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Spontaneous Release of Endogenous Ecotropic Type C Virus from Rat Embryo Cultures

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Type C viruses were isolated from embryo cultures of two different rat strains, Sprague-Dawley and Fischer. Both viruses (termed rat leukemia virus, RaLV) were released spontaneously from rat embryo cells, have a density of 1.14 to 1.15 g/cm^3 based on equilibrium sedimentation in sucrose gradients, contain 60-70*S* RNA, RNA-directed DNA polymerase, and rat type C virus-specific 30,000 molecular-weight-protein determinants. Molecular hybridization studies using the Sprague-Dawley RaLV 60-70*S* RNA show that the virus-specific nucleotide sequences are present in the DNA of rat embryos. Both Sprague-Dawley and Fischer RaLV can rescue the murine sarcoma virus genome from Kirsten murine sarcoma virus-transformed nonproducer cells and are neutralized by antisera to the RPL strain of RaLV. In contrast to previous RaLV's, these viruses propagate in their own cells of origin as well as in cells of heterologous rat strains.

Type C viruses have been described in spontaneous or chemically induced rat tumors (3, 32, 33), in rat cells treated by chemical carcinogens and halogenated pyrimidines alone or in combination (4, 9, 10, 25, 30), in rat tumors induced by Moloney sarcoma virus, and in rat cells transformed by polyoma virus (6, 22). Although type C viruses are produced by several long-term propagated rat cell lines (1, 5, 11, 15, 19, 24, 27), they have not heretofore been isolated from rat embryo cells in relatively early passage. In the present report we describe the spontaneous release of endogenous type C virus from whole embryo cultures of Sprague-Dawley and Fischer rats at passages 13 and 23, respectively. Of interest is the ability of these isolates (termed SD-RaLV and F-RaLV, respectively) to infect and replicate in cells of homologous and heterologous rat species, i.e., ecotropic behavior (13) and to accelerate malignant transformation of the infected çells (20).

Rat cells used included normal rat kidney (NRK-9) (11), wild-rat (*Rattus norwegicus*) embryo fibroblasts, and primary cultures of two random-bred Sprague-Dawley (SD-1 and SD-2) and two random-bred Fischer rat embryos (F-1 and F-2) of 14 to 20 days gestation obtained from Flow Laboratories, Inc., Rockville, Md. Cultures were grown at 37 C in minimum essential medium supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum, and 50 μg of gentamicin per ml.

Type C virus production was measured by an assay for RNA-directed DNA polymerase (RDP) activity in culture fluids by using exogenous template-primer poly(rA)oligo(dT) (8), electron microscopy of the cells and fluids, [^{3}H]uridine labeling and banding of virus by isopycnic centrifugation, and detection of viral antigen in sonicated tissue culture cell packs by radioimmunoassay (RIA) (2, 26) or complement fixation (CF) test. Highly specific antisera made in guinea pigs against isoelectric focus purified p30 proteins of rat leukemia virus (RaLV) (16), murine leukemia virus, hamster leukemia virus, feline leukemia virus (6), RD-114 virus (17), gibbon ape leukemia virus (7), and simian sarcoma and associated virus (28) were used for detection of p30 proteins. The RIA employed ^{125}I-labeled RaLV p30 from the RPL strain of RaLV (11). The interspecies specificity of p30 was detected by using a serum prepared by sequential immunization of a goat with feline leukemia virus, RD-114, simian sarcoma and associated virus, and gibbon ape leukemia virus p30 proteins (2).

For host range study 20 different cell lines/strains of human and other animal species, Osborne-Mendel (NRK) and five other secondary rat embryo cultures (Table 1, footnote *a*), preincubated for 18 to 24 h with 2 μg of polybrene per ml were separately exposed to SD-

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TABLE 1. *Growth of SD-RaLV and F-RaLV in different rat cells*[a]

Virus[b]	Cells					
	Sprague-Dawley (SD-1)		Fischer (F-1)		Osborne-Mendel (NRK)	
	RDP[c]	RIA[d]	RDP	RIA	RDP	RIA
Control (uninfected)	203	<2	40	<3.9	256	<3.3
SD-RaLV	1,811	75	4,046	360	6,350	270
F-RaLV	7,737	1,350	5,785	280	4,978	1,065

[a] SD-1 cells passage 8, F-1 cells passage 6, and NRK cells passage 35. Similar results were obtained with F-2 passage 4, SD-2 passage 2, and wild-rat embryo cultures passage 2. Cell lines nonpermissive for replication of SD- and F-RaLV included human embryo and adult fibroblasts, HT-1080 (21), RD (14), and BEWO cells (Naval Biomedical Research Facility [NBL], Oakland, Calif.), monkey, DBS-FRhL-1 (31), dog D-17 (Riggs), two cat embryo fibroblasts, BALB/c, NIH and wild-mouse embryo cultures, rabbit cornea (SIRC), guinea pig embryo kidney (Flow Laboratories), and mink MUL-Lu (NBL).

