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Continuous Propagation of Radiation Leukemia Virus on a C57BL Mouse-Embryo Fibroblast Line, with Attenuation of Leukemogenic Activity

(murine leukemia virus/thymic lymphomas/5-bromodeoxyuridine)

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ABSTRACT **The radiation leukemia virus (RadLV), a murine leukemia virus derived from thymic lymphomas induced by x-irradiation in strain C57BL/Ka mice, has been successfully propagated in sustained high titer *in vitro* in a newly established line, BL-5, of C57BL/Ka mouse-embryo fibroblasts. In addition, the production of endogenous virus, presumed to be RadLV, has been induced and sustained through multiple serial passages after treatment of BL-5 cell cultures with 5-bromodeoxyuridine. The chronically RadLV-infected subline, designated BL-5 (RadLV), sheds virus into the supernatant culture fluids that is biologically active *in vitro* in the XC cell plaque assay, in interference assays for focus-formation by murine sarcoma virus, and in the intracellular induction of group-specific antigens detectable by immunofluorescence, but is apparently devoid of leukemogenic activity after intrathymic inoculation into neonatal or immunosuppressed C57BL/Ka mice. Although BL-5 cells exhibited morphological alterations suggestive of transformation *in vitro* and gave rise to fibrosarcomatous ascites tumors after intraperitoneal inoculation with C57BL/Ka mice, the chronically infected BL-5(RadLV) cells remained normal in morphology and failed to yield fibrosarcomas *in vivo*.**

The numerous murine leukemia viruses (MuLV) reported to date may be divided into two broad categories: a "virulent" group and a "temperate" group (1). The virulent agents, of which the Friend (2), Moloney (3), and Rauscher (4) viruses are prototypes, were extracted initially from long-transplanted epithelial or sarcomatous neoplasms, rather than from the leukemias and lymphomas to which they give rise on inoculation into susceptible strains of mice. They are highly antigenic, induce leukemias after a very short latent period, and are not known to be transmitted vertically in any strain of mouse, nor is there evidence that they have ever been responsible for "spontaneous" leukemogenesis in the natural state.

In contrast, the temperate viruses were initially extracted from lymphatic leukemias or lymphomas developing spontaneously (5) or after exposure to ionizing radiation (6–8) or chemical agents (9) and are vertically transmitted in their strains of origin. Before their prolonged serial passage, they induce lymphomas and lymphatic leukemias with a relatively long latent period after neonatal inoculation into susceptible strains of mice and tend to have a restricted host range (8).

Virulent viruses were rather readily adapted to continuous replication in leukemic cells and in established mouse cell lines *in vitro* about a decade ago (10–14). The large quantities of virus particles that could be harvested from the supernatant fluids of such cultures greatly facilitated their purification, biochemical characterization, and the development of various quantitative *in vitro* assays. In contrast, it has proven appreciably more difficult to cultivate the temperate murine viruses continuously *in vitro* in nonleukemic cells of the strain of origin. Some success with short-term propagation of the Gross virus in mouse cells was reported some years ago (15, 16), and its continuous replication *in vitro* has been achieved in mouse and rat lymphoma cells (17–19) and in rat thymic cell cultures (20). The B/T-L virus, originally extracted from a presumably "spontaneous" lymphoma of an old strain BALB/c mouse (21), grows well in two morphologically distinct cell lines, established from infant BALB/c thymus tissue initially infected with the virus (22).

The radiation leukemia virus (RadLV), which is regularly extractable from the thymic lymphomas induced in strain C57BL mice by wholebody x-irradiation, and which is serially propagated *in vivo* in the identical thymic lymphomas that it induces after neonatal inoculation into mice of this and related strains (6, 8), has been particularly difficult to adapt to *in vitro* cultivation. Release of a leukemogenic C-type virus, which may well have been RadLV, occurred when normal mammary and embryo cell cultures from strain C57BL/Haag mice were infected with mammary tumor virus (23). Fischinger and O'Connor (24) reported that RadLV derived by *in vivo* passage from a preparation supplied by this laboratory could not be propagated on C57BL or Swiss mouse embryo cell cultures, but the virus was later successfully cultivated in BALB/c 3T3 and S + L-cells by Nomura *et al.* (25). However, the highest titers they obtained after several passages were only about 2×10^3 focus-inducing units/ml, leading these investigators to postulate that RadLV "may consist of several populations of virus of varying replicative potential," the predominant feature being defective growth in normal cells.

