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Biophysical, Biological, and Cytochemical Features of a Virus Associated with Transplantable Hamster Tumors[1]

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A virus resembling type C murine leukemia viruses, which is associated with transplantable hamster tumors, was partially characterized with respect to certain biological, biophysical, and cytochemical features. As determined by electron microscopy, high concentrations of the virus appeared in the blood plasmas of tumor-bearing hamsters. Hamsters inoculated with virus concentrates did not show gross evidence of disease, and preliminary attempts to infect various hamster cells in tissue culture were unsuccessful. A line of cells from a virus-containing tumor which had been established in tissue culture released large numbers of the virus into the supernatant fluids by budding. The buoyant density peak of virus concentrates in potassium tartrate density gradients was 1.13 g/cm^3 by ultraviolet absorption and electron microscopic analysis. Acridine orange staining and nuclease digestion methods have indicated that the virus is probably a ribonucleic acid virus.

Previously, we reported the presence of virus particles in several transplantable hamster tumors as revealed by electron microscopy (12). One of the two types of particles found resembles type C murine leukemia virus, the first report of such an agent in hamsters. One of our transplantable tumors (D-9), a malignant lymphoma, consistently contained many budding type C particles, as revealed by electron microscopy, and a second type particle which appears to be morphologically identical with the particle first described by Bernhard and Tournier (2) and recently studied by Thomas et al. (13). Blood plasma pellets from animals bearing this tumor contain large numbers of mature and immature type C particles but no "Bernhard" type particles. The present report is concerned primarily with studies of the type C virus.

MATERIALS AND METHODS

In vivo virus. Groups of twenty-five 6- to 8-week-old hamsters received transplants of about 1 mm^3 of tumor subcutaneously by trocar. This tumor, a malignant lymphoma, grows rapidly and usually kills within 12 to 14 days. At about 12 days, the bloods of the moribund animals were withdrawn by cardiac puncture into syringes rinsed with 1,000 units of heparin per ml, and were pooled in a chilled container in an ice bath. The blood cells were removed by low-speed centrifugation, and the plasma was stored at 4 C until concentration of the virus was performed (within 1 day).

Tissue-culture virus. Cells obtained by trypsinization from a freshly excised D-9 tumor were placed in Eagle's minimal essential medium supplemented with 10% fetal calf serum in 32-oz (0.946 liter) bottles and were incubated under 5% CO_2 at 37 C. After approximately 1 month, with minimal disturbance and feeding only once a week, a luxuriant growth of cells was evident. These cells were subsequently serially passed at approximately 10- to 12-day intervals. Thin sections of cell pellets and pellets of supernatant fluids showed large numbers of mature and immature type C particles, many in the process of budding from the cytoplasmic membranes. The "Bernhard" type particles, present in the original tumor, were not evident in the cultured cells.

Virus concentration. Virus-rich hamster plasmas or supernatant tissue culture fluids were centrifuged at 10,000 × *g* for 2 min and were then filtered through a thin layer of Celite (10). The virus was then pelleted from the clarified fluids at 30,000 rev/min for 1 hr in the no. 50 rotor of the Spinco model L ultracentrifuge or was collected on a cushion of potassium tartrate (density, 1.25 g/cm^3) in the SW 25.1 rotor at 25,000 rev/min for 1 hr; the supernatant fluids were discarded.

Density gradient centrifugation. Virus pellets were gently resuspended in about 0.5 ml of single strength phosphate-buffered saline (PBS) with a Potter-Elvehjem homogenizer (8). The material was layered over linear(1.04 to 1.25 g/ml) potassium tartrate gradients (6) preformed with the aid of a layering device (4), and was centrifuged at 37,500 rev/min for 4 hr in the SW-39 rotor of a Spinco model L ultracentrifuge. The

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gradient was then sampled either by collecting four-drop fractions after piercing the bottom of the tube or, for ultraviolet (UV) analysis, by utilizing a top-unloading piercing unit (Buchler Instruments, Inc., Fort Lee, N.J.). For further purification, the fluid above the opalescent band near the center of the gradient was removed into a syringe equipped with a 2-inch, 19-gauge needle bent at right angles. The band was then similarly removed with a fresh syringe and was submitted to a second cycle in a potassium tartrate gradient after dialysis against single strength PBS. Densities were determined by weighing 20-μliter samples (11) or by measurement of the refractive index (8).

