

Cell-Associated Nature of Cottontail Rabbit Herpesvirus In Vitro¹

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The cottontail rabbit herpesvirus is presently classified in subgroup A of the herpesviruses, whereas the virus is strongly cell associated and belongs in subgroup B.

In a generally accepted scheme for the classification of herpesviruses, Melnick et al. (1, 2) proposed that these deoxyribonucleic acid-containing viruses be subdivided into two groups solely on the basis of viral release. Subgroup A contains herpesviruses which are readily released from infected cells into the surrounding medium in an infectious form, and subgroup B is composed of those herpesviruses which are highly cell associated and are not freely released into the culture medium. This type of classification is of some conceptual importance, since three group B herpesviruses have been found associated with tumors in certain animals [namely, Marek's disease virus, Burkitt lymphoma virus, and Lucké tumor virus (5)]. At least one classification of human and animal viruses (5) has listed the cottontail rabbit herpesvirus isolated by H. C. Hinze (Bacteriol Proc., p. 149, 1968) under subgroup A. We present evidence which suggests that the cottontail rabbit herpesvirus (CRH) is strongly cell associated and, therefore, should not be classified as a subgroup A virus but instead belongs with subgroup B viruses.

The CRH virus was kindly supplied by H. C. Hinze of the University of Wisconsin. The fraction of cell-free and cell-associated infectivity of CRH virus was determined after infection of LLC-RK₁ rabbit kidney cell cultures. These cells were propagated in medium 199 (Earle's base) which was supplemented with 10% bovine serum, penicillin (250 units/ml), streptomycin (125 µg/ml), and 0.112% NaHCO₃. Monolayers were grown in shell vials (21 by 70 mm) and contained about 10⁶ cells. The cultures were infected with

10⁶ plaque-forming units of CRH virus; after adsorption for 2 hr, the monolayers were rinsed with tris(hydroxymethyl)aminomethane-buffered saline (TBS), pH 7.4, and re-fed with 1 ml of fresh medium; the cultures were incubated at 37 C. Samples for viral infectivity were taken in triplicate at intervals between 4 and 85 hr after infection, and cell debris was separated from the medium by filtration through 0.45-µm membrane

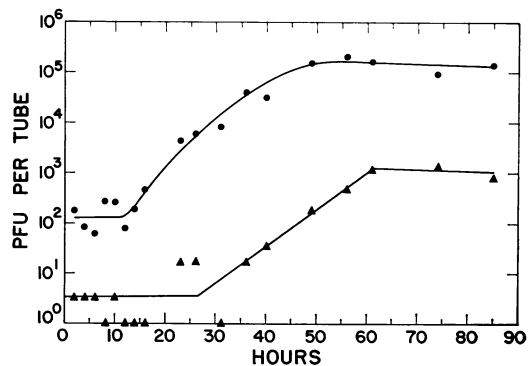


FIG. 1. Growth curve of CRH virus in LLC-RK₁ rabbit kidney cells. Symbols: O, cell-associated virus; Δ, cell-free virus.

filters (Millipore Corp., Bedford, Mass.). Cell-free virus (CFV) was assayed directly from the filtrate, and cell-associated virus (CAV) was determined by assaying disrupted cell material prepared from washed and sonicated monolayers.

Infectivity of CRH was measured by a plaque-assay method that utilized LLC-RK₁ cells grown with medium 199 in 60-mm plastic tissue culture dishes in a humidified CO₂ incubator at 37 C. Monolayers containing about 10⁶ cells and covered with 3 ml of TBS were inoculated with 0.2 ml of the virus sample appropriately diluted in TBS. The adsorption fluid was decanted after 2 hr

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and replaced with 8 ml of an overlay medium containing 0.75% methylcellulose, Temin's modified Eagle's medium (4), antibiotics, and 1% bovine serum. After 6 days of incubation, small plaques (about 0.5 mm) were visible when the cell sheets were fixed with a Formalin-acetic acid-ethyl alcohol solution (3) and then stained with crystal violet.

Data presented in Fig. 1 indicate that the titer of CAV remained constant during the first 12 to 14 hr after infection, increased steadily over the ensuing 35 to 40 hr, and remained constant thereafter. In contrast, CFV increased during the interval from 25 to 60 hr postinfection and constituted a very minor fraction of the total infective virus present. In repeated experiments, the fraction of CFV never exceeded 1% of the total infectious virus; for this reason, we believe that the CRH

virus should be considered a cell-bound virus and, therefore, classified in subgroup B of the herpesviruses.

LITERATURE CITED

1. Melnick, J. L., M. Midulla, I. Wimberly, J. G. Barrera-Oro, and B. M. Levy. 1964. A new member of the herpesvirus group isolated from South American marmosets. *J. Immunol.* **92**:595-601.
2. Melnick, J. L., and R. M. McCombs. 1966. Classification and nomenclature of animal viruses. *Progr. Med. Virol.* **8**:400-409.
3. Merchant, D. J., R. H. Kahn, and W. H. Murphy, Jr. 1964. *Handbook of cell and organ culture*. Burgess Publishing Co., Minneapolis.
4. Temin, H. M., and H. Rubin. 1958. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. *Virology* **6**:669-688.
5. Wilner, B. I. 1969. *A classification of the major groups of human and other animal viruses*. Burgess Publishing Co., Minneapolis.