We report here the successful continuous propagation of tumor-derived RadLV in sustained high titer, as well as the induction of replication of the endogenous virus, in established mouse-embryo fibroblast cell lines of its strain of origin, C57BL/Ka. Some of the altered properties of the infected cells and of the *in vitro*-derived virus are described. RadLV harvested from the supernatant tissue culture fluids of these cell lines has been used successfully for the development of new *in vitro* assays for this virus, and for studies of the viral coat proteins by polyacrylamide gel electrophoresis and lactoperoxidase radioiodination (26).

ESTABLISHMENT OF THE BL-5 CELL LINE

Primary fibroblast cultures were prepared from C57BL/Ka embryos at 14–16 days of gestation; pooled embryos taken from one pregnant female were used for each culture. The suspended cells were seeded at a concentration of 1×10^6 per Falcon culture flask (75 cm²) in a growth medium consisting of Eagle's minimal essential medium (MEM) with 10% heat-

Abbreviations: MuLV, murine leukemia virus; MSV, murine sarcoma virus; RadLV, radiation leukemia virus.

inactivated fetal-bovine serum and antibiotics added (100 units of penicillin/ml and 50 μg of streptomycin/ml). Cells were subcultured when they reached confluence (at 4 days for the first two passages and at weekly or longer intervals thereafter), until the culture became established and grew well enough to reach confluence in about 5 days.

Cultures were started from several embryo pools; some were lost during adaptation to *in vitro* existence; others became established as permanent lines. The line that has been used in the present study originated from the fifth embryo pool used; hence the designation BL-5.

The morphological appearance of BL-5 cells is fibroblastic, and their growth pattern exhibits contact inhibition only at high cell density. From time to time, temporary alterations, such as decrease in the size of cells and disordered orientation, have been observed. Several clones were isolated from the original culture, but these also showed a tendency to morphological instability, which first became apparent after a period of several weeks in culture and gradually increased thereafter. For this reason, fresh cultures are started periodically from a frozen stock of early-passage cells. In addition to the development of morphological instability, the BL-5 line has also acquired the ability to induce fibrosarcomas at the site of injection, as described below.

BL-5 cells have not spontaneously released a leukemogenic virus, and cell-free extracts prepared from them and inoculated into newborn or infant C57BL/Ka mice have induced no lymphomas. No plaques appeared when BL-5 cells were used as infective centers in the XC cell assay (27), and immunofluorescence tests with potent rat antisera to MuLV group-specific antigen have been negative. This is consistent with the report by Hall *et al.* (28) that cell lines derived from two C57BL/KaLw mouse embryo cell pools remained virus-free by electron microscopy and complement fixation after 412 and 598 days, respectively, *in vitro*. However, the presence of the viral genome in BL-5 cells was demonstrable by "activation" with 300 μg/ml of 5-bromodeoxyuridine (29), to which the cells were exposed for 24 hr, leading to the production of virus, presumably RadLV, which was detectable by the XC cell assay. The rate of spread of the endogenous virus and/or of the replication of virus-producing cells in serial passages of the culture was relatively slow. It required 9 to 10 passages to reach a peak of virus titer, at which time about 30% of the cells were infected (Fig. 1). There was then a decrease to 4.5% at the twelfth passage, at which time the cells were frozen and stored for future use.

