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Consecutive Activation of a Type C RNA and Polyoma Virus from Tumors Induced by Polyoma Virus-Transformed Rat Cells[1] (38427)

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Type C particles have been observed on rare occasions in rats with naturally occurring leukemia (1) and in transplantable rat tumors (2,3). The particles were also found in chemically induced tumors in rats (4,5), and in spontaneously transformed rat cell lines (6,7). Chemical induction of type C virus from rat cell lines have been reported (8–10). We recently reported an induction of a type C RNA virus from tumors resulting from transplantation of chemically transformed rat cells (11).

With one exception, attempts to activate virus synthesis in cells transformed by polyoma virus have previously been unsuccessful (12–14), although the presence of viral genes in polyoma virus-transformed cells has been suggested—Fogel and Sachs (15) have reported the successful activation of virus synthesis in polyoma-transformed rat cells; the line of rat embryo muscle cells transformed by the large plaque strain of polyoma virus was found to release polyoma virus spontaneously at low frequency. Various physical and chemical agents increased the frequency of induction of virus synthesis (16, 17).

We describe here activation of a type C RNA virus from cell lines derived from tumors induced by polyoma virus-transformed rat embryo cells and further induction of polyoma virus synthesis from these rat tumor cells following 5-bromodeoxyuridine treatment.

Materials and Methods. Cell cultures and media. The polyoma virus-transformed rat embryo cell line (R-667-3) used in this experiment has been described in detail (18, 19). The current experiment utilized this line at the 39th and 45th subcultures. The line was obtained after infection of cultures from Fischer rat embryo with a mixed-plaque-type strain of polyoma virus. Polyoma T antigens were demonstrated in the transformed cells by complement-fixation (CF) and fluorescent antibody (FA) tests, but polyoma viral (structural) antigens were not detected. Neither type C virus, virus-like particles, nor viral antigens were observed in the transformed line when examined by electron microscopy and CF tests. Progressive, transplantable tumors were readily produced in newborn rats by inoculation of the transformed cells. Both the cultured tumor cells and rat tumors contained polyoma T antigen but not the polyoma viral antigen (19). Growth and maintenance medium consisted of 10% fetal bovine serum in Eagle's minimum essential medium with 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Virus assay. A combination of the following procedures was used to detect the replication of type C virus in infected cultures: (a) CF test using rat type C virus group reactive guinea pig serum (20, 21); (b) RNA-dependent DNA polymerase assay using poly rA:oligo dT; (c) electron microscopy. The replication of polyoma virus in infected cultures was determined by the following methods: (a) the presence of cytopathic effects in mouse embryo cells; (b) induction of polyoma virion and hemagglutination (HA) antigens in infected mouse embryo cells.

Complement fixation. Complement-fixation tests were carried out by the microtiter technique as described for tumor antigen studies (22). Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement. Cell pack preparations for CF test-

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ing were made as previously described (19). Guinea pig antiserum specific for the major internal protein of rat type C viruses used in the CF test has been described in detail (20, 21). Purified rat gs-1 antigen (21) was used as a positive control. Polyoma T antisera were obtained from hamsters bearing polyoma tumors.

Reverse transcriptase assay. The procedure of assaying supernatant viral RNA-dependent DNA polymerase, which is a highly sensitive method for the detection of replication of type C RNA virus (23), has been described previously (11). A synthetic template, poly rA:oligo dT, was used to enhance enzyme detection.

Results. As previously reported (19) the polyoma virus-transformed rat embryo cells (R-667-3) were highly tumorigenic; all newborn Fischer rats inoculated with 1×10^6 cells developed tumors within a week (Table I). The tumors grew progressively and reached a diameter of 1–2 cm within 3 weeks. The induced tumors were trypsinized, and the cells were reestablished in culture (Table I).

The cultured tumor cell lines (R-964 and R-976) contained type C virus particles. Electron-microscopic examination revealed budding and free extracellular particles possessing characteristic type C morphology. In addition, high levels of RNA-dependent DNA polymerase activity were further demonstrated in these lines. The polymerase assay has been successfully applied as a rapid and sensitive means of type C RNA virus detection by many laboratories and is an accepted standard procedure (24, 25). The tumor cells contained polyoma T antigen but not the polyoma viral antigen as previously described (19). Further, the cultured rat tumor cells were assayed and found positive for the major rat species-specific antigen (gs-1) in CF tests (Table I). The presence of rat type C virus specific and mammalian interspecies (gs-3) antigen in tumor cell lines was verified in gel diffusion assays.

It has been reported that the treatment of polyoma virus-transformed rat cells with halogen derivatives of uridine (16) induced polyoma virus synthesis with increased frequency. One-day-old tumor cells in 16-oz cultures were grown for 24 hr in the presence of medium containing various concentrations of bromodeoxyuridine (BrdU). The fluid was then removed, and fresh medium was added. The cultures were incubated for 20 days at 37° under 5% CO_2 in air. At various intervals the medium was collected and

TABLE I. Activation of Type C RNA Virus From Tumors Inoculated Subcutaneously with Rat Embryo Cells Transformed by Polyoma Virus (1×10^6 Cells Inoculated/Animal).