Electron microscopy. Low-speed cell pellets and high-speed virus pellets were fixed in 3% phosphate-buffered glutaraldehyde for 1 hr or, if necessary, were stored in this solution for several days at 4 C before processing as previously described (12). Briefly, the pellets were postfixed in osmium tetroxide, dehydrated in graded alcohols, and embedded in Araldite resin. Thin sections were double-stained with uranyl acetate and lead citrate and were examined in the Siemens Elmiskop 1A electron microscope.

Cytochemical staining technique. The virus band from the second cycle of density gradient centrifugation was dialyzed against single strength PBS and then was pelleted at 30,000 rev/min for 1 hr. Small droplets of the concentrated virus were placed on the center of small cover slips and were allowed to dry thoroughly in air. After fixation in Carnoy's fixative, acridine orange fluorochrome staining and nuclease digestion procedures were performed according to the methods of Mayor (5).

Fluorescence microscopy. The Leitz Ortholux fluorescence microscope equipped for dark-field illumination was used. For observation of acridine orange-stained virus materials, the BG-38 (heat) and BG-12 (blue) filters and the OG-1 (orange) eye piece barrier filter were employed.

RESULTS

Lack of serological cross-reactivity with known murine viruses. To help rule out the possibility that the agent might be a murine leukemia virus inadvertently introduced into our hamster colony, 3 frozen, virus-rich tumor samples and 30 sera from tumor-bearing hamsters and 60 sera from normal control hamsters were sent to the laboratories of Janet Hartley and W. Rowe, National Institute of Allergy and Infectious Diseases, to whom we are indebted for testing these materials. No specific cross-reactivity was found in the complement-fixing (CF) test with their murine leukemia group-reactive rat tumor antigen and antiserum reagents (3).

Attempts to demonstrate biological activity. High-speed pellets from the blood plasma of D-9 tumor-bearing hamsters contained large numbers of type C virus particles as revealed by electron microscopic examination of thin sections (Fig. 1). These particles consist of an electron-dense nucleoid surrounded by a loose membranous envelope. Virus samples concentrated on a cushion of potassium tartrate and dialyzed overnight against PBS were inoculated into the following hamster cells in tissue culture: hamster embryo, hamster mixed spleen-thymus cells, adeno-12 induced tumor cells, and BHK-21 cells. No cytopathic effects (CPE) were seen, and thin sections of cell pellets examined by electron microscopy showed no evidence of type C viral replication, although the "Bernhard" type particles were observed in the BHK-21 cells, as reported by others (2, 13), as well as in the spleen-thymus cells and adeno-12 tumor cells. Supernatant fluid pellets from the above cells contained type C particles only on the first medium change, most probably representing the inoculum.

Newborn and weanling hamsters were inoculated with cell-free virus concentrated from tumor-bearing hamster plasma. Tumors were found in two animals; because of the low incidence and advanced age of the animals at the time of autopsy, the relationship of the tumors to the inoculum is uncertain. Membranous glomerulonephritis was also observed in the older animals coming to autopsy. The possible etiological relationship between the virus and the kidney lesions is under investigation.

Virus production in tissue culture. Immature type C virus particles were seen budding from cytoplasmic membranes of the D-9 tumor cells in tissue culture (Fig. 2). These immature particles were observed at a frequency of about 3 to 8 per standard 200 mesh electron microscope grid square, and the frequency remained the same between 2 and 8 days in culture. When 10^5 tissue-cultured cells were inoculated into weanling hamsters, tumors were produced in four of four hamsters within 50 days; these tumors also contained budding type C virus particles in thin sections at a frequency of 0 to 6 per 200 mesh grid square. The "Bernhard" type particles were not evident in the D-9 cells in tissue culture nor in the tumors that we obtained from these cells. Pellets of supernatant fluids from cell cultures contained many type C particles in thin sections (Fig. 3).