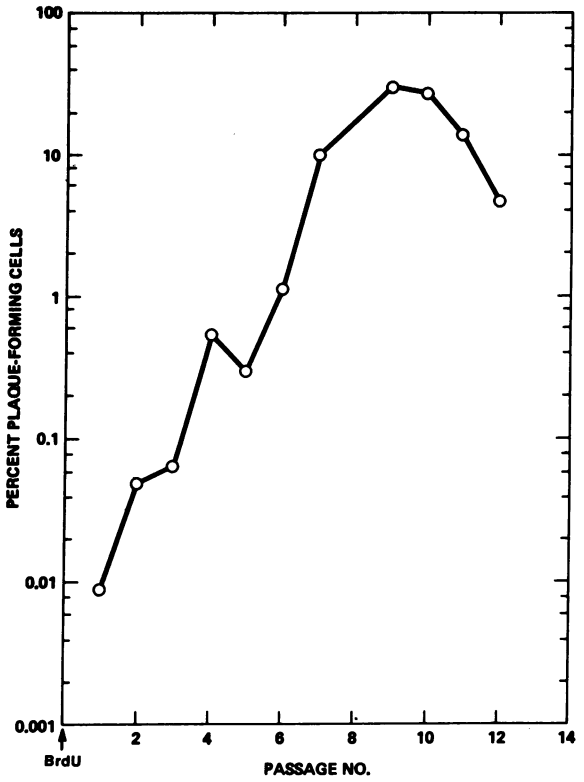

FIG. 1. Induction in BL-5 cells incubated with 5-bromodeoxyuridine, and successful propagation through multiple serial passages, of an endogenous virus, presumed to be RadLV, as measured by plaque-forming capacity in the XC cell assay.

ESTABLISHMENT OF THE BL-5(RadLV) VIRUS-INFECTED SUBLINE

BL-5 cells were seeded in Falcon culture flasks, at a density of 1×10^6 cells per 75-cm^2 flask. The following day, the medium was removed, and the cells were exposed for 45 min to 25 μg of DEAE-dextran per ml of MEM, at 37°. After one washing with MEM, 2 ml of a cell-free extract of C57BL/Ka lymphoma tissue containing "wild-type" RadLV was added, and the virus was allowed to adsorb for 1 hr at 37°. Complete medium was then added, and the flasks were returned to the incubator. Medium was changed every 3–4 days, and the culture was trypsinized and divided at weekly intervals.

The infected cultures revealed no evidence of a cytopathic effect. Accordingly, indirect methods had to be used to reveal the presence of replicating virus. At the second, fourth, and sixth subcultures, the RadLV-infected cells, as well as the uninfected BL-5 line, were tested for their capacity to support focus formation by the RadLV pseudotype of murine sarcoma virus, MSV(RadLV). Reduction in the number of foci formed by a murine sarcoma virus pseudotype occurs in the presence of interfering murine leukemia virus in the test culture (30, 31); the assay can thus be used to detect the presence of replicating RadLV in the infected cultures. The results of experiments with two different preparations of RadLV are shown in Table 1. It is apparent that 4 or 5 subcultures sufficed to render the infected cells completely refractory to focus formation by MSV(RadLV).

Cells from the sixth subculture were used as infective centers in the XC cell plaque assay. Aliquots containing about 10–100 infected cells were added to 2×10^5 BL-5 control cells and seeded in 5-cm Falcon culture dishes. After six days, 1×10^6 XC cells were added to each dish, and plaques were counted 3 days later. Within experimental error, there was a 1:1 correspondence between the number of cells plated and the number of resulting plaques, indicating that every cell in the RadLV-infected culture acted as an infective center. This assay has been repeated numerous times and has consistently yielded an excellent correlation between the number of cells seeded and the number of plaques formed. It appears, therefore, that infection of the culture is complete and that every cell carries replicating RadLV. This infected subline has been designated BL-5(RadLV). Since its establishment, the BL-5(RadLV) subline has maintained its original appearance with remarkable constancy, in contrast to the tendency to transformation of individual cells and of the culture as a whole in the uninfected BL-5 line.

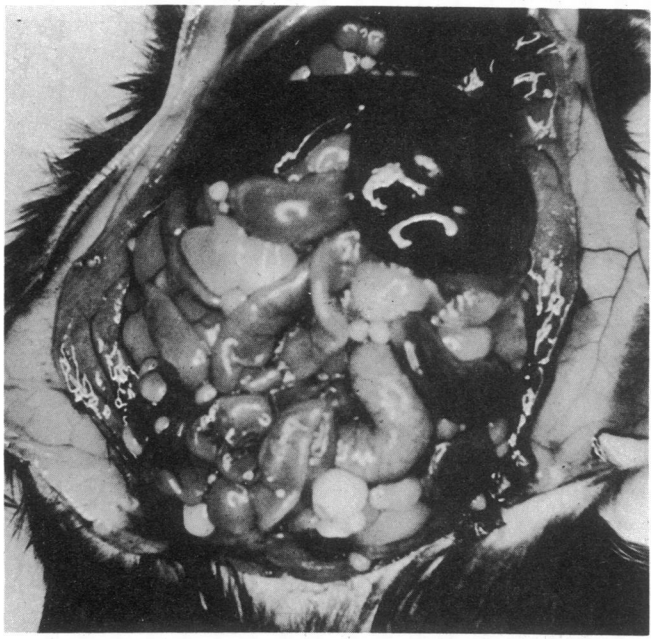

FIG. 2. Abdomen of a C57BL/Ka mouse revealing ascites and white spheres of fibrosarcomatous tissue resulting from the prior intraperitoneal inoculation of late-passage BL-5 cells.

