Long-Term *in Vitro* Cultivation of Rauscher Leukemia Virus in Rat Embryo Cells[1] (35010)

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The long-term cultivation of Rauscher leukemia virus (RLV) in established cultures derived from mouse spleen cells (1), from a mixture of BALB/c mouse spleen and thymus cells (2), and from BALB/c mouse embryo cells (3) has been described. Multiplication of RLV has also been shown in mouse kidney cells (4). Recently, the propagation of RLV in a kidney cell line derived from a rat with lymphoid leukemia has been reported (5). The present study reports results of experiments showing that rat embryo cells can be capable of supporting RLV multiplication *in vitro*, and carrier cultures can be established.

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). The CF antigen titer of a cell pack preparation made with this virus in NIH-METC was 1:16 when it was tested against anti-RLV rat serum. Supernatant fluid titering $10^{3.0}$ TCD_{50}/ml in secondary NIH-METC was used.

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 U of penicillin and 100 mg of streptomycin/ml.

Establishment of rat embryo cell cultures. Approximately 20 ml of primary rat embryo cell suspension (1×10^6 cells/ml) in growth medium were infected with 1.0 ml of undiluted virus and were incubated at 4° for 30 min, with gentle shaking. This mixture was then planted in a B flask (approximately 50-cm^2 surface area) and incubated at 37° under 5% CO_2 in air. By the third day after planting, a complete monolayer was obtained. Fourteen days after inoculation, the cultures were subdivided by trypsin treatment. This was repeated every 7–10 days. The medium was renewed at 3 to 4 day intervals. For the first 4 subcultures the cells were maintained in Eagle's minimum essential medium with 0.1 mM calcium, supplemented with 5% dialyzed calf serum, 2% fetal bovine serum, 2 m*M* glutamine, 0.1 m*M* nonessential amino acids, and antibiotics (6). Thereafter, the cells were maintained in the medium described above.

Uninoculated normal RE cultures were established by the same technique.

Tests for infectious virus. Cell lines were tested for infectious virus by the complement fixation (CF) test for murine leukemia virus, *i.e.*, the COMuL test (3, 7). To insure that supernatant fluids from infected RE cells were cell-free, they were filtered through HA (0.45 μ) Millipore filters. Infectivity titers were determined by CF induction assays in disposable 60-mm Falcon plastic petri dishes of secondary NIH-METC, with 2 plates/tenfold dilution (3).

Cell pack preparation of CF antigen from

[1] This work was supported by NIH Contract PH 43-68-705 from the National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014.

