Type C Virus from Cell Cultures of Chemically Induced Rat Hepatomas

Abstract. *Type C RNA viruses are present in cell cultures from transplantable and primary hepatomas induced by aromatic amine carcinogens. Virus yield was markedly enhanced by treating the cells with bromodeoxyuridine. Preparations of rat hepatoma–associated virus obtained from cultures treated with this compound were deficient in DNA polymerase activity.*

Naturally occurring tumors are frequently caused by exposure to chemical carcinogens, but the possible interaction between these agents and oncogenic viruses in the causation of cancer is poorly understood. We have detected type C RNA virus, designated "rat hepatoma–associated virus" (RHAV), in tissue cultures established from transplantable or primary hepatomas induced in the rat by aromatic amine carcinogens. The precise role of this virus is not known, but its presence must be considered in studies on the mechanism of hepatic carcinogenesis and the biochemistry of hepatomas.

Hepatoma 5123C, induced with *N*-2-fluorenylphthalamic acid and maintained in Buffalo rats (*1*), was obtained from H. P. Morris. The tumor was dissected free of necrotic tissue, minced with a scissors, pressed through a fine stainless steel screen, and plated in enriched Ham's F12 medium containing antibiotics, amphotericin B, and 5 percent fetal calf serum (*2, 3*). Distinct epithelial clones were seen after 12 days. The present studies were done with the clone designated W-7. Primary hepatomas were obtained from Fisher 344 rats fed a pyridoxine-deficient diet containing *N*-2-acetylaminofluorine (*4*). The liver contained multiple nodules (2 to 5 mm), which were dissected free of surrounding liver and processed for culture as described above. Several clones appeared after 3 to 4 weeks, and two of these, W-14 and W-15, were studied. Two epithelial cell lines, K-16 and K-22, established from normal livers of adult Sprague-Dawley rats (*3*), and the cell line H-4-II-E (H-4) were provided by M. E. Kaighn. The H-4 line was established by Pitot *et al.* (*5*) from the Reuber H-35 hepatoma induced in an AXC rat by the carcinogen *N*-2-fluorenyldiacetamide (*6*). The B-1 cell line, established by C. Borek from the 5123C hepatoma in 1969 (*7*) and stored frozen, was also used. All cell lines were maintained in enriched F-12 medium with 5 percent fetal calf serum as described (*2, 3*).

The normal cell lines K-16 and K-22 formed epithelial colonies with sharply circumscribed borders and grew into a monolayer of tightly packed but nonoverlapping cells. The transformed cells W-7, W-14, W-15, B-1, and H-4 had a fusiform to fibroblast-like appearance at low density and formed colonies with ragged edges. At high density they had a more epithelial appearance, tended to form multilayers, and, in contrast to the normal cells, formed colonies in soft agar.

Evidence for virus production was obtained by labeling W-7 cells with [^{3}H]uridine and examining the media on a sucrose density gradient for labeled material banding at the density characteristic of the RNA tumor viruses (Fig. 1). A reproducible peak was obtained in the gradient region containing 35 percent sucrose, corresponding to a density of 1.16 g/cm^3. The yield of virus was markedly enhanced when cells were first treated with bromodeoxyuridine (BrdU) (20 μg/ml) for 40 hours, a procedure described for activating type C viruses in other cell lines (*8, 9*). A further two- to threefold enhancement of virus yield was obtained when BrdU-treated cells were then treated with dimethyl sulfoxide (DMSO), by a modification of the procedure of Stewart *et al.* (*9*). In the absence of BrdU treatment DMSO did not enhance virus yield. The cell line B-1 gave results similar to those obtained in Fig. 1. To exclude the possibility that virus in W-7 and B-1 cells was simply a contaminant during transplantation, we developed epithelial cell cultures from primary hepatomas. Clones W-14 and W-15 were tested for virus production within 3 to 6 weeks after the cells were established in culture. After cells were treated with BrdU, labeled material with a density of 1.16 g/cm^3 was again detected in the media, and the yield was enhanced when the cells were treated with both BrdU and DMSO. The normal cell lines K-16 and K-22 were repeatedly negative for virus release, even after treatment with BrdU alone or together with DMSO.

