*MAREK'S DISEASE HERPES VIRUS: A CYTOMEGALOVIRUS?**

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Abstract.—The DNA of herpes viruses associated with Marek's disease of fowl contains 56–57 moles of guanine and cytosine per 100. The composition of its DNA and lack of infectiousness of cell-free preparations suggest that the herpes virus associated with Marek's disease belongs to the herpes virus group B which contains predominantly cytomegaloviruses.

Introduction.—Marek's disease of domestic fowl is characterized by lymphoid proliferation in the nervous system and other organs and by the formation of visceral tumors. A virus, Marek's disease virus, structurally resembling herpes virus is almost invariably present and has been isolated from the blood and tumors of birds afflicted with the disease.[1–4] The classification of this virus within the herpes virus group was uncertain. The outstanding property of this virus is that extracts of infected cells are not infectious to fowl.[5–7] The evidence suggesting that this virus is the causative agent of Marek's disease is indirect and based on observations that (*1*) chicks inoculated with infected cells maintained *in vitro* regularly develop Marek's disease[8–9] and (*2*) high-passage tissue culture virus immunizes birds against the disease.[10] It is also noteworthy that birds afflicted with Marek's disease make antibody against antigens present in infected cell culture.[11]

The purpose of this paper is to report that MDV-DNA has a buoyant density in CsCl corresponding to 56 to 57 moles guanine + cytosine per 100 and that on the basis of two criteria, i.e., DNA composition and the lack of infectiousness of cell-free preparations, Marek's disease virus, is tentatively classified in the same group with cytomegaloviruses.

Materials and Methods.—Media, solutions and chemicals: EMEM-10 consisted of Eagle's[12] minimal essential medium fortified with 10% inactivated calf serum; it was used for growth of primary cells. EMEM-4 contained 4% inactivated calf serum; it was used as maintenance medium and for initiation of viral infection. Approximately 24 hr after infection, EMEM-4 was replaced with medium consisting of mixture 199 and 1% calf serum.

TES buffer consisted of 0.01 *M* Tris(hydroxymethyl)aminomethane, 0.001 *M* disodium-(ethylenedinitrilo)tetraacetate (EDTA) and 0.1 *M* sodium chloride, pH 8.0, and was used as a general buffer for suspending cells and viruses. Optical grade cesium chloride obtained from Nutritional Biochemical Company, Cleveland, Ohio, was used in equilibrium centrifugation studies. ^{3}H-(methyl)thymidine (specific activity 18 c/mmole) and thymidine-^{14}C (spec. act. 57 mc/mmole) were obtained from Schwarz BioResearch, Orangeburg, New York.

Stock virus: The KN variant of JM strain of Marek's disease virus used in these studies was developed at the USDA Regional Poultry Research Laboratory. It grows in chick or duck embryo fibroblasts. The propagation and properties of the MP strain of herpes simplex virus have also been described.[13]

Preparation of primary duck embryo and chick embryo fibroblasts: The fertilized chick eggs were obtained from the inbred lines maintained at the USDA Regional Poultry Research Laboratory. The duck eggs were obtained from Truslow Farm, Chesterton, Maryland. The fertilized duck and chick eggs were incubated for 14 and 11 days, respectively, at 33°C prior to cultivation. The primary duck and chick embryo fibroblast cultures were prepared according to the published procedures.[14]

Growth of Marek's disease virus on duck embryo fibroblast with roller bottles: Duck and chick embryo fibroblasts were grown in roller bottle cultures. Briefly, cylindrical bottles 10 cm in diameter × 40 cm in length were seeded with 2×10^8 primary embryo fibroblasts in 200 ml of pre-warmed EMEM-10. The bottles were rolled at 0.25 rpm in an apparatus[15] maintained at 37°C. The EMEM-10 was replaced 24 hr after seeding. When monolayer cultures became nearly confluent, they were seeded with stock Marek's disease virus-infected cells suspended in EMEM-4 at a ratio of 1 infected cell per 10 to 20 uninfected cells. After incubation at 37°C overnight, the culture fluid was replaced with mixture 199 lacking thymine, but fortified with 1% dialyzed calf serum and sufficient ^{3}H thymidine to yield 5 μc/ml. The cells were then incubated at 35°C for several days. The cells were scraped or shaken off the glass surface when cellular degeneration was complete.

Growth of herpes simplex virus in chick embryo fibroblasts: Primary chick embryo fibroblasts grown in 32 oz prescription bottles in medium EMEM-10 were exposed to 50 PFU of herpes simplex virus per cell for 2 hr at 37°C. The inoculum was then aspirated and replaced with EMEM-1% calf serum. After 4 hr of incubation, the medium was replaced with mixture 199 lacking thymine but fortified with 1% dialyzed calf serum and sufficient ^{14}C thymidine to yield 0.2 μc/ml. After 24 hr of incubation, the cells were scraped and resuspended in TES buffer.

Purification of Marek's disease virus and Herpes simplex virus: The purification of Marek's disease and herpes simplex viruses consisted of 2 steps: sedimentation and then flotation through discontinuous sucrose gradients in a centrifugal field. Both steps were monitored with the aid of electron microscopy.