BIOLOGICAL ACTIVITY OF BL-5(RadLV) CELLS AND OF THE VIRUS PROPAGATED *IN VITRO*

As described above, the BL-5(RadLV) cells produce plaques on cocultivation with rat XC cells to an extent indicative of complete infection of the line. On cocultivation with hamster HT-1 cells, rescue of the defective MSV genome in HT-1 cells (32) occurs readily; the resulting MSV(RadLV) pseudotype induces sarcomas on injection into neonatal C57BL/Ka mice and produces foci of transformed cells when plated on BL-5 cells or on secondary C57BL/Ka embryo fibroblasts. The BL-5(RadLV) cells also contain cytoplasmic group-specific antigen, which can be detected by immunofluorescence with rat antiserum to RadLV or to MSV(RadLV).

The virus released by BL-5(RadLV) cells into the supernatant fluid replicates in secondary cultures of C57BL/Ka mouse-embryo fibroblasts, as well as in continuous cell lines (BL-5 and BALB/c-3T3), in which it can be detected and assayed by the immunofluorescence method (A. Declève *et al.*, manuscript in preparation). Using a modification of the XC cell assay, which we have called the reverse XC cell assay (O. Niwa *et al.*, manuscript in preparation), we have consistently obtained titers of 5×10^7 to $>10^8$ plaque-forming units per ml of culture fluid, whereas tumor-derived RadLV seldom exceeds a titer of 10^5. Thus, contrary to the experience reported by others (24, 25), there is no evidence that RadLV is defective when it is grown in cells of its strain of origin.

The leukemogenicity of RadLV propagated *in vitro* has been tested by bioassay in C57BL/Ka mice. In the first experiment, 1×10^6 BL-5(RadLV) cells were injected intrathymically into 2-week-old mice; 1 week later, the recipients were exposed to a single 200-R dose of wholebody x-irradiation to potentiate viral leukemogenesis (33). The control group was injected with uninfected cells. Of 28 mice receiving virus-infected cells, 11 (39%) developed lymphomas, whereas none of the 31 control mice that received uninfected BL-5 cells became leukemic. In this experiment, the BL-5(RadLV) cells were used less than 2 months after establishment of the infected subline. When the leukemogenicity of these cells was assessed in several later subpassages, with or without added irradiation of the host, negative results were consistently obtained.

The leukemogenicity of the free virus released into the culture fluid by BL-5(RadLV) cells has also been tested by intrathymic inoculation of infant mice, or of weanlings immunosuppressed by injection of an adrenal corticosteroid, Synalar (Syntex Laboratories). No lymphomas have developed in any of the 94 injected animals. We have previously observed that C57BL/Ka bone marrow cells are highly susceptible to infection and neoplastic transformation by tumor-derived RadLV. For this reason, bone-marrow cell suspensions from C57BL/Ka mice were incubated in a second experiment with virus-containing culture fluids fron BL-5(RadLV) cultures, then washed and injected intrathymically into irradiated adult recipients. Of 40 injected mice, only one has become leukemic to date (7–8 months). Experiments are currently in progress to assess the leukemogenic potential of concentrated and purified culture-derived virus.

We had observed earlier (35) that injection into C57BL/Ka mice of murine leukemia viruses with a low leukemogenic potential for this strain renders the host animals less susceptible to subsequent lymphoma induction by RadLV. A series of experiments has been performed to test the possibility that the same protective effect might result from inoculation of culture-attenuated RadLV. These experiments, which will be reported fully in a separate publication, demonstrate conclusively that BL-5(RadLV) cells can immunize C57BL/Ka mice against virus-induced, cell-bound antigens and thus strongly inhibit the induction of lymphomas by either "wild-type" RadLV or x-irradiation. Prior inoculation of C57BL/Ka mice with BL-5(RadLV) cells, as well as adoptive transfer of their lymphoid cells to secondary hosts, also reduces significantly the incidence of "takes" of isogenic lymphoma cells.