Equilibrium density gradient centrifugation. In potassium tartrate gradient centrifugation of virus-containing materials, two light-scattering bands were produced (Fig. 4A). The upper band was a diffuse, opalescent band with a 260-nm absorption peak at a density of 1.13 g/cm^3, whereas the lower band was flocculent with a UV absorption peak at a density of 1.19 g/cm^3 (Fig. 5). Control materials contained only the lower band (Fig. 4B). When the two bands were collected and pelleted for thin sectioning after

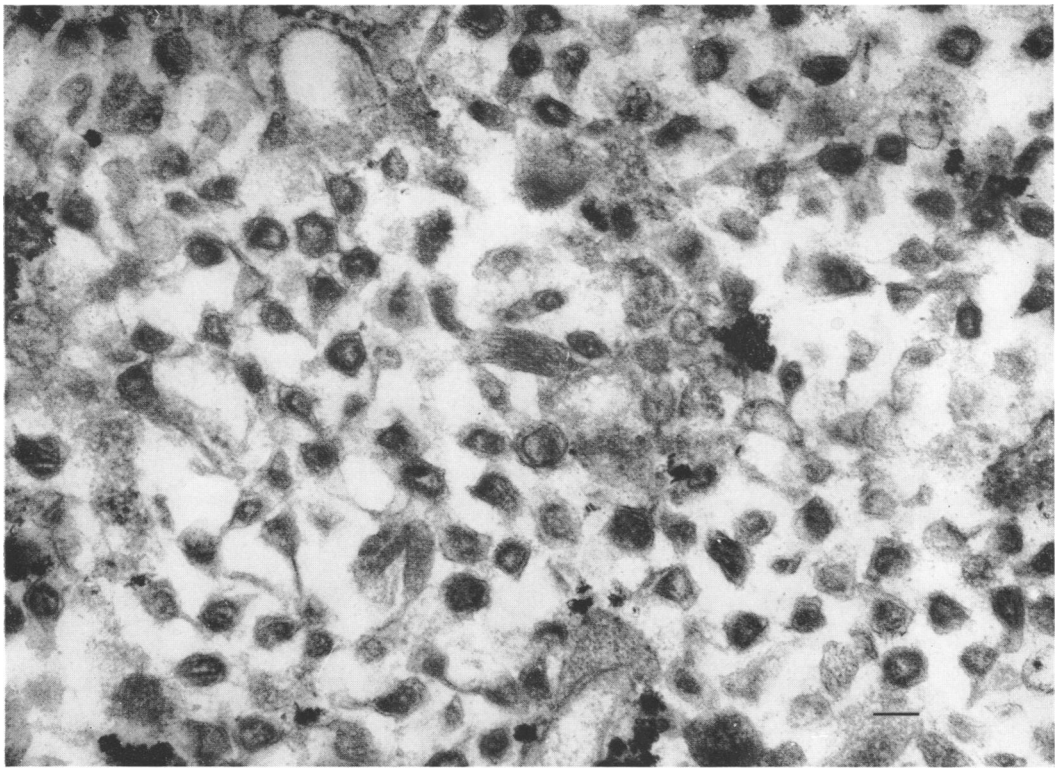

FIG. 1. *Thin section through a high-speed pellet of pooled blood plasmas from 25 tumor-bearing hamsters. Many type C virus particles may be seen throughout the section. The bar in all micrographs represents 100 nm.*