Briefly, cells infected with Marek's disease virus or herpes simplex virus were sedimented by centrifugation at 2000 rpm for 2 min and resuspended in TES buffer. The cells were lysed by 80 strokes in a tight-fitting Dounce homogenizer. Cell debris was collected by centrifugation in a Sorvall RC-2B centrifuge at 3000 rpm for 15 min, washed, then discarded. The supernatant fluid containing the cell extract and the wash were pooled and adjusted to 0.3 *M* potassium citrate. In addition, the spent tissue culture fluid was then centrifuged in a Spinco 42 rotor at 40,000 rpm for 1 hr. The pellets were suspended in 0.3 *M* potassium citrate.

The solutions containing 0.3 *M* potassium citrate were then layered on top of discontinuous sucrose gradient consisting of approximately 3 ml of 65% w/w sucrose solution on the bottom and 5–8 ml of 0.15 *M* potassium citrate in a 20% w/w sucrose solution on top. The gradients were then centrifuged in a Spinco SW25.3 rotor at 25,000 rpm for 16 to 24 hr. In this centrifugation the virus formed two bands immediately above the 65% w/w sucrose cushion. Both bands were harvested and pooled.

In the second purification step, the partially purified virus was floated in a centrifugal field through a sucrose density gradient. The sucrose concentration in material obtained in step 1 was adjusted to 50% w/w. The material was then placed in the bottom of a cellulose nitrate tube and overlayed with equal volumes of 45, 35, 30, and 25% w/w sucrose, respectively, and centrifuged for 16 to 24 hr in a Spinco SW27 rotor. During this centrifugation the virus again formed 2 bands in the layer containing 35% w/w sucrose. The bands containing the virus were removed and dialyzed overnight against TES buffer.

DNA extraction: Sodium dodecyl sulfate (final concentration 2%) and heat-inactivated pronase (final concentration 2 mg/ml) were added to the concentrated virus preparation and incubated in a 37°C water bath for 2 hr with slow shaking. Redistilled phenol saturated with TES buffer was added to the mixture. After shaking at room tempera-

ture for 1 hr, the phenol-extracted mixture was centrifuged at 10,000 rpm for 10 min in a Sorvall centrifuge. The aqueous layer was then removed, extracted once with a mixture of chloroform and 2% isoamyl alcohol, centrifuged, and dialyzed overnight against TES buffer. Host DNA was extracted as described.[16]

Isopycnic centrifugation of DNA in cesium chloride solution: Sufficient optical grade cesium chloride was added to DNA solutions to yield a density of 1.710 gm/cm.[3] The solution was then centrifuged in a Spinco SW39 rotor at 35,000 rpm for 48 hr at 25°C. At the end of equilibrium centrifugation, 5-drop and 1-drop fractions were collected from the bottom of the tube for assay of radioactivity and refractive index, respectively.

Assay of radioactivity: The various fractions collected at the end of the isopycnic centrifugation in CsCl solutions were diluted with 1 ml of 1 × SSC. Carrier herring sperm DNA and three volumes of absolute ethyl alcohol were added to each fraction. The precipitate was filtered onto a Millipore filter, washed once with 75% ethyl alcohol, and then dried in an oven at 60°C. The filters were immersed in vials containing scintillation fluid. The radioactivity was measured in a Packard scintillation counter.

Electron microscopy: Aliquots of bands harvested during the purification procedures were stained with sodium silicotungstate and examined in an AEI electron microscope.

Results.—Purification of Marek's disease virus: The top bands obtained in both the sedimentation and flotation parts of the purification procedure contained more enveloped Marek's disease virus nucleocapsids than the bottom

