Neoplastic Transformation of Rat Embryo Cells Induced *in Vitro* by Rauscher Leukemia Virus[1] (34594)

JOHNG S. RHIM, ROBERT J. HUEBNER, W. T. LANE, AND M. L. VERNON

Department of Virus Research, Microbiological Associates, Inc., and National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

Morphological transformation by Rauscher leukemia virus (RLV) has been reported in mouse (1), rat (2), and hamster cells (3). Duc-Nguyen *et al.* (4) described persistent infection by RLV in a rat kidney cell line (NCl-RR) derived from a rat with lymphoid leukemia and also observed morphological changes in sublines of the NCl-RR (2).

Long-term replication of RLV *in vitro* can be achieved in cultures of normal rat embryo cells (5). In our laboratory, normal rat embryo cultures, infected once with RLV, have been replicating the virus and producing complement-fixing (CF) antigen characteristic of the murine leukemia-sarcoma virus complex for more than 18 months (5). Production of complete virus has been demonstrated by means of electron microscopy, and of infectious virus by the *in vitro* assay technic (COMUL test) based on detection of the group-specific CF antigen of murine leukemia viruses (6). The present communication describes the *in vitro* neoplastic transformation observed in rat embryo cell cultures infected with RLV and the neoplastic lesions induced by these altered cells in newborn rats.

Materials and Methods. Virus. RLV was obtained from Dr. J. W. Hartley, National Institute of Allergy and Infectious Diseases, as 22nd passage tissue culture fluid pool (No. 1254) and was passed twice in secondary Swiss NIH mouse-embryo tissue cultures (NIH-METC).

Cell cultures and media. Primary cultures of NIH-METC and Fisher rat embryo tissue cultures (RETC) were prepared as previously described (6) or obtained from Microbiological Associates, Inc., Bethesda, Maryland. Growth and maintenance medium consisted of 10% fetal bovine serum in Eagle's minimal essential medium with 2 m*M* glutamine and 100 units of penicillin and 100 mg of streptomycin/ml.

Rat embryo cell lines. The establishment of RLV infected and uninfected rat embryo cell lines have been dsecribed in detail (5).

Test for infectious virus. Cell lines were tested for infectious virus by the COMUL test (6). To insure that supernatant fluids from infected rat embryo cells were cell free, they were filtered through HA (0.45 μ) Millipore filters.

Cell pack preparation of CF antigen from infected and normal rat embryo cells was made as previously described (7).

Complement fixation. CF tests were carried out in the microtiter technic described for tumor antigen studies (8). Titers were recorded at reciprocals of the highest dilution giving 3 to 4 + fixation of 1.8 units of complement.

Antiviral sera. Rat antisera used in the CF test were obtained from Fisher rats carrying transplanted sarcomas induced by the Moloney strain of murine sarcoma virus (M-MSV) (9). RLV and Gross leukemia virus (GLV) antisera used in the neutralization test were obtained from adult (Lewis X BN) F_1 rats. The rats were given injections of virus-infected rat cells three times at intervals of 2 weeks. All sera were heat inactivated at 56° for 30 min and passed through 0.45-μ Millipore filters.

Neutralization test. Two-tenths ml of virus, containing an estimated 50 focus-forming units (FFU), was mixed with an equal

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amount of serum diluted 1:5 in medium. The mixture was held at 37° for 30 min. Two-tenths ml of the mixture was used to infect monolayers of NIH-METC in order to determine FFU.

MSV assay. MSV was assayed in NIH-METC by the method of Hartley and Rowe (10), and the titer was expressed as FFU per