TRANSFORMATION OF THE BL-5 LINE

In the course of the experiments described in the preceding section, it was observed that among the control C57BL/Ka mice that had received several intraperitoneal injections of BL-5 cells from late passages (i.e., over 6 months after the establishment of the line), several developed ascites 5–6 months after injection. At autopsy, the peritoneal cavity appeared filled with tiny (1–3 mm) white spheres of tumor tissue, most of which were floating free in the ascites fluid, though some were attached to tissue (Fig. 2). Histologically, these spheres are composed of masses of fibrosarcoma cells, presumably representing an *in vivo* suspension culture of transformed BL-5 cells. On subcutaneous transplantation, the cells grew slowly and killed the host C57BL/Ka animals after several weeks or even months; growth remained localized to the site of injection. No such ascites fibrosarcomas have occurred in any of the groups injected with BL-5(RadLV) cells.

The tumor cells grow readily *in vitro* in a "network" pattern, and their ability to produce tumors *in vivo* persists. Mice in which RadLV replication was induced *in vivo* by x-irradiation (36, 37), after injection of BL-5 cells, yielded ascites tumor cells of similar morphology that had become infected *in vivo* with RadLV and that released the virus into the culture fluids as actively as BL-5(RadLV) cells. Extracts from these cells have produced no lymphomas to date in C57BL/Ka mice, nor has it been possible to detect a sarcomagenic virus in extracts

TABLE 1. *Reduction of MSV (RadLV) focus formation on BL-5(RadLV) versus BL-5 cells*

	Subculture no.	No. of foci on BL-5 cells	No. of foci on BL-5(RadLV) cells
Expt. 1	2	87	50
	4	26	0
	6	144	0
Expt. 2	2	38	23
	4	47	2
	6	126	0

of the tumor cells, whether or not they carry RadLV, either by bioassay or by tests for focus formation on C57BL/Ka mouse-embryo fibroblast cultures. If an endogenous C57BL/-Ka MSV genome is indeed present in these tumor cells, RadLV is apparently incapable of acting as a "helper" for its replication and release, despite the fact that the helper function of RadLV for MSV in other systems is well-established (24, 31, 38).

DISCUSSION

The development of an established line of C57BL/Ka mouse embryo fibroblasts has permitted the continuous propagation of RadLV at high titer *in vitro*, and thus greatly facilitated the purification, assay, and partial biochemical characterization of this virus. The culture-derived virus has undergone virtually complete attenuation of its leukemogenicity, as measured by bioassay in young C57BL/Ka mice, even with the aid of immunosuppression. Attenuation after sustained *in vitro* propagation on fibroblastic cell lines has previously been reported for the Friend (39), Moloney (11, 12), and Rauscher (40) viruses. However, the B/T-L virus, propagated in two cell lines established from infant BALB/c thymus tissue (22), and the Gross virus, propagated in rat lymphoma or rat thymus cells (17, 20), were still capable of inducing a high lymphoma incidence after inoculation into susceptible suckling mice. This result suggests the testable hypothesis that the cell type may be the relevant determinant, with thymic or lymphoma cells favoring retention, and fibroblastic cells favoring loss, of leukemogenicity.

The ascites fibrosarcomas induced by late pasages of BL-5 cells in C57BL/Ka mice were an unexpected byproduct of these investigations. The free-floating clones of tumor cells in the ascites fluid were apparently capable of becoming infected with RadLV induced *in vivo* by x-irradiation and supported the continued propagation of the virus after return to an *in vitro* environment. However, no evidence of the presence of MSV genome in these tumor cells has been elicited to date, even in cultures superinfected with RadLV, which has established helper activity in other MSV systems (24, 31, 38). It is particularly intriguing that BL-5 cells infected with RadLV *in vitro* have not yielded these ascites fibrosarcomas after inoculation *in vivo*, nor have the morphological variations suggestive of transformation been observed in BL-5 cells infected with RadLV *in vitro*, suggesting that superinfection of these cell cultures with this MuLV may inhibit their neoplastic transformation.

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