capsid, became coated with a layer of amorphous substance (compare *A, B,* and *C* of Fig. 1 with *D, E,* and *F*; also *H* and *I* with *J* and *K*). Virus particles found in cells at early stages of virus production are never coated; the coat is removed by enzyme treatments used in the purification procedures. However, its composition and role are yet to be determined. Only naked virus particles, empty or nucleated, either single or in

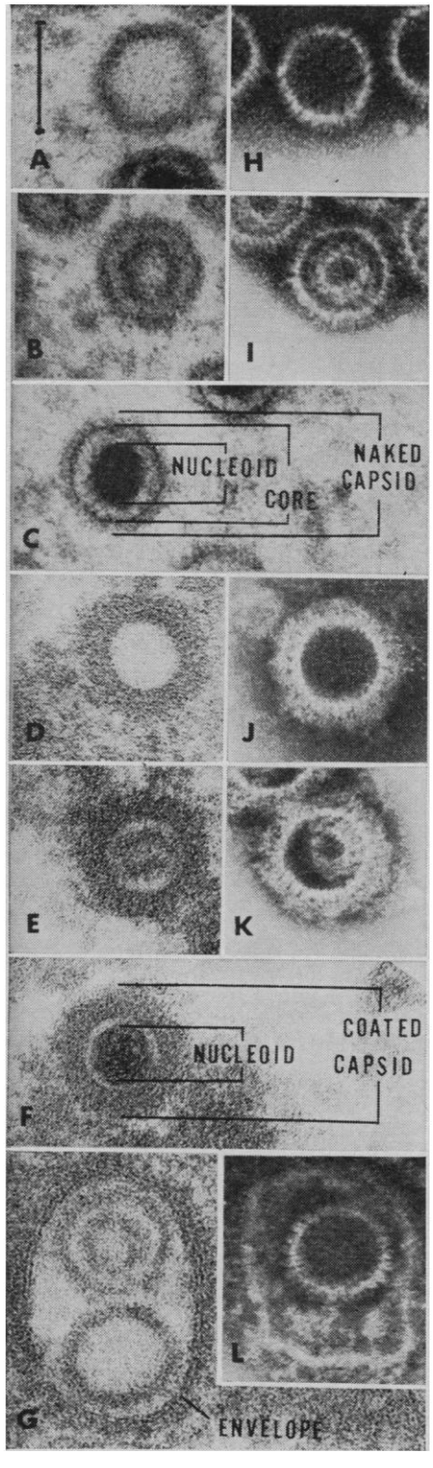

Fig. 1. *A* to *G*: Morphological entities of virus from EB-3 cells as seen in sections. *H* to *L*: Same, as seen in PTA negative stains. *A* and *H*, Empty-naked particles; *B* and *I*, nucleated-naked particles; *C*, nucleated-naked particle; *D* and *J*, empty-coated particle; *E* and *K*, nucleated-coated particle; *F*, nucleated-coated particle; *G*, an empty- and a nucleated-naked particle enveloped by a membrane of unit membrane structure; *L*, empty-naked-enveloped particle. The bar in *A* represents 0.1 μm. (Magnification for *A* to *L*, approximately × 146,000.)

groups of two, three, or more, become enveloped by membranes of nuclear or cytoplasmic origin by a pseudobudding process. This envelope does not appear to become an integral part of the virus particles although it could influence their infectivity and serological properties, as has been shown for herpes simplex virus (*9*). The capsid core (Fig. 1*C*) has a diameter of approximately 700 Å. The capsomeres at the periphery of negatively stained particles are approximately 100 Å long by 100 Å wide, with a central bore approximately 30 Å in diameter. The overall diameter of the capsid is therefore approximately 900 Å. Although the coat probably penetrates in between the capsomeres its thickness is in the order of 150 to 200 Å, which brings the overall diameter of coated particles to approximately 1200 Å.

The largest amounts of virus were consistently observed in flocculent bands (density 1.19 to 1.21) from enzyme-treated EB-3 preparations; there was also a predominance of nucleated viral forms in this heavier band. Comparable flocculent zones from undigested preparations of EB-3 cells also contained virus particles, but associated with the virus was an increased amount of contaminating cell debris. Virus particles were observed in the band with a density of 1.13 to 1.16 from enzyme-treated EB-3 suspensions and in the lighter opalescent zone from undigested EB-3 preparations. There appeared to be a much higher percentage of empty viral forms in these zones, which may account for the lighter densities of these particles.

This study indicates that the particles in EB-3 cells resemble members of the herpesvirus group, such as the virus of bovine malignant catarrhal fever (*10*), cytomegalovirus (*11*), pseudorabies virus (*12*), infectious laryngotracheitis virus of chickens (*13*), feline rhinotracheitis virus (*14*), and a canine herpesvirus (*15*). Capsids of these herpes-group viruses appear to be composed of 162 capsomeres arranged in a regular icosahedral lattice. However, evidence presented here on particles in EB-3 cells does not exclude the possibility that the geometry of their capsid surfaces may be that of a skewed rather than a regular icosadeltahedral lattice. In future attempts to determine the total number of capsomeres and their disposition on the surface of capsids of the virus in EB-3 cells it will be necessary to consider the possibility that this virus may belong to one of the following three classes of symmetry (*16*): (i) $T = 13$, 132 capsomeres, skewed lattice; (ii) $T = 16$, 162 capsomeres, regular lattice; and (iii) $T = 19$, 192 capsomeres, skewed lattice.

Note added in proof: While this report was in press, Hummeler *et al.* (*17*) described the morphology of the same virus in EB-3 cells using a technique of negative staining of whole cells only. Their observations are similar to ours except for their failure to note the presence of *coated* virus particles. Their description is concerned only with *naked* particles.

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