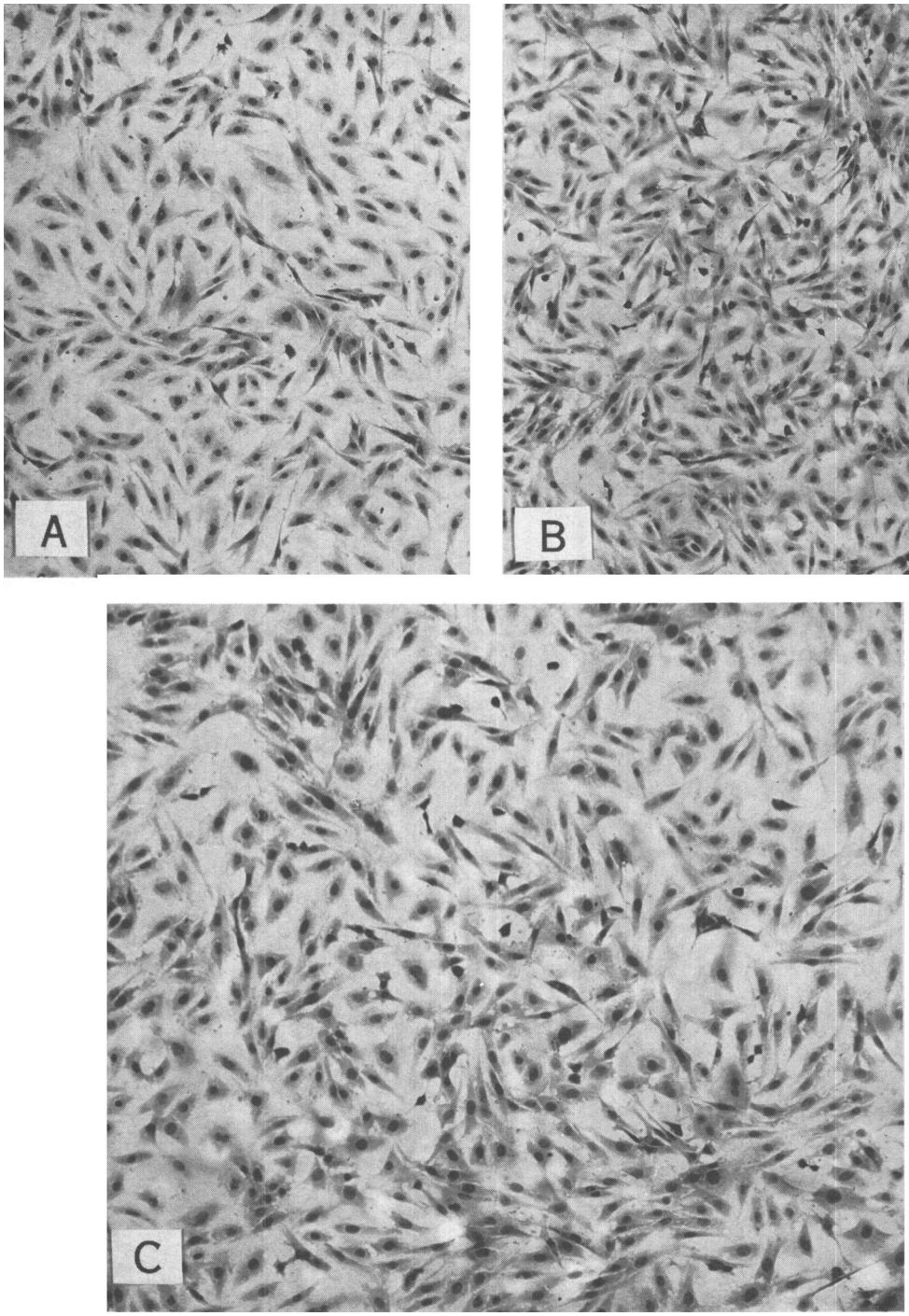


FIG. 1A. Normal rat embryo cultures at the 28th passage; Giemsa stain (×62); (B) Rauscher leukemia virus infected rat embryo cultures at the 28th passage; Giemsa stain (×62); (C) Same culture as in (×100).

infected and normal RE cells was made as previously described (8).

Complement fixation. CF tests were carried out in the microtiter technic described for tumor antigen studies (9). Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement.

Anitiviral 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) (10). 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 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 (11), and the titer was expressed as FFU per ml.

Marker rescue. A genome rescue experiment was done with a mixed culture of a virus-free hamster tumor (HT-1) and mouse embryo cells superinfected with leukemia virus, as described by Huebner *et al.* (12).

Animal inoculation. Newborn NIH mice and Fisher rats were inoculated subcutaneously with freshly trypsinized cell suspensions of RE cells.

Preparation and examination of cells by electron microscopy was made as previously described (13).

Assay of interferon. Interferon (IF) production and assay were essentially the same as previously described (14). The culture fluids to be tested for IF activity were centrifuged (1500 rpm for 20 min) and acidified (pH 2.0) for 24 hr and realkalized to pH 7.0 with concentrated HCl and NaOH, respectively. Interferon was assayed in secondary plate cultures of RETC. Test materials were preincubated with cells at 37°. After 24 hr, following the removal of culture supernatant fluids, the cells in each plate were challenged with vesicular stomatitis virus (VSV). In control culture complete destruction of cells occurred regularly within 48 hr.