Direct evidence for type C virus in hepatoma cell cultures was obtained by electron microscopy. Figure 2 shows W-7 cells cultured in the absence of BrdU and DMSO. Characteristic budding and free particles were associated with the plasma membrane. Particles were also occasionally seen budding into or lying free within dilated cisternae of rough endoplasmic reticulum and cytoplasmic vacuoles. Particles were first recognizable as an electron-dense

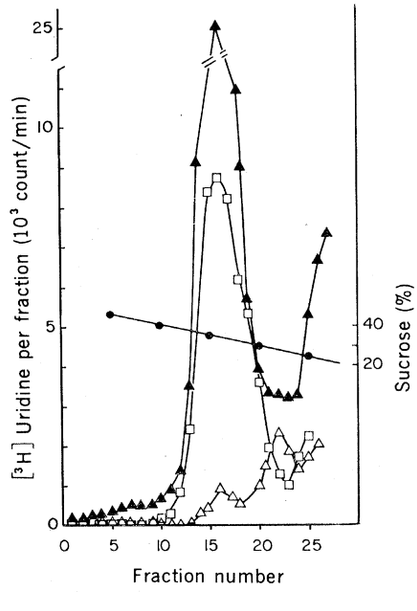

Fig. 1. Isopycnic centrifugation of the medium from W-7 cells labeled with [^{3}H]uridine. One day after 1×10^6 cells were plated per 250-ml T flask (Falcon), six flasks received BrdU (20 μg/ml) and three were left untreated. After this time the cells were protected from bright light. After 40 hours the BrdU media were removed, and 2 percent DMSO was added to three of the flasks treated with BrdU. After 3 days the media were replaced with normal media containing [^{3}H]uridine (20 μc/ml, 26 c/mmole). After 18 hours the media were harvested and clarified by centrifugation, first at 2,500 rev/min for 10 minutes and then at 10,000 rev/min for 10 minutes. The supernatants were centrifuged at 25,000 rev/min for 40 minutes at 4°C. The pellets were suspended in 1 ml of TNE buffer [containing 0.01*M* tris(hydroxymethyl)aminomethane-HCl (tris-HCl) at *p*H 8.3; 0.15*M* NaCl, and 0.005*M* ethylenediaminetetraacetic acid] and centrifuged on a preformed linear sucrose gradient (20 to 50 percent, in TNE buffer) for 3.5 hours at 26,000 rev/min at 4°C in a Spinco model L2-65B centrifuge and a SW-27 rotor. Fractions (1.2 ml) were collected, trichloroacetic acid and carrier RNA were added, and radioactivity of the precipitates was determined. Sucrose concentrations (●) were determined in every fifth fraction by refractive index. Symbols are △, untreated cells; □, cells treated with BrdU; ▲, cells treated with BrdU and DMSO.