Donor (passage no.)	No. with tumor/ No. inoculated	Tumor cell lines	Subculture no. when tested	Polymerase activity[a] (cpm)	CF titers[b] against:			Gel diffusion test for RaLV gs-1 and gs-3	Type C Virus particles
					Polyoma T	Polyoma V	RaLV GPS[c]		
R-667-3			11	5,905	1:2	0	1:2	positive	positive
	5/5 (7)	R-964							
(p-39)			14	21,740	ND[e]	0	1:16	positive	ND
R-667-3			5	13,798	1:4	0	1:2	positive	positive
	5/5 (6)	R-976							
(p-45)			8	32,826	ND	0	1:16	positive	ND

[a] Virus pellet suspended in 0.01 *M* Tris buffer pH 7.4 in 0.01 supernatant volume after centrifugation at 105,000g for 2 hr.
[b] CF titers = reciprocal of dilution giving 3^+ to 4^+ fixation of 1.8 units of complement; 0 = 0/2.
[c] RaLV = Rat leukemia virus; GPS = guinea pig serum.
[d] Day tumor first seen.
[e] ND = Not done.

assayed for infectious polyoma virus and for the presence of type C virus gs antigen by the CF test.

The course of induction of polyoma virus synthesis in culture fluids of tumor cells after exposure to various BrdU doses for 24 hr is shown in Table II. There was no evidence of polyoma virus synthesis in untreated cultures (Table II). The induction of virus synthesis reached a maximum between 6 and 8 days after the cells were exposed; virus synthesis was still observed even 20 days after the cells were exposed. Higher yields of virus were obtained in fluids on the sixth day after exposure to 100 μg BrdU/ml for 1 day (Table II). Cell pack preparations from infected mouse cells on the 10th day after inoculation reacted with polyoma mouse anti-viral serum. Moreover, tissue culture fluids of infected mouse embryo cells gave hemagglutination titers of 1:16–1:32, which were inhibited by anti-polyoma serum (Table III).

In addition, both rat gs-1 as well as mammalian interspecies reactivities were detected in cell suspensions prepared from untreated or treated tumor cells. However, the titers of rat gs-1 antigen increased after BrdU treatment. Ribonucleic acid-dependent DNA polymerase activities in supernatants of treated cells at Day 6 after exposure are also shown in Table II. Treatment with BrdU always decreased their activity

Discussion. The experimental results described above showed that a rat type C RNA virus could be detected in tumors resulting from transplantation of polyoma virus-transformed rat embryo cells and that the polyoma viral genome from these rat tumor cells could further be inducible by BrdU treatment. Thus this is the first example of "double" or consecutive activation of RNA and DNA tumor viruses from DNA virus-induced tumor cells.

Of special interest in the present study was the polyoma virus synthesis from tumors induced by polyoma virus-transformed rat embryo cells following chemical treatment. There has been only one reported article by Fogel and Sachs (15) to date on activation of virus synthesis in polyoma-transformed rat cells. The established line of rat embryo muscle cells transformed by the large plaque strain of polyoma virus was found to release polyoma virus spontaneously at a frequency of about 1 in 10^4. Furthermore, various physical and chemical agents which cause DNA breakage increase the frequency of induction of polyoma virus synthesis. Thus, our data indeed support Fogel and Sach's findings (15): the successful induction of polyoma viral genome from the transformed cells following chemical treatment. As mentioned above, the transformed line used in this study was obtained after infection of Fischer rat embryo cultures with a mixed-plaque-type strain of polyoma virus.

The present study describes also the coincident activation of type C RNA virus in tumors induced by rat cells transformed by polyoma virus. Although the transformed rat cells were negative for infectious virus before inoculation into animals, budding type C virus particles were activated from the cell line derived from the transformed cell-induced tumors. A type C RNA virus in the tumor cell lines was further demonstrated by reverse transcriptase. These lines did also contain rat type C virus group-specific (gs-1) and mammalian interspecies (gs-3) antigens. Thus these data suggest that the virus was indeed of rat origin. Since recent findings (8, 9) indicated that rat cell clone in culture contains the genetic information for producing a type C RNA virus in an unexpressed form in their somatic cells as well as in their germ cells, and type C particles are not usually demonstrable in rat tissues of normal or tumor origin, we suggest that the polyoma virus and activation of the virus may be related as proposed previously (26). We have recently observed similar findings: A rat-specific type C RNA virus was activated from tumors induced by rat cells transformed by a chemical carcinogen (11). Spontaneous *in vitro* induction of type C RNA virus in rat cells is known (6, 7, 10, 27). Therefore, possible low-grade virus production occurred *in vitro* before *in vivo* transplantation. Because *in vivo* tumor induction often represents clonal selection, the transplantation might have increased the probability of virus detection by eliminating non-transformed cells. On the other hand, the possibility that the virus was "picked" in the host during transplantation could not be excluded.