Results and Discussion. Growth characteristics of RLV infected RE (RLV-RE) cells. The cultures of RLV-RE cells (S-1193c) (Fig. 1B, C) consisted of a smooth monolayer of fibroblastic cells with occasional multilayered areas caused by local retraction of the cell sheet similar to those in the uninoculated RE cultures (Fig. 1A). Observation in the light microscope revealed the presence of living cells floating in the culture medium. The cell population of uninoculated RE cell cultures (S-1193h) was composed of fibroblast-like cells (Fig. 1A). The time required to disperse the monolayer by trypsin treatment was shorter for the RLV-RE cell cultures than for the controls. No cytopathic effect and no sign of morphological transformation was observed in these cultures for over the period of 1 year. However, morphological transformation was observed in the 14th subculture of the subline (R-26) derived from the RLV-RE (S-1193c) cells (15). The R-26 subline was derived from floating living cells obtained by centrifugation (1500 rpm for 20 min) of supernatant fluid of the fifth subculture of the RLV-RE (S-1193c) cells. The neoplastic transformation observed in the R-26 cultures and the neoplastic lesions induced by these altered cells in newborn rats have been described in detail (15).

These cultures appeared to grow indefinitely *in vitro.* Subsequently, cell lines of both the RLV-RE and uninfected RE cultures were obtained. A the present time they have been carried through more than 41 cell transfer passages during a period of over 12 months.

Presence of CF antigens of murine leukemia-sarcoma virus in the RLV-RE cell line. The group-specific murine leukemia-sarcoma virus CF antigen was demonstrated in cell pack preparations of all the subcultures tested. As shown in Table I, the CF titers

TABLE I. Complement-Fixing (CF) Antigen Titers of the Two Established Cell Lines Tested Against MSV Rat Antiserum.

Passage	Cumulative no. of days in tissue culture	CF titer[a]	
		RLV infected RE cell (S-1193 c)	Uninoculated RE cell (S-1193 h)
3	34	8	0[b]
5	47	16	0
8	70	16–32	0
13	110	32	0
17	137	>16	0
18	146	>32	—[c]
20	159	>4	0
24	189	>8	0
27	214	>4	0
32	264	>4	—
41	354	>4	—

[a] CF titer = reciprocal of dilution giving 3+ to 4+ fixation of 1.8 units of complement.
[b] 0 = <4.
[c] Not done.

varied between 1:8 and 1:32. CF antigen was demonstrated in 30 to 34-day old cultures which were tested for the first time. Perhaps CF antigen could have been detected earlier in these cultures. No positive reactions were found in similar preparations of the normal, uninfected RE cell line.

CF antigen was not directly demonstratable in supernatant fluids of the RLV-RE cells. However, a CF titer of more than 1:16 was detected in the fluids of a number of different subcultures (5th, 6th, and 10th) after they had been concentrated 20 times in volume by the Diaflo ultrafiltration (8).

Presence of infectious virus in the infected RE cell line. Unfiltered, clarified, or filtered cell-free supernatant fluids of different passages of RLV-RE cell cultures were positive in the COMuL test in NIH-METC. The cultures yielded infectious virus, the yield increasing with higher passage levels (Table II). Infectious virus was also detected by an MSV genome rescue procedure described by Huebner *et al.* (12). The rescued infectious MSV was neutralized by type-specific RLV immune serum, but not by GLV immune serum.

Comparative sensitivity of mouse and rat embryo cell cultures for detection of infectious virus from the RLV infected cell line.

TABLE II. CF Antigen Induction Titers of Supernatant Fluids of RLV Infected Rat Embryo Cells.

Passage	Supernatant	Infectivity titer[a]
2	Unfiltered	3.4
	Clarified	
3	Unfiltered	3.4
	Fltered, cell-free	4.4
8	Unfiltered	5.4
13	Filtered, cell-free	4.4
23	Unfiltered	>3.4
25	Unfiltered	5.4

[a] Infectivity titers were determined by CF induction assays in disposable 60-mm Falcon plastic petri dishes of secondary NIH-METC (3). CF induction titer ($\log_{10}$ TCD_{50}/ml).

Comparative sensitivity for the detection of infectious virus in mouse and rat embryo cells inoculated with supernatants of RLV-RE cells were examined.

Clarified supernatant fluid from RLV-RE cells in their third subculture was inoculated into two sets of 1-day-old monolayers prepared from primary NIH-METC and normal cell line of RE (6th passage). Fourteen days after inoculation, the first set of cells was harvested for CF testing. The second set

was subdivided weekly for further subcultures and tested for CF antigens in each subculture.