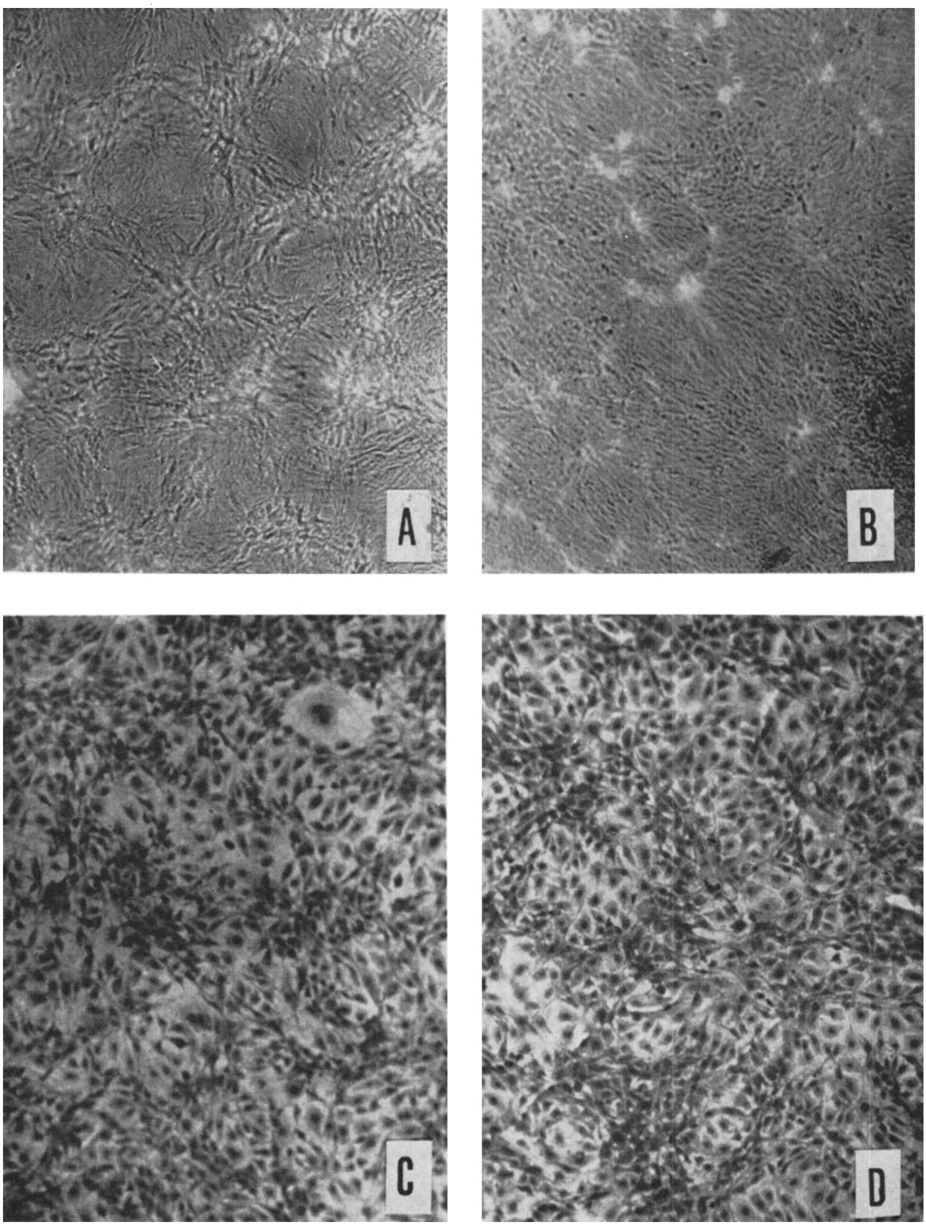

FIG. 1A. Rauscher leukemia virus—transformed rat embryo culture a the 37th passage. (× 40). Note spindle-shaped cells growing in a disorganized pattern and forming foci. 1B. Normal rat embryo culture at the 37th passage (× 40). 1C. The transformed culture at the 38th passage. Giemsa stain (× 72). Note foci formation by spindle-shaped cells. A few multinucleated giant cells are present. 1D. The transformed culture at the 38th passage. Giemsa stain (× 72). Note crisscrossing spindle-shaped cells and nuclear and cytoplasmic overlapping. Foci of epithelioid cells are also present.

TABLE I. Presence of CF Antigen and Virus in Tumors and Organs of Newborn Rats Inoculated Subcutaneously with Rauscher Leukemia Virus Transformed Rat Embryo Cells (R-26) and a Rat Tumor Cell Line (RRT #1).

Donor	Inoculum (per animal)	No. with tumor/no. inoculated	Pathology	Specimen	CF titers	Virus recovery (COMUL test)	
						METC	RETC
R-26, 33rd subculture	6.5×10^6	7/7 (31)[a]	A mixed tumor	20% tumor	>16	+[b]	+
				20% spleen	>16	+	+
				20% thymus	16	+	+
				20% tail	>16	+	+
R-26, 36th subculture	8×10^6	6/6 (14)			ND[c]	ND	ND
RRT #1, 12th subculture	4×10^4	6/10 (14)		20% tumor	>4	+	ND

[a] No. of days, average latent period.
[b] + = CF titer >2.
[c] ND = not done.

ml.

