Propagation of Rauscher Leukemia Virus to Human Lymphoblastoid Cells by *in Vivo* Diffusion Chamber Cultivation (37427)

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(Introduced by R. J. Huebner)

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It is known that some animal sarcoma viruses can cross species barriers, causing tumors even in monkeys (1–3). Furthermore, leukemia (4–10) and sarcoma (7, 8, 11–13) viruses of murine or feline origin have been shown to infect human cell cultures. In a course of studies on the interactions between human cultured leukocytes and murine leukemia virus, we have demonstrated that Epstein–Barr virus (EBV)-bearing lymphoblastoid cells can be co-infected with Rauscher leukemia virus (RLV) *in vitro* (14). The present paper describes propagation of RLV to human lymphoblastoid cells by temporarily implanting in diffusion chambers within the abdominal cavity of Rauscher leukemic mice.

Materials and Methods. A clonal subline of lymphoblastoid cell line (OUMS-6C1) established from lymph node of a patient with Hodgkin's disease was used. The derivation and morphology of this suspension culture line has been described elsewhere (15).

Cells were maintained in medium RPMI 1640 with 20% fetal calf serum and antibiotics. Cultures were incubated at 37° in a 5% CO_2 atmosphere and serially passaged every 5–7 days.

RLV used was obtained as 10% leukemic spleen extract (HL-67-1117H) from Dr. F. J. Rauscher of the National Cancer Institute. Young adult BALB/c mice were inoculated with 0.1 ml of the virus preparation prior to diffusion chamber implantation.

By a slight modification of the method of Algire *et al.* (16), diffusion chambers were constructed of Plexiglass rings (14 mm o.d., 10 mm i.d., and 2 mm thick) and Millipore membranes (0.14 mm thick and 0.45 μm average porosity). The membranes were sealed to the rings with MF cement. The constructed chambers having the capacity of 0.157 ml were sterilized in ethylene oxide vapor. OUMS-6C1 cells from the 80th passage after 696 days of continuous cultivation were centrifuged at 1000 rpm for 5 min and resuspended in fresh medium so as to provide approximately 4×10^5 cells per chamber. The cells were placed into the chambers through a 0.59 mm hole of the ring and the hole was sealed with Nylon thread and MF cement. The cell-loaded chambers were immediately inserted through a small midventral incision within the peritoneal cavity of BALB/c mice that had developed Rauscher leukemia by inoculation of RLV 19 days previously. The chambers were removed from the peritoneal cavity one and two weeks later and transferred into 35 mm Petri dishes containing 3 ml of fresh medium. The membranes were ruptured with forceps and scissors so as to allow free release of cells out of the chambers. Until the cells began to grow actively again, half of the medium was replaced twice a week.

Electron microscopy was performed by the method already described (17).

For bioassay, newborn BALB/c mice less than 48 hr old were inoculated ip with 0.2 ml of culture materials either as mixtures of supernatant culture fluids and cells or as centrifuged (3000 rpm for 20 min) culture fluids.

Results. In the Petri dishes containing diffusion chambers removed from the mouse abdominal cavity, two types of cells were observed; free-floating cells which appeared predominantly in the vicinity of the chambers and elongated or polygonal cells which were attached to the glass surface. These two populations persisted for 1–2 months before the suspended cells began to proliferate and it took another month for these cells to be subculturable. In the meantime, the attached cells were gradually decreased in number and after a few cell transfers, they were no longer observable. The mouse-passaged cultures thus became indistinguishable from the parent cultures in morphology and growth behavior. The two lines deriving from recultivation of cells that were kept for one and two weeks within the abdominal cavity of Rauscher leukemic mice were designated as OUMS-6C1-R1 and OUMS-6C1-R2, respectively. Chromosome analysis performed several months after recultivation showed both cell lines to be of human origin.