[b] The inocula consisted of undiluted filtered (0.45-μm, Millipore) culture fluid containing 10^7 F-RaLV and 10^8 SD-RaLV type C particles/ml as determined by negative-stain electron microscopy.

[c] RDP, RNA-directed DNA polymerase activity in the tissue culture fluid ([^{3}H]dTMP incorporation values, counts per minute per milliliter), tested 20 days after infection.

[d] RIA, Radioimmunoassay. Measured in nanograms of RaLV p30 per milligram of cell protein.

RaLV and F-RaLV. The infected and control uninfected cells were subcultured once a week and tested for RDP activity at 3 weeks. If the cultures were negative they were further passaged and tested again at 4, 6, and 8 weeks. Sonicated cell packs of cultures which were RDP positive, together with RDP-negative controls, were tested for RaLV p30 by CF and RIA. Titration end points of the two viruses were determined in F-2 cells (Table 2).

Rabbit-immune serum to banded RPL-RaLV (2) was tested in vitro for its ability to inhibit focus formation by the murine sarcoma virus (MSV) pseudotype of F- and SD-RaLV. Approximately 100 focus-forming units of the virus (0.2 ml) were mixed with equal volumes of each of the serum dilutions (1:10 to 1:640), left at room temperature for 30 min, and incubated with target cells for the next 30 min at 37 C in a humidified atmosphere containing 5% CO_2 before adding medium containing 10% calf serum and 1% dimethyl sulfoxide. Foci were counted after 7 to 8 days.

DNA was extracted from various embryos (Table 3), and hybridization to viral RNA was performed as previously described (23).

Spontaneous type C virus activity was first confirmed by RDP and RIA or CF in Sprague-Dawley (SD-1) embryo cultures at passage 13 and in Fischer (F-1) rat embryo cultures at passage 23. The SD-1 cells at passage 24 and F-1 cells at passage 23 showed budding type C particles by electron microscopy and reacted by CF with an antiserum to RaLV p30 but not with antisera to murine leukemia virus, feline leukemia virus, hamster leukemia virus, gibbon ape leukemia virus, simian sarcoma and associated virus, or RD-114 virus p30 proteins. Culture fluids from these cells also incorporated [^{3}H]uridine at a density of 1.14 to 1.15 g/cm^3 in the sucrose gradients. Both cultures, with continued in vitro passage of the cells, showed increased levels of RDP activity (90,000 counts/min per ml at passages 60 to 70) and gs antigen detectable by CF (antigen titer, 1:16 to 1:32) and RIA (690 to 800 ng p30 per mg of cell protein). Interspecies p30 antigen was also detected by CF in the SD-1 and F-1 cells at passages 56 and 87, respectively.

Both SD-RaLV and F-RaLV rescued the MSV genome from Kirsten-MSV-transformed

TABLE 2. *Titration of SD-RaLV and F-RaLV on Fischer rat cells*[a]

Virus dilutions	RDP[b]	RIA[c]
Control (uninfected)	40	<3.9
F-RaLV		
10^0	2,176	320
10^{-1}	1,415	135
10^{-2}	NT[d]	11
10^{-3}	61	<2.4
SD-RaLV		
10^0	2,437	300
10^{-1}	429	70
10^{-2}	NT	6.7
10^{-3}	57	<2.5

[a] Tissue culture fluids (0.4 ml) were tested on F-2 cells (3×10^5 cells) (passage 4) from passage 42 (SD-RaLV) and passage 45 (F-RaLV).

[b] RNA-directed DNA polymerase activity (counts per minute per milliliter) in the culture fluid on the 20th day after infection with the virus.

[c] RIA, Radioimmunoassay. Cell packs were tested against RaLV gs antisera in radioimmunoassay as described (2). Levels of sensitivity for this assay were >4.5 ng of RaLV p30 per mg of cell protein.

[d] NT, Not tested.

nonproducer rat cells. The pseudotype virus produced foci on NRK and low-passage Fischer rat cells but not on NIH Swiss mouse cells. The MSV pseudotypes of F- and SD-RaLV had a titer of $10^{3.3}$ and $10^{4.3}$ focus-forming units/ml on NRK cells, respectively, and were neutralized by antiserum (titer, 1:160 and 1:320, respectively) to RPL-RaLV, but not by normal NIH Swiss and wild mouse, guinea pig, or rabbit sera.

Zone sedimentation in 5 to 20% sucrose density gradients of the RNA from SD-RaLV showed two major species of RNA, a fast sedimenting band in the vicinity of 60–70S and a slower sedimenting band in the vicinity of 4S. The 70S RNA structure was dissociated into a heterogenous lower-molecular-weight RNA species with a major peak at about 35S by heating for 2 min at 80 C in 20 mM Tris-hydrochloride (pH 7.4) containing 10 mM EDTA.