Marker rescue. A genome rescue experiment was done with a mixed culture of a virus-free hamster tumor (HT-l) and mouse embryo cells superinfected with leukemia virus, as described by Huebner *et al.* (11).

Transplantation of transformed cells in rats. Newborn Fisher rats were inoculated subcutaneously with freshly trypsinized transformed cells to determine the oncogenicity of the transformed cells.

Results and discussion. An abnormal pattern of growth was first noted in the 14th subculture of subline R-26. This line was derived from floating live cells obtained by centrifugation (1500 rpm for 20 min) of supernatant fluid of the fifth subculture of Fisher rat embryo cells which had been inoculated with $10^3 TCD_{50}$ of RLV. In most of the flask, crisscrossing spindle-shaped cells gave the cultures a whorled appearance (Fig. 1A, C, D). Several proliferative foci of tightly packed epitheloid cells had been also observed (Fig. 1D). These cells had a tendency to grow on top of one another, forming thick, whitish areas that stained deeply and were also readily visible macroscopically. There was nuclear and cytoplasmic overlapping. A few large giant cells with atypical nuclei, sometimes multinucleated, were also noted (Fig. 1, C, D). Thus, the transformed culture which contained foci of both spindle-shaped cells and epithelioid cells gave the appearance of "stars in sky" and were readily recognized. (Fig. 1A). There was an increased rate of acid production in the culture medium. In contrast, the cellular morphology remained unchanged in a control, uninoculated line, which grew in continuous monolayers of fibroblast-like cells (Fig. 1B). The morphological differences between the infected and the control cells were maintained when both lines were serially subcultured for more than 12 months.

This cell line yielded infectious virus continuously and produced group-specific CF antigen characteristic of the murine leukemia-sarcoma virus complex. The CF antigen titers at different passage levels fluctuated between 1:8 and 1:32. Complement-fixing antigen was not directly demonstrable in supernatant fluids of the transformed cells. However, a CF titer of 1:16 was detected in the fluids of the ninth subculture after they had been concentrated 20 times in volume by Diaflo ultrafiltration (7). Cell-free supernates from the cultures were infectious for NIH-METC and RETC as determined by the COMUL test (6, 9). Infectious virus was also detected by an MSV genome rescue procedure described by Huebner *et al.* (11).

The neoplastic effect of transformed R-26 cells was evidenced by their induction within a few weeks (average latent period, 14–31 days) of subcutaneous tumors (Fig. 2) in newborn rats each receiving 6.5 to 8 $\times$ 10^6 cells. No tumors developed during more than 3 months of observation in groups of rats inoculated with cell suspensions prepared from the normal control line.

The subcutaneous tumors, readily transplantable in newborn rats but not in weanling rats, were histologically mixed tumors with some elements of sarcoma and other areas of adenomatous (Fig. 2-1) and epithelioid (Fig. 2-2) differentiation. Other parts of the tumor illustrated the small fusiform sarcoma cells and the large round cells with acidophilic cytoplasm, large clear nuclei, and small central nucleoli.

A 20 % extract of tumor, spleen, thymus, and tail tissues yielded CF antigen titers of $\geqq$ 16. Infectious virus was detected in the COMUL test when extracts of these rat tumors, spleens, thymuses, and tails were inoculated into mouse and rat embryo cells. The tumors were trypsinized and the cells reestablished in culture. These cells also con-