The results indicate that the group-specific CF antigen was induced in the NIH-METC and the continuous RE cell line within 2 weeks after exposure to the supernatant fluids of the RLV-RE cultures (Table III).

Electron microscope observation. The RLV-RE cell line contained numerous virus particles that were readily seen in the electron microscope to be principally in the intercellular spaces (Fig. 2A). The particles were approximately 100 mμ in diameter, and demonstrated general morphological features comparable with those of the murine-leukemia C-type particles described by other investigators (16, 17). An occasional budding form was seen (Fig. 2B).

Induction of leukemogenic diseases in animals by RLV-RE cells. When fresh cell suspension from the third subculture and supernatant fluids from the eighth subculture of the RLV-RE cell line were inoculated into newborn NIH Swiss mice and Fischer rats, only the mice developed leukemogenic diseases indistinguishable from the original diseases described by Rauscher (18). Control animals inoculated with normal RE cells suspension were free from the diseases.

Interferon production. Sinkovics and Howe (1) described an interferon-like substance in leukemic mouse spleen cultures. On the other hand, no evidence of IF in a mouse cell line infected with RLV was reported by Peries *et al.* (4) or Sarma *et al.* (19). Therefore, we examined IF production in RLV-RE cells. The supernatant fluids from three cultures of RLV-RE cells at the 20th, 21st, and 32nd subcultures were tested for the presence of IF. No delay in the CPE of VSV in plates pretreated with these supernatant fluids was noted as compared with the plates inoculated with culture fluids from the control lines. To test for direct viral interference, subcultures from 20th and 32nd passages of RLV-RE and RE line were challenged with VSV in serial 10-fold dilutions. With 100 TCD_{50} of virus/ cell, extensive CPE was observed in both RLV-RE and RE monolayers within 48 hr. The data suggested that, under the present testing conditions, detectable IF was not produced in RLV-RE cells.

The findings of this study suggest that RE cells, as well as NIH-METC could be used for cultivation of RLV. 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 RE cultures. Thus, viral replication took place constantly in RE cells *in vitro* during a total period of more than 12 months.

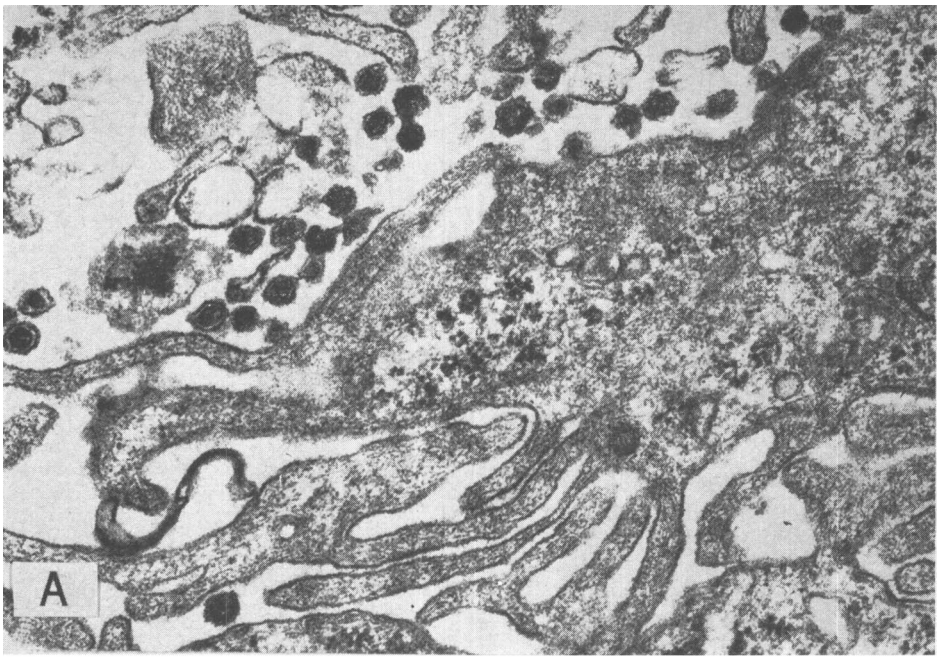
Further comparative studies of CF antigen production and virus yields, and assay of RLV and other murine leukemia and sarcoma viruses in COMuL tests, indicated that RE cells appeared to be a useful *in vitro* system for propagation and assay of tissue culture

TABLE III. Comparative Sensitivity of Rat and Mouse Embryo Cells for Detection of Infectious Virus from Supernatant Fluid[a] of RLV-Infected Rat Embryo Cells.