The ^{3}H-labeled 70S RNA was used as a probe to detect complementary nucleotide sequences in cellular DNA by liquid hybridization technique in DNA excess. At C_0t values of 10^4, about 77 to 82% of the SD-RaLV RNA hybridized with DNA from Sprague-Dawley, Fischer, and Wistar-Furth rat embryos (Table 3).

None of the human, primate, dog, cat, mouse, guinea pig, mink, or rabbit cells (Table 1, footnote *a*) exposed to the two different stocks of freshly harvested tissue culture viruses (SD-RaLV and F-RaLV) showed detectable levels of RDP activity in their fluids after 30 to 65 days.

SD-RaLV and F-RaLV were added separately to NRK and to five different early-passage rat embryo cultures which were not producing virus based upon lack of detectable RDP and gs antigen by RIA and CF. After 20 days all virus-exposed rat cells, including wild-rat cultures, showed significant levels of RDP and rat gs antigen as detected by RIA (Table 1). Infection of early-passage Fischer embryo cells with serial 10-fold dilutions indicated that after 21 days both viruses had a titer of $10^{2.9}$ mean (50%) tissue culture infective doses/ml (Table 2).

TABLE 3. *Characterization of SD-RaLV by RNA-DNA hybridization*

Animal source of DNA	Animal tissue	Hybridization (%)[a]
Rat	Embryos (Sprague-Dawley)	82.2 ± 5.0
	Embryos (Fischer)	76.8 ± 8.3
	Embryos (Wistar Furth)	81.2 ± 4.3
Mouse	Embryos (Swiss-Webster)	24.8 ± 2.9
	Embryos (AKR)	27.7 ± 2.0
Cat	Spleen	29.5 ± 3.0

[a] Expressed as percentage of acquisition of resistance to pancreatic ribonuclease. Experiments were done in triplicate with 1 mg of DNA fragments and 250 to 300 counts/min (2.5×10^{-4} to 3.0×10^{-4} μg) of Sprague-Dawley virus 70S [^{3}H]RNA in 0.4 M salt containing 0.05% sodium dodecyl sulfate at uncorrected C_0t value of 10^4.

The SD-1 and F-1 cultures were fibroblastic and formed contact-inhibited monolayers at the passage levels when release of RaLV was first detected. After 20 further subpassages, i.e., passage 33 and passage 43 respectively, both virus-producing cultures transformed as evidenced by rounded morphology, increased growth rate, and tumorigenicity upon transplantation into newborn rats (20). Inoculation of F- and SD-RaLV into newborn syngeneic rats did not induce systemic infection or any disease in 6 months.

The infectivity of these viruses for homologous and heterologous strains of rat embryo cells, albeit with only a modest infectivity titer, established them as so-called ecotropic viruses (13). The molecular hybridization data indicate the endogenous nature of the viruses, consistent with the observation of Tsuchida et al. (29), who found RaLV-related sequences in rat cellular RNA and DNA. Elsewhere, we have shown that the major polypeptides of SD-RaLV show the typical pattern of other mammalian type C viruses and that both p10 and p12 structural proteins are phosphorylated, which is characteristic of the rat species (18). Thus, these isolates fulfill morphological, immunological, and biochemical criteria for designation as endogenous ecotropic rat type C viruses.

Spontaneous release of ecotropic RaLV from relatively early-passage cultures of Sprague-Dawley and Fischer rat embryo cells has not previously been reported despite the frequent use of cultures from these two rat strains in a number of laboratories (4, 12). The phenomenon of spontaneous activation of RaLV from SD-1 and F-1 cells was repeatable. Moreover, we have recently isolated RaLV from other Sprague Dawley (SD-2) passage 38 and Fischer (F-2) passage 42 rat embryo cultures. Perhaps an environmental or genetic change in the breeding stock or a particularly early embryonic age may have influenced the spontaneous release of endogenous type C viruses in the rat embryo cultures we studied.

It is puzzling that RaLV was recovered repeatedly from two different rat embryo cell strains in the same laboratory. Although handled by different persons in separate biological hoods, the possibility of cross contamination cannot be absolutely excluded. However, no known rat virus was in the laboratory prior to isolation of the SD-RaLV. Thus, any possible contamination would have to be with the SD-

RaLV, since this virus was isolated first. Sensitive biochemical and immunological comparisons should help settle this question.

The SD-RaLV and F-RaLV resemble Wistar-Furth and RPL-RaLV's (11, 24) in their ability to propagate in heterologous strains of rat cells. They differ from the previous isolates in that they can also replicate well in their respective cells of origin. They are thus amenable to further study, including tests of in vivo pathogenicity using different virus concentrations. The ability of these RaLV isolates to accelerate transformation of productively infected rat cells is described in the accompanying paper (20).

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