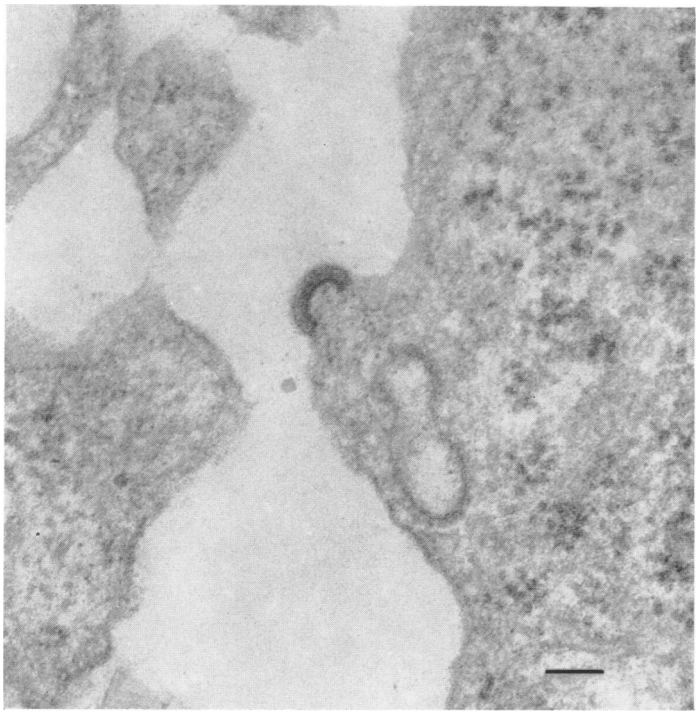

FIG. 2. *Immature type C virus particle budding from the cytoplasmic membrane of a D-9 tumor cell in tissue culture.*

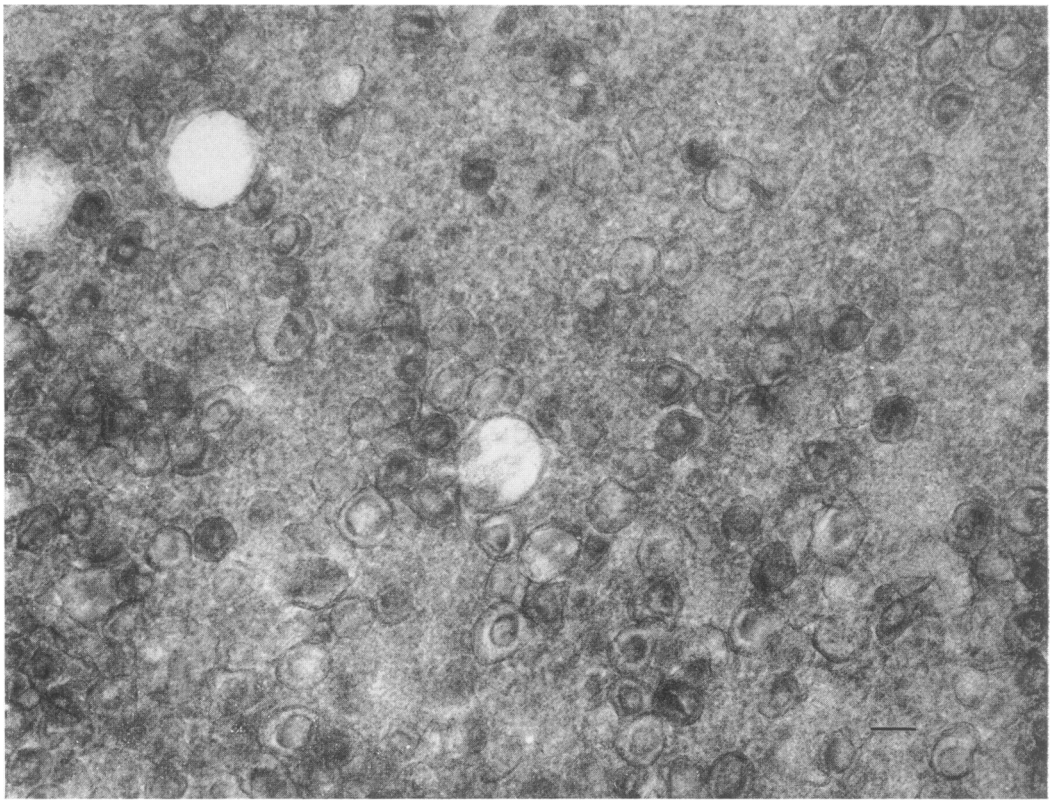

FIG. 3. *Thin section through a high-speed pellet of 6-day supernatant fluids from D-9 tumor cells growing in tissue culture.*

dialysis against single strength PBS, the upper band (density, 1.13 g/cm³) was found to contain type C particles in large numbers (Fig. 6). No virus particles were associated with the lower band, and the nature of its amorphous constituents is unknown. When the upper band was collected and submitted to a second cycle of density gradient centrifugation, a lower flocculent band was still produced but to a lesser degree.