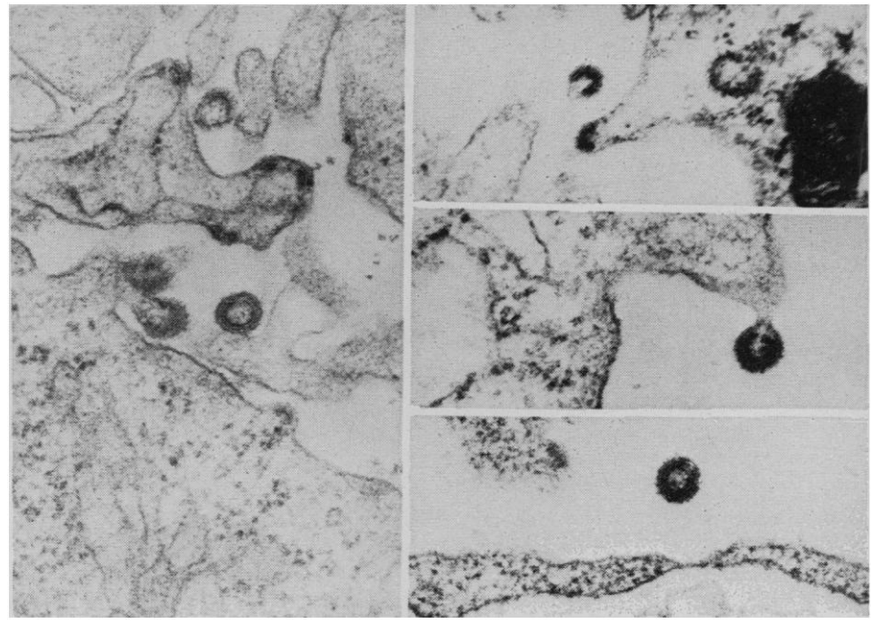

Fig. 2. Evidence obtained by electron microscopy for type C particles in rat hepatoma cells. Cells from clone W-7 were grown to confluency, rinsed with phosphate-buffered saline, removed from the flask by gentle scraping, and pelleted by centrifugation at 1500 rev/min for 10 minutes. The pellets were fixed successively for 1 hour each in buffered 2.5 percent glutaraldehyde and 1 percent osmium tetroxide dehydrated in acetone and embedded in Durcupan. Thin sections were stained with uranyl acetate and lead nitrate and examined with a Siemens I electron microscope. Particles in varying stages of budding and "immature C particles" are demonstrated. (See text for description.)

horseshoe-shaped layer separated from the unit membrane by a less dense intermediate zone. At later stages of development all concentric layers were complete; and the particles rested either on a fine stalk or a microvillus before detachment. The outer diameter of the particles was approximately 100 nm, and the inner concentric rings measured approximately 75 and 50 nm, respectively. The free forms correspond with the "enveloped A" or "immature C" particles discussed by de Harven (*10*). No intracytoplasmic particles and no "mature C" particles (that is, particles with a dense central nucleoid) were detected in several preparations from W-7 cells. Identical type C particles in various stages of maturation were also found in W-14, W-15, and H-4 cells. In addition, the H-4 cells had mature type C particles in the extracellular spaces; and W-7, W-14, and W-15 cells treated with BrdU and DMSO contained numerous elongated or filamentous forms of budding particles. Virus particles have not been detected in thin sections of the normal cells K-16 and K-22.

Activities of DNA polymerase of RHAV and avian myeloblastosis virus (AMV) were compared (Table 1). Detergent-treated RHAV had extremely low endogenous activity and a negligible response to 70*S* RNA from AMV or to the synthetic templates $(dT)_{10} \cdot (A)_n$ or $(dT)_{10} \cdot (dA)_n$ (*11*). There was a significant response to $(dA\text{-}dT)_n$ and high activity with $(dT)_n \cdot (A)_n$. A kinetic analysis gave qualitatively similar results. As previously described (*12*), AMV had significant endogenous activity and a marked response to both $(dT)_{10} \cdot (A)_n$ and $(dT)_n \cdot (A)_n$ (Table 1). The low endogenous activity and lack of response to $(dT)_{10} \cdot (A)_n$ and 70*S* RNA from AMV suggested that RHAV may be deficient in the "reverse transcriptase" activity characteristic of other type C RNA viruses (*12*). To exclude the possibility that this was due to a nuclease or other inhibitor, $(dT)_{10} \cdot (A)_n$ or 70*S* RNA from AMV were first incubated with detergent-treated RHAV, and then purified DNA polymerase from AMV (*12*) and other components of the polymerase reaction were added. Incubation with detergent-treated RHAV did not impair the capacity of $(dT)_{10} \cdot (A)_n$ to stimulate AMV enzyme activity and only partially inhibited the response to RNA from AMV.