It has already been shown that type C or type C-like viruses can be activated in virus-free cells of four species, the mouse, rat, cat, and guinea pig, *in vitro* by the presence of BrdU or iododeoxyuridine (IdU) and that the chemically induced viruses were endogenous helper virus of the host cells rather than any helper virus of the exogenous virus (8, 9, 28–30). More recent re-

TABLE II. Activation by BrdU of Polyoma Virus from Tumor Cells Induced by Polyoma Virus-Transformed Rat Cells.[a]

Rat tumor cells	Treatment (μg/ml)	Polyoma virus induction (days after exposure)					Type C virus		Gel diffusion for RaLV gs-1 and -3
		3	6	8	15	20	Reverse[c] transcriptase activity	CF titers[b] against RaLV GPS	
R-964 (p-12)	None	–	–	–	ND	–	8,215	1:2	+
	BrdU-25	–	ND	ND	ND	ND	7,729	1:4	+
	BrdU-50	–	+(1.3)[d]	ND	ND	ND	5,937	1:4	+
	BrdU-100	–	+(2.3)	+	+(1.3)	+(> 0.3)	5,554	1:4	+
R-976 (p-6)	None	–	–	ND	ND	–	32,732	1:2	+
	BrdU-25	–	ND	ND	ND	ND	22,402	1:8	+
	BrdU-50	–	+	ND	ND	ND	25,895	1:8	+
	BrdU-100	–	+	ND	ND	ND	13,600	1:8	+

[a] After treatment of BrdU for 24 hr, cell were washed and fresh medium was added. At various intervals the medium was collected. The supernatant fluids and cells were examined for the presence of polyoma virus, reverse transcriptase and rat leukemia virus (RaLV) CF antigen. Minus signs indicate no cytopathic effects in mouse embryo cells inoculated with undiluted virus.

[b] CF titers = reciprocal of dilution giving 3^+ to 4^+ fixation of 1.8 units of complement.

[c] Counts per minute of incorporated (^{3}H) TMP.

[d] Infectivity titer in mouse embryo cells expressed as $\log_{10}$ TCD_{50}/ml.

TABLE III. Infectious Polyoma Virus and Viral Antigen Synthesis in Tumor Cells Induced by Polyoma Virus-Transformed Rat Cells After BrdU Treatment.[a]

Tumor cell lines	Treatment (μg/ml)	Polyoma virus assayed in mouse embryo cells		
		Infectivity titers[b]	CF titers[c]	Hemagglutination titer[d]
	None	0	0	No titer
R-964	BrdU-50	1.3	> 4	1:64
(p-12)	BrdU-100	2.3	> 4	1:32
	None	0	0	No titer
R-976	BrdU-50	> 0.3	1:2	1:16
(p-6)	BrdU-100	> 0.3	1:4	1:16

[a] After treatment with BrdU for 24 hr, cells were washed and fresh medium was added. On sixth day after treatment fluids were inoculated into mouse embryo cells for the presence of polyoma virus. The mouse cultures were observed for cytopathic effects for 10 days. Fluids and cells were examined for the polyoma virus by CF and hemagglutination tests.

[b] Infectivity titer expressed as $\log_{10}$ TCD_{50}/ml.

[c] CF titer = reciprocal of dilution giving 3–4 fixation of 1.8 units of complement; 0 = 0/2.

[d] Hemagglutination titer with guinea pig erythrocytes at 4°.

ports indicate that the type C virus can be activated in the tissue cultures isolated from a human pulmonary adenocarcinoma with IdU (31). Activation of virus synthesis in polyoma-transformed cells by chemicals has been reported (15–17). Together with our findings and those reported with murine leukemic sarcoma viruses and DNA tumor virus, it seems that chemical induction may be extremely useful for the activation and enhancement of both certain RNA and DNA tumor viruses in normal and malignant cells of animals and man. The mechanism by which chemical compounds induce viruses from normal or virus-negative cells is not known. Lowy *et al.* (29) have reported that incorporation of BrdU or IdU into cellular DNA plays a vital role in the activation of murine leukemia virus production in the AKR cell lines. Fogel (17) presented data indicating that the replication of polyoma virus in the chemically treated cells is induced by single strand breakage of the cellular DNA.

Studies are in progress to determine whether the induced polyoma virus in rat tumor cells after BrdU treatment is different from the original virus, and to further induce polyoma virus from the rat transformed cells before implantation. The polyoma-transformed rat cells might have either an increased or decreased efficiency in activating or picking up endogenous type C particles. Or it could be the type C particles which facilitate BrdU activation of polyoma virus. Which came first? Are they synergistic or independent events? Thus the further information on the interactions of DNA and RNA tumor viruses in the process of cell transformation is important and should be explored.

Summary. A type C RNA virus was activated from cell lines derived from tumors induced by polyoma virus-transformed rat embryo cells and further induction of polyoma virus synthesis from these rat tumor cells was obtained following 5-bromodeoxyuridine treatment.

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