Acridine orange staining. When fixed and stained with acridine orange, the partially purified virus concentrate appeared brilliant orange-red under the fluorescence microscope. Prior treatment with ribonuclease prevented the red fluorescence, whereas treatment with deoxyribonuclease or buffer alone had no effect. These results are consistent with a single-stranded ribonucleic acid (RNA)-containing virus (5).

DISCUSSION

Lack of a system for assaying biological activity has limited the extent to which the characteristics of this virus, which is associated with transplantable hamster tumors, can be studied.

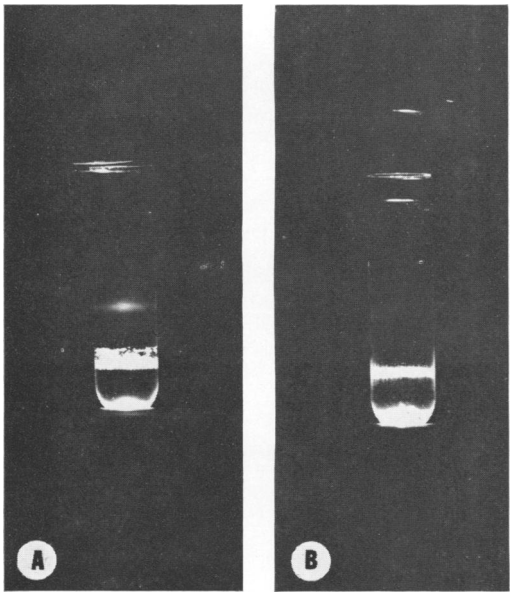

FIG. 4. *Distribution of light-scattering zones of virus-containing and control materials in linear potassium tartrate density gradients. Resuspended virus pellets were layered over preformed gradients and were centrifuged at 150,000 × g for 4 hr in the SW-39 rotor of a Beckman Spinco ultracentrifuge. (A) Virus band, derived from tissue culture fluids of D-9 tumor cells, is visible near the middle of the tube. (B) Control fluids from hamster embryo cells were devoid of virus.*

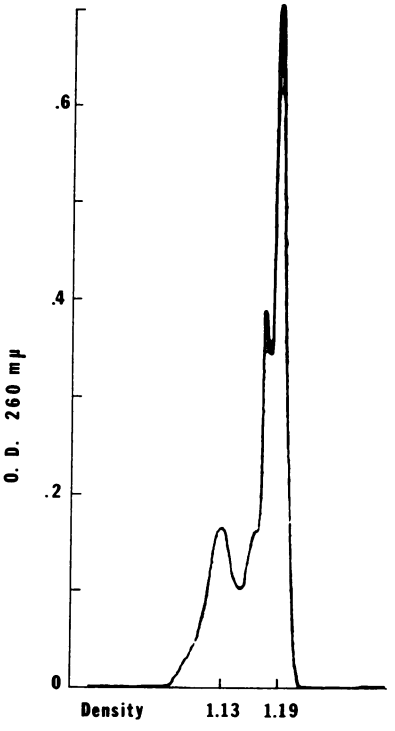

FIG. 5. *Photographic reproduction of a tracing made during UV absorption analysis of a potassium tartrate density gradient run similar to that shown in Fig. 4A. The initial virus pellet was derived from tumor-bearing hamster plasma. The peak at the 1.13 g/cm³ density level contained the virus. The 1.19 g/cm³ density peak consisted of amorphous material devoid of virus.*

Nevertheless, some important characteristics have been determined by physical and cytochemical means. These characteristics include: (i) possession of a loose sac-like envelope, (ii) possession of high water content as evidenced by morphological alteration in concentrated potassium tartrate, (iii) particle formation by budding from the plasma membrane with a lack of CPE, and (iv) cytochemical evidence that the nucleic acid of this virus is RNA. All of these characteristics are common to oncogenic RNA viruses (1). The buoyant density of 1.13 g/cm³ may be slightly less than that of known leukemia viruses (8); however, this density was determined by physical means rather than by assay of biological activity. Possible destruction of biological activity by the potassium tartrate has not been ruled out; however, there is no reason to assume that brief exposure of this virus to the salt should be any more detrimental than such exposure is to other similar viruses (6, 8).