Passage	Cumulative no. of days from initiation of exp.	CF titer[b] vs MSV rat serum pool no. 6			
		Rat embryo cells		Mouse embryo cells	
		Uninoculated	Inoculated	Uninoculated	Inoculated
1	14	0[c]	8	0	8
2	21	0	8	0	16
3	28	0	8	0	8
4	35	0	>16	0	8
5	42	0	16	0	8

[a] Clarified supernatant fluid of RLV infected rat embryo cells (passage 3).
[b] CF titer = reciprocal of dilution giving 3+ to 4+ fixation of 1.8 units of complement.
[c] 0 = <4.

grown murine leukemia and sarcoma viruses (20). Complement-fixing antigen titers and yields of virus produced in RE cells are similar to those obtained in NIH-METC, the most sensitive host cell system thus far reported (3). Ting (21) observed that M-MSV induced morphological transformation of RE cells *in vitro*, and infectious viruses were found in the culture media of the transformed cultures. Continuous viral replication



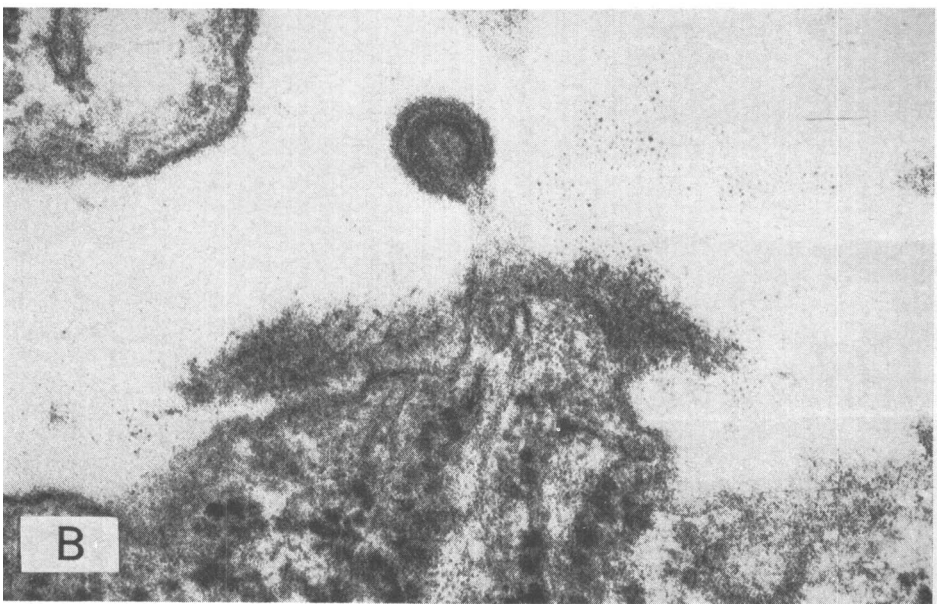


FIG. 2A. Typical C-type particles in the intracellular spaces (×48,600); (B) A budding C-type particle is seen (×115,500).

and malignant cellular transformation by Finkel's osteosarcoma virus has recently been reported in RE cells (22).

Summary. The long-term *in vitro* cultivation of Rauscher leukemia virus (RLV) in rat embryo (RE) cells is reported. RE 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 12 months. 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. Interferon production was not demonstrated in infected cultures. The data indicate that RE cells could be used for cultivation of RLV in a carrier state.

We wish to thank Mr. H. C. Turner, National Institutes of Health, for supervising the complement-fixing tests, and Mrs. Betty Creasy for her excellent technical assistance.

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Received March 31, 1970. P.S.E.B.M., 1970, Vol. 135.