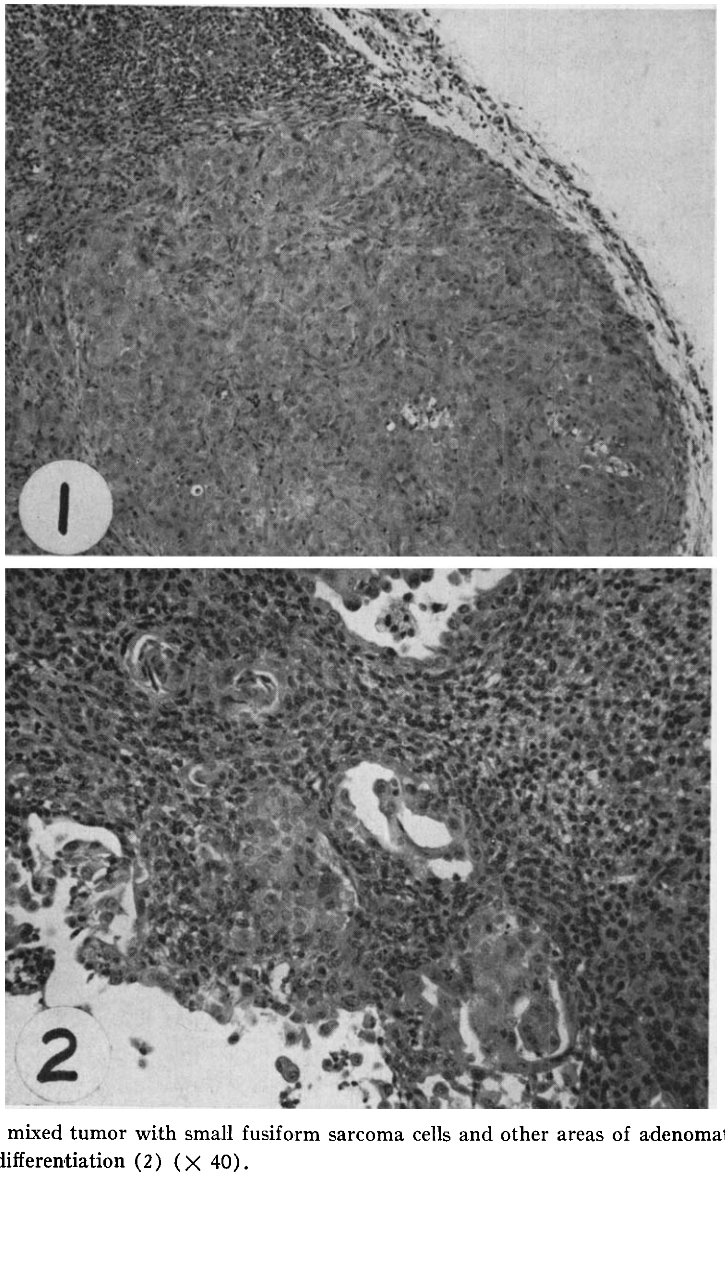

FIG. 2. A mixed tumor with small fusiform sarcoma cells and other areas of adenomatous (1) and epithelioid differentiation (2) ($\times$ 40).