There is no evidence that murine agents

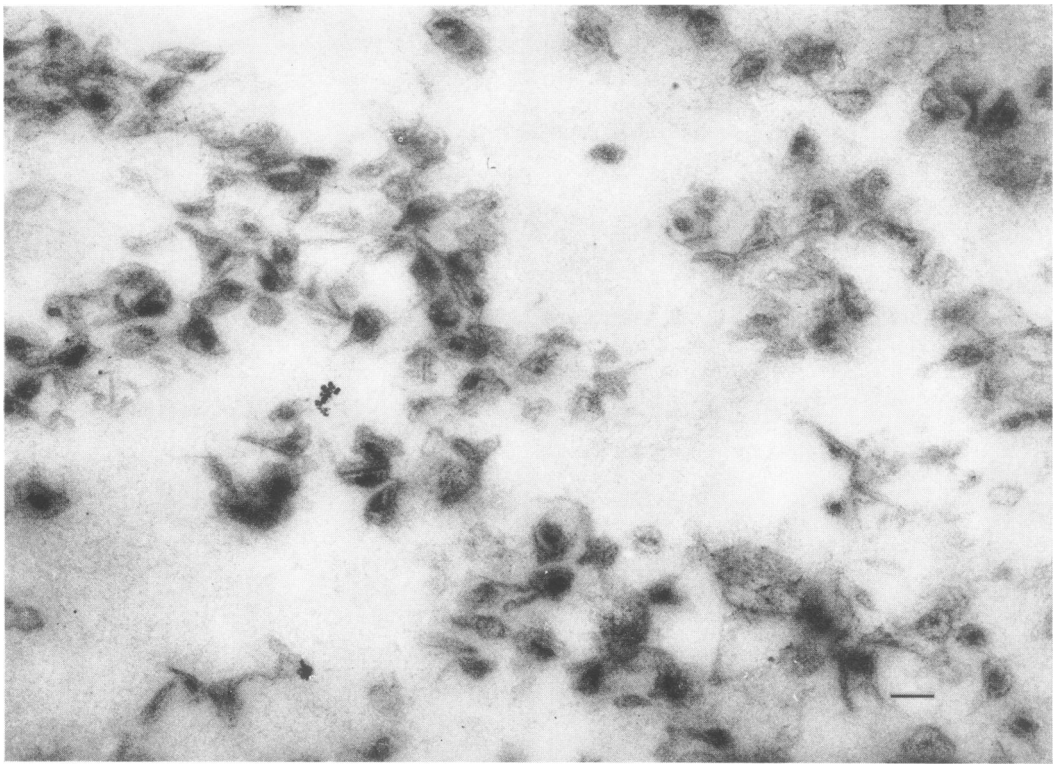

FIG. 6. *Thin section through a high-speed pellet of a 1.13 g/cm³ density band isolated from a potassium tartrate density gradient. The pellet consisted almost exclusively of type C virus particles. Most of the particles were distorted as a result of osmotic effects of the concentrated potassium tartrate.*

replicate in hamsters. It has been reported that inoculation of the Moloney agent into hamsters produces a reticulum cell sarcoma (7); however, to the best of our knowledge, the agent was never recovered from the tumor or from subsequent transplants. The lack of CF cross-reactivity with murine agents in the present study suggests that the virus is a new hamster virus. The fact that the hamster harbors a virus which resembles murine leukemia agents is important in light of the extensive role played by the hamster in cancer research. The possibility has not been ruled out that the agent is merely a passenger in these tumors, but if the virus should prove to be causally related it would provide a useful new laboratory model for study.

In studies currently in progress, we are attempting to produce specific antisera for further serological studies, to apply radioisotope techniques to confirm the nucleic acid type, and to disclose biological activity which might be employed for viral assay.

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