Table 1. A comparison of the template responses of DNA polymerases associated with rat hepatoma–associated virus (RHAV) and avian myeloblastosis virus (AMV). The RHAV was prepared from the media of W-7 cells grown in the presence of BrdU and DMSO as described for Fig. 1. The virus pellet obtained after centrifugation at 25,000 rev/min was resuspended in 1/2500 the original volume of medium, and 50 μl of this suspension or 50 μg of purified AMV virus was first incubated in 0.1 percent NP-40 detergent (Shell) and 0.02*M* dithiothreitol (total volume, 70 μl) at 0°C for 10 minutes. To this mixture was added 5 μmole of tris-HCl (*p*H 8.3); 4 μmole of KCl; 0.6 μmole of $MgCl_2$; 0.2 μg of bovine serum albumin; 0.08 μmole each of dGTP, dATP, and dCTP (*11*); 0.1 mc of [^{3}H]dTTP (16.7 c/mmole); and 1 μg of the indicated templates [final volume, 100 μl; templates and other materials obtained as described (*12*)]. After incubation at 37°C for 30 minutes, acid-precipitable radioactivity of a 10-μl portion was determined as described (*12*). Values are expressed as picomoles of [^{3}H]-dTMP incorporated in 30 minutes per 10-μl portion after subtraction of the zero-time blank.

Template	[^{3}H]dTMP incorporated (pmole)	
	AMV	RHAV
Endogenous	0.70	0.02
$(dT)_{10} \cdot (dA)_n$	1.16	0.03
$(dT)_{10} \cdot (A)_n$	25.50	0.04
$(dT)_n \cdot (A)_n$	155.50	54.95
$(dA\text{-}dT)_n$		4.01

These results suggest that the DNA polymerase associated with RHAV is deficient in reverse transcriptase activity. We have not, however, excluded the possibility that treatment of the cells with BrdU and DMSO, which was necessary to enhance virus yield, led to the synthesis of a defective RHAV particle. It is also possible that the high activity detected with RHAV and $(dT)_n \cdot (A)_n$ was caused by contamination with a normal host DNA polymerase (*13*). The only other reported examples of RNA tumor viruses deficient in reverse transcriptase activity are Rous sarcoma virus $\alpha(0)$ in the avian system and the murine viruses S+L− and S+H− (*14*). The apparent deficiency of enzyme activity in RHAV and its possible relation to infectivity and transformation need further study.

Our results do not indicate whether RHAV is simply a passenger virus or is causally related to the induction of hepatomas. Type C particles have been previously detected by electron microscopy in chemically induced rat hepatomas (*15*). To our knowledge, none of these viruses have been previously isolated or characterized. The evidence that irradiation and chemical carcinogens can activate latent viral information in cells has been reviewed by Todaro and Huebner (*16*) and is largely confined to leukemias, lymphomas,

and sarcomas. Our results raise the possibility that activation of type C viruses may also play a role in the induction of hepatomas by chemical carcinogens.

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11. Abbreviations are as follows. The center dot indicates that the polynucleotide chains before and after the dot are hybridized; $(dT)_{10}$, a ten-unit oligomer of deoxythymidylate; $(dA)_n$, a polymer (length unspecified) of deoxyadenylate; $(A)_n$, a polymer of adenylate; $(dT)_n$, a polymer of deoxythymidylate; $(dA\text{-}dT)_n$, a polymer of deoxyadenylate and deoxythymidylate units in alternating sequence; dGTP, deoxyguanosine triphosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dTTP, deoxythymidine triphosphate; dTMP, deoxythymidine monophosphate.
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Ceramidase Deficiency in Farber's Disease (Lipogranulomatosis)

Abstract. *Ceramidase activity could not be demonstrated in the kidney and cerebellum from a deceased patient with Farber's disease, whereas the activities of six control acid hydrolase enzymes appeared normal. This enzyme defect presumably accounts for the accumulation that has been described in two patients and may represent the biochemical basis of this disorder.*

Farber's disease is a disorder that affects young children and is characterized by progressive arthropathy, subcutaneous nodules, nutritional failure, and, in most instances, psychomotor retardation. The basic lesion is a histiocytic granuloma which contains variable numbers of foam cells. In the nervous system the pathological changes resemble those seen in the neuronal lipidoses (*1–3*). In one family the illness occurred in a brother and a sister (*1*); all the other eight reported patients were the only affected members of their families (*3*).