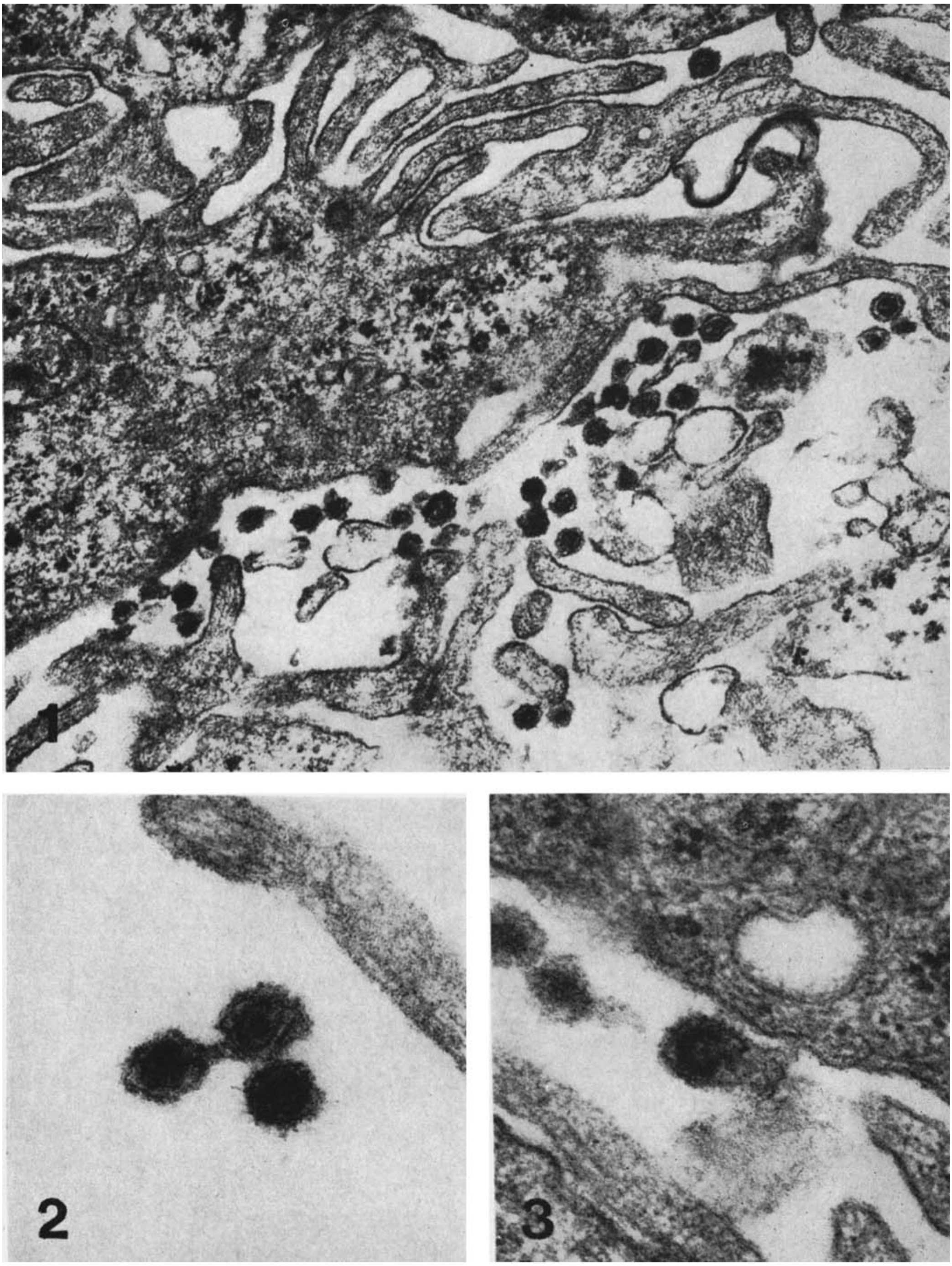

FIG. 3-1. Typical mature C-type particles in the intracellular spaces (× 48,600). 3-2. Higher magnification of extracellular mature C-type particles (× 109,000). 3-3. A budding C-type particle (× 110,000).

tained CF antigen, and continuously released infectious virus. The virus isolated from a rat tumor cell line (RRT No. 1) had MSV genome rescue activity. The rescued infectious MSV was neutralized by type-specific RLV immune serum, but not by GLV immune serum. The rat tumor cell line (RRT No. 1) induced tumors rapidly also in newborn rats (Table I).

This cell line contained numerous virus particles that were readily seen in the electron microscope to be principally in the intercellular spaces (Fig. 3-1). The particles were 100 to 110 mμ in diameter, with a nucleoid approximately 65 mμ in diameter (Figs. 3-2, 3-3). Morphologically, the majority of the particles were comparable to the mature and immature C-type virus particles described by other investigators (12, 13).

Since normal rats usually do not reveal the presence of overt C-type leukemia viruses, it is assumed that the C-type virus particles represented continuous replication of the RLV that was used initially to infect the rat embryo cultures. Thus, viral replication took place constantly in the embryo cells, both *in vitro* and *in vivo*, during a total period of more than 12 months.

Concomitantly, one subline of infected rat embryo cells acquired a malignant potential during their culture *in vitro*, which was revealed upon their transplantation into isologous recipients. The transformed cultures also produced large amounts of viral particles and yielded CF antigens. Since the sequence of virus growth and transformation events in the RLV rat system is analogous to rat cell transformation induced by GLV (14) and also by RLV in hamster cells (3), it seems logical to attribute the transformation observed to RLV. Since, however, only one subline transformed in one attempt, this assumption requires further investigation.

Tumors induced by transformed cultures also yielded virus production. Osato *et al.* (15) reported transformation of cells infected *in vitro* with Friend leukemia virus. However, the cultures lost their leukemogenic potential after subculture, and the presence of leukemogenic virus could not be demonstrated either in cultures or in the fibrosarcomas that arose after grafting cultured cells in mice. Similar findings were described by Duc-Nguyen *et al.* (2) for transformation in sublines of cells derived from rats infected with RLV.

The process of transformation by RLV apparently can be accelerated by the concurrent use of a chemical carcinogen (DENA) (16).

Summary. Neoplastic transformation of rat embryo cells *in vitro* by Rauscher leukemia virus (RLV) is reported. Rat embryo cells infected initially with RLV have replicated infectious virus and produced complement-fixing (CF) antigen characteristic of the murine leukemia-sarcoma virus complex for more than 18 months. Cells from these cultures underwent neoplastic transformation *in vitro* and produced tumors when injected into homologous hosts. The tumors were serially transplantable, and subsequent transplants continued to carry the initial RLV and to yield CF antigen.

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