Serial electron microscopic observations were carried out on cells from these two lines; OUMS-6C1-R1 cells were examined at 164, 247, and 327 days and OUMS-6C1-R2 cells at 118, 163, 240, and 320 days after recultivation. In every sample from each line, abundant C type particles were seen extracellularly (Fig. 1) and within the cytoplasmic vacuoles. Virus particles in the process of budding from the plasma membranes were also observed. In addition, both lines revealed, although rare, the presence of EBV particles (Fig. 2) along with C type particles. Ultrastructurally, RLV-infected cells were similar to control uninfected cells that were positive only for EBV.

FIG. 1. OUMS-6C1-R2 cell line showing intercellular C type particles at 240 days after recultivation. ×35,000.

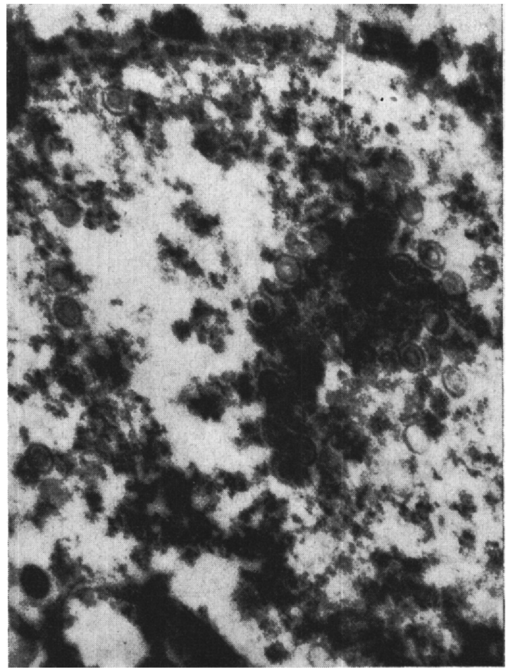

FIG. 2. OUMS-6C1-R2 cell line showing intranuclear EBV particles at 118 days after recultivation. ×35,000.

To examine the identity of C type viral antigens in OUMS-6C1-R1 and OUMS-6C1-R2 cell lines, acetone-fixed cell smears were periodically prepared from each line and stained by the indirect immunofluorescence technique with anti-RLV goat serum and anti-murine gs-1 rat serum. Both cell lines were shown to contain immunofluorescence-positive cells reacting in approximately equal percentages with either antiserum.

The leukemogenicity of C type particles continuously produced by human lymphoblastoid cells was tested by inoculation into newborn BALB/c mice. The OUMS-6C1-R1 culture materials harvested later than 6 months after recultivation were all negative (Table I). On the other hand, similar bioassays of the OUMS-6C1-R2 culture materials performed within 3 months after recultivation gave rise to Rauscher leukemia in 8 of 14 mice inoculated but subsequent

TABLE I. Bioassays of OUMS-6C1-R1 Culture Materials in Newborn BALB/c Mice.

Days after recultivation	Culture materials	No. of leuk./no. of inoc.
189	C + SF[a]	0/6
189	CF[b]	0/4
193	C + SF	0/10
193	CF	0/7

[a] Approximately 1×10^5 cells suspended in 0.2 ml of supernatant culture fluids.
[b] 0.2 ml of centrifuged culture fluids.

bioassays were consistently negative during the observation period of 2 months (Table II).

Mice that did not develop Rauscher leukemia at 2 months after inoculation of human cell-passaged C type virus were challenged with ip inoculation of 0.1 ml of 10-fold dilution of 10% Rauscher leukemic spleen extract. One month later, the challenged as well as control mice inoculated with challenge virus only were sacrificed and mice with spleens weighing more than 0.4 g were considered positive for the development of Rauscher leukemia. Of 27 mice inoculated with OUMS-6C1-R1 C type particles and subsequently challenged with spleen-derived RLV, 5 (19%) developed Rauscher leukemia (Table III). The leukemia developed in 7 (30%) of 23 mice similarly treated with OUMS-6C1-R2C type particles and challenge virus. In contrast, 14 (70%) of 20 control mice inoculated with challenge virus only manifested Rauscher leukemia.