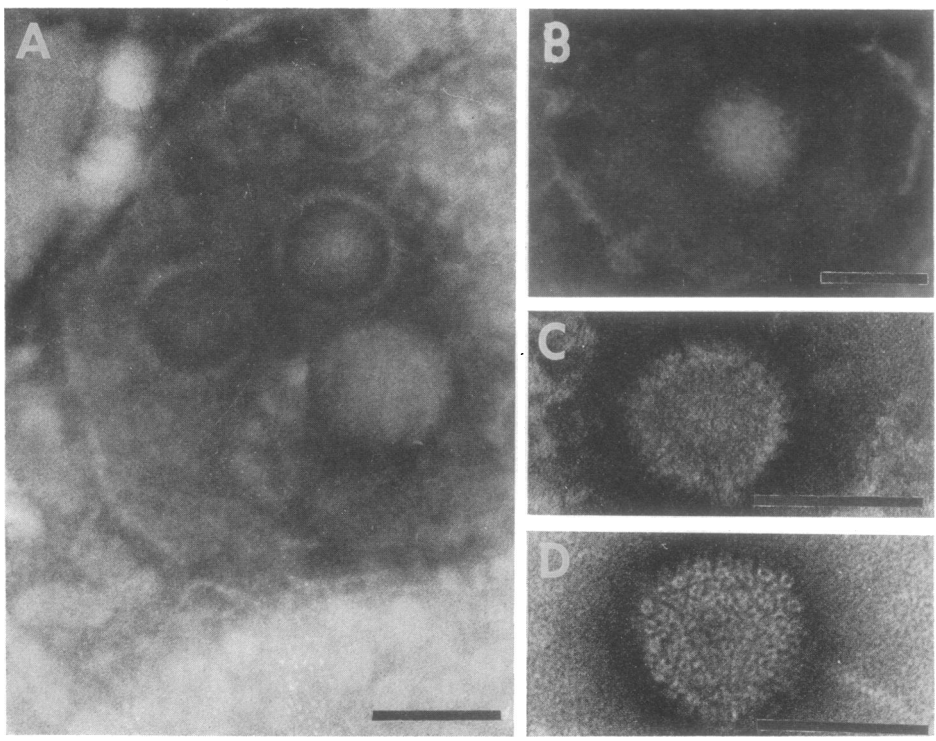

FIG. 1.—Partially purified Marek's disease and herpes simplex viruses stained with silicotungstic acid. *A*, 3 Marek's disease virus nucleocapsids in a single envelope; *B*, enveloped Marek's disease virus; *C*, naked HSV nucleocapsid; *D*, naked Marek's disease virus nucleocapsid. The bar at the bottom right hand corner of each electron photomicrograph represents 100 nm.

It is convenient to classify herpes viruses into 2 groups[23] differing with respect to physical and biologic properties. The viruses in group A, exemplified by herpes simplex and pseudorabies, have a DNA containing 67 and 74 moles of band and, moreover, the virus bands obtained after both sedimentation and flotation contained less host debris than after sedimentation alone. Electron photomicrographs of Marek's disease virus obtained by procedures described in this paper are shown in Figure 1. As previously reported,[1-4] the structure of the Marek's disease virus nucleocapsid cannot be differentiated from that of the herpes simplex virus nucleocapsid.

The properties of MDV-DNA: We have examined the MDV-DNA labeled with ^{3}H-thymidine and extracted from two preparations of Marek's disease virus. The MDV-DNA was centrifuged to equilibrium in CsCl solutions alone and in artificial mixtures with ^{14}C-labeled host DNA or with ^{14}C-labeled herpes simplex DNA. The profiles of Marek's disease virus, duck embryo, and herpes simplex DNA centrifuged singly to equilibrium are shown in Figure 2. The buoyant densities of the MDV-DNA in CsCl solutions ranged from 1.715 to 1.716 gm/cm^3 corresponding to a linear DNA molecule with guanine and cytosine content of 56 to 57 moles per hundred. The host DNA banded at a density of 1.702 gm/cm^3, whereas herpes simplex virus DNA banded at a density of 1.726 gm/cm^3 corresponding to guanine and cytosine content of 43 and 67 moles per hundred, respectively.

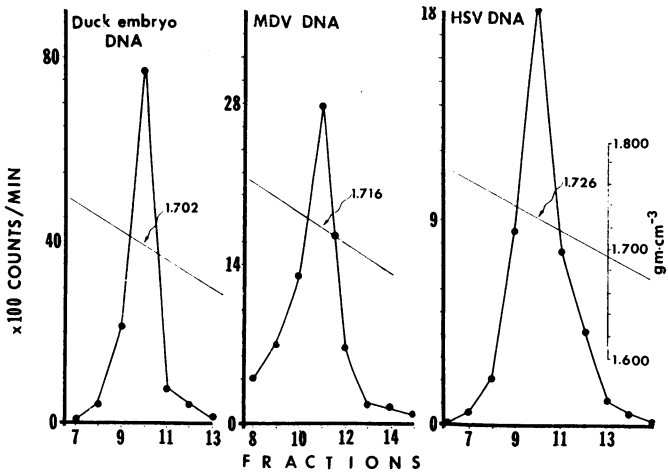

FIG. 2.—The profile of duck embryo fibroblast, Marek's disease virus, and herpes simplex virus-labeled DNA centrifuged to equilibrium in CsCl solutions.

Discussion.—A number of herpes-like viruses have been associated recently with tumors of man, fowl, frogs, guinea pigs, and monkeys[1-4, 17-22] The nature of these viruses and particularly the relationship of these viruses to known herpes viruses is uncertain. A characteristic of all herpes viruses associated with tumors is that they grow poorly in cell culture and are not readily transmissible from cell to cell as cell-free filtrates.

guanine and cytosine per hundred, respectively. The viruses comprising this group multiply readily in cell cultures and produce progeny which are infectious as cell-free filtrates. The group B consists largely of cytomegaloviruses. The DNA of these viruses contains 56–58 moles of guanine and cytosine per hundred and, moreover, they are not readily transmissible by cell-free filtrates.

In the work described in this paper, we have produced and purified Marek's disease virus and extracted its DNA. Based on the buoyant density of the MDV-DNA, we concluded that it contains approximately 56–57 moles of guanine and cytosine per hundred. On the basis of its guanine and cytosine content and lack of infectiousness as a cell-free filtrate, we tentatively classify the virus into group B which contains predominantly cytomegalovirus subgroup.

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