A 10- to 60-fold excess of ceramide was demonstrated in the visceral organs of a 9-month-old girl with Farber's disease who died in 1965, and a high concentration of ceramide was found in a subcutaneous nodule (*3*). Comparable ceramide accumulation has been demonstrated in the kidney and subcutaneous nodule, but not in the other tissues, of another patient with Farber's disease who had survived until he was 16 years old, and who appeared to be more mildly affected than other patients (*4*). The observation that ceramide was found to be accumulated in two patients prompted us to examine ceramidase activity in this disease.

We have now examined the stored postmortem tissues of the above-mentioned 9-month-old girl with Farber's disease; other biochemical findings in this patient have been reported (*3*). Immediately after autopsy, which had been performed 6 hours after death, the tissues were placed in sealed plastic bags and stored at −60°C. As controls we used tissues stored under identical conditions for various periods from patients whose ages and causes of death are listed in Table 1.

The substrate for ceramidase, *N*-[1-^{14}C]oleoylsphingosine, was synthesized chemically from [1-^{14}C]oleoyl chloride and sphingosine (*5*). After treatment with 0.1*N* methanolic NaOH for 30 minutes, the labeled ceramide was purified by silicic acid column chromatography and preparative thin layer chromatography (*3, 6*).

Ceramidase activity was measured in homogenates of tissue in two volumes of a solution of 0.25*M* sucrose containing 1 m*M* EDTA. Incubations were performed for 1 hour at 37°C at the optimum *p*H for each organ with 0.25 m*M* *N*-[1-^{14}C]oleoylsphingosine (specific activity, 2678 count min^{-1} nmole^{-1}), except for those shown in Fig. 1, where the concentration of the substrate was 0.38 m*M* (specific activity, 1019 count min^{-1} nmole^{1-}). Each assay mixture also contained 25 μl of 1*M* citric acid–phosphate buffer, *p*H 4.5 (for kidney) or *p*H 4.0 (for cerebellum), 0.1 mg of Tween 20, 0.25 mg of Triton X-100, 0.4 mg of sodium cholate, and 0.3 to 4.0 mg of protein in a final volume of 200 μl. The data in Table 1 were obtained with 0.7 to 1.3 mg of protein. Under these conditions, enzyme activity was linear as a function of the amount of protein added. Control assays were performed with each tissue, and contained homogenate that had been heated at 100°C for 5 minutes. Before addition of buffer and homogenate, the radioactive ceramide and an aqueous solution of the detergents were mixed and subjected to sonication for 10 minutes. After the incubation, carrier oleic acid, 500 μg in a mixture of $CHCl_3$ and CH_3OH (2 : 1, by volume), was added, and the reaction mixture was partitioned with Dole's solvents (*7*). The fatty acid fraction including [1-^{14}C]oleic acid released was further purified by preparative thin layer chromatography (silica gel G), with $CHCl_3$, CH_3OH, and CH_3COOH (94 : 1 : 5, by volume) as the solvent system. The fatty acid band was located by means of iodine vapor, with authentic standards for comparison; the band of silica gel containing the fatty acid region was scraped off and the radioactivity was counted in Aquasol (New England Nuclear) in a liquid scintillation counter.

Galactocerebrosidase and glucocerebrosidase were assayed in the supernatant remaining after the cell nuclei were removed, according to the method of Hajra *et al.* (*8*) and Radin and Arora (*9*) with 0.1 m*M* ^{3}H-labeled galactocerebroside (specific activity, 2473 count min^{-1} nmole^{-1}) and 0.1 to 1.4 mg of protein per assay in 0.1*M* citric acid–phosphate buffer, *p*H 4.0, or 0.1 m*M* ^{14}C-labeled glucocerebroside (spe-