Discussion. Prior to the initiation of the present experiment, we tested reculturability of OUMS-6C1 cells after implantation in diffusion chambers within the peritoneal cavity of normal BALB/c mice and it was found that continuously growing cell cultures can be reestablished from cells that were kept in the heterologous hosts for up to 4 weeks (unpublished data). It was anticipated, in the present experiment, by similarly implanting these cells in diffusion chambers within the abdominal cavity of Rauscher leukemic BALB/c mice, spontaneous propagation of RLV from viremic mice to implanted cells would take place through cell-impermeable Millipore membranes. The result obtained proved this to be the case; the human lymphoblastoid cells became chronically infected with RLV after one and two weeks' growth in diffusion chambers within the heterologous peritoneal cavity. The EBV carrier state of these cultures was not apparently altered by this heterograft procedure nor by super-infection of RLV. This was indicated by the coexistence of both C type and EBV particles in the same electron microscopic field. We have also observed the same dual infection in a human lymphoblastoid cell line inoculated with RLV *in vitro* (14). It remains to be seen if there are any interactions between these two RNA and DNA viruses in human leukocyte cell lines.

TABLE II. Bioassays of OUMS-6C1-R2 Culture Materials in Newborn BALB/c Mice.

Days after recultivation	Culture materials	No. of leuk./no. of inoc.
63	C + SF[a]	7/7
90	CF[b]	1/7
211	C + SF	0/8
211	CF	0/3
216	C + SF	0/4
216	CF	0/8

[a] Approximately 1×10^5 cells suspended in 0.2 ml of supernatant culture fluids.
[b] 0.2 ml of centrifuged culture fluids.

TABLE III. Effect of Immunization of BALB/c Mice with Human Cell-Passaged RLV Against Challenge with Spleen-Derived RLV.

Virus treatment	No. of leuk./ no. of inoc.	Incidence of leukemia (%)
OUMS-6C1-R1 + Challenge	5/27[a]	19
OUMS-6C1-R2 + Challenge	7/23[a]	30
Challenge only	14/20[b]	70

[a] Mice that did not develop Rauscher leukemia at 2 mo in bioassays shown in Tables I and II were challenged with 0.1 ml of 10-fold dilution of 10% Rauscher leukemic spleen extract.
[b] 2-mo-old control mice were used.

Bioassays of RLV-infected culture materials were mostly negative except for mice inoculated with culture materials harvested within 3 months of recultivation. The reason for this is unclear; in the early stage of recultivation, however, scattered fibroblastlike cells of presumed mouse origin were seen on the glass surface and these cells, being carried into culture together with the diffusion chambers, possibly liberated leukemogenic RLV until they were eventually outgrown by human lymphoblastoid cells that released only nonleukemogenic C type virus. Alternatively, it may be explained by the attenuation of leukemogenicity of RLV during long-term propagation *in vitro*, as has been reported in RLV-infected mouse cell cultures (18, 19). It cannot be inferred which of these two mechanisms is right for the present experiment, because both are known to occur; the leukemogenicity of RLV can be either lost (4, 6, 20) or reduced (21) when it is propagated in human embryo cell cultures. The fact that some protection was afforded in mice inoculated with human cell-passaged RLV against challenge infection with spleen-derived RLV indicates that there are antigenic similarities between these two viruses. Such protective immunization was also achieved with attenuated RLV grown in murine cells (22, 23).

The diffusion chamber technique herein described may be useful for isolating or rescuing oncogenic viruses that are difficult to propagate in tissue culture by the ordinary method of *in vitro* infection. The advantage of this technique appears to be in the point that cells are exposed to virus continually under more physiologic circumstances. The ease with which established human leukocyte cultures can be productively infected with murine leukemia virus strengthen the need for further studies on the possible relationship of animal leukemia viruses to human leukemia.

Summary. Diffusion chambers containing EBV-bearing human lymphoblastoid cells were placed temporarily within the peritoneal cavity of BALB/c mice with Rauscher leukemia. Reestablished human leukocyte cultures became dually infected with C type and EBV particles and have continuously produced abundant C type particles. These virus particles were nonleukemogenic when inoculated into BALB/c mice but afforded some protection against challenge infection with spleen-derived leukemogenic RLV.

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