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Transformation of DBA/2 mouse fetal liver cells infected *in vitro* by the anemic strain of Friend leukemia virus

(transformation *in vitro*/erythropoietin/spleen focus-forming virus)

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ABSTRACT **Fetal liver cells of DBA/2 mice were infected with the anemic strain of Friend leukemia virus (FLV-A), which has no spleen focus-forming virus (SFFV) activity. The infected cells were grown in medium with or without erythropoietin. Transformed lines were isolated only from the infected cultures that had been treated with erythropoietin at the time of their initiation. The properties of three permanent cell lines in serial passage for over 2 years are described. Each has an aneuploid karyotype. Only the immature hematopoietic cells of the first line have metacentric chromosomes. They grow in suspension, as do the erythroleukemic lines derived from leukemic spleens of FLV-infected mice, and clone on agar. They produce tumors resembling reticulum cell sarcomas upon subcutaneous inoculation into syngeneic hosts. Stimulation of differentiation induced after treatment with dimethyl sulfoxide identifies the cells of the first line as being erythroid in origin. The two other lines are adherent and epithelioid in appearance. These lines may have originated from the nonhematopoietic cells present in fetal liver. No tumors were produced after the subcutaneous inoculation of 10^6 cells. All three lines synthesize virus. The virus is attenuated for leukemogenicity and has no SFFV activity. The transforming event appears to be specific, because fetal liver cells from C57BL/6 mice, which are resistant to the induction of leukemia by FLV, were not affected by the virus. Malignant transformation of erythroid cells by FLV-A *in vitro* confirms the *in vivo* findings that SFFV may not be a necessary prerequisite for the induction of erythroleukemia in susceptible hosts.**

The mechanisms involved in leukemogenesis induced by Friend leukemia virus (FLV) are not clear, primarily because some strains of the virus consist of a complex of viruses (for review, see ref. 1). The prototype virus (FLV-A) induces erythroleukemia associated with anemia, as did the original isolate (2, 3). The FLV-P strains, which induce erythroleukemia associated with polycythemia, contain a second virus, the defective spleen focus-forming virus (SFFV) (4). Both strains are comparable in regard to incubation period and mean lethal dose (LD_{50}) for the induction of the rapid, fulminating erythroleukemia in susceptible strains of adult mice (1). Their similarities, attributable to FLV, and their differences, presumed to be due to SFFV, are listed in Table 1. SFFV appears to be a recombinant between the FLV genome and either a xenotropic virus (5) or normal cell components (6, 7). The expression of SFFV results in polycythemia and in the formation of spleen foci detectable 9 days after inoculation (4). SFFV in FLV-P strains stimulates erythropoiesis in hypertransfused mice and FLV-A does not (8). The genetic control for FLV and SFFV is different, because the *FV-1* and *FV-2* genes controlling susceptibility to the viruses segregate independently (9). Finally, preliminary results obtained in a collaborative study with R. Lerner indicate that the viral gp71 envelope glycoproteins of FLV-A and FLV-P are different, but the p30 core proteins are similar.

Table 1. Comparison of effects of FLV-A and FLV-P *in vivo*

	FLV-A	FLV-P
Similarities		
Incubation period, weeks	1–2	1–2
Titer*	3.8	3.5
Pathology	Erythroleukemia	Erythroleukemia
Differences		
Hematocrit, %	29	63
Spleen foci	−	+
Defective	−	+
Stimulation of erythropoiesis	−	+
Genetic control	*FV-1*	*FV-2*

* Expressed as the reciprocal of the LD_{50}.

The studies on FLV-A described here and on FLV-P in a following report (10) were designed to answer two questions: (*i*) whether FLV could transform hematopoietic cells *in vitro*, and (*ii*) whether SFFV was essential for that event to occur. The transformation by FLV-A of mouse fetal liver cells of the virus-susceptible DBA/2 mice is described here. The criteria for transformation were altered morphology, establishment of the cells in continuous culture, growth on soft agar, and tumorigenicity in syngeneic hosts. Three permanent lines originated from the infected cultures that had also been treated with erythropoietin (EP). The undifferentiated hematopoietic cells of one line grow in suspension and are malignant. The stimulation of differentiation after treatment with dimethyl sulfoxide (Me_2SO) (11) identify them as being erythroid in origin. The cells of the two other lines are adherent and do not produce tumors at the site of inoculation. They may have originated from nonhematopoietic liver cells. The three cell lines, in continuous culture for over 2 years, synthesize virus that is attenuated for leukemogenicity.

MATERIALS AND METHODS

Virus. Stock FLV-A virus was prepared from leukemic spleens of anemic Swiss female mice (Taconic Farms, Germantown, NY), as described (2), and similar preparations were made from the spleens of normal, uninfected mice to provide control material. Aliquots were stored in ampoules at −100°C until use. The virus titer for leukemogenicity in 6- to 8-week-old Swiss mice, expressed as the reciprocal of the LD_{50}, was 3.8/0.2 ml intraperitoneally. The spleen focus-formation assays (4) were negative in BALB/c mice (kindly provided by the National

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Abbreviations: CFU-E, colony-forming unit—erythroid; Me_2SO, dimethyl sulfoxide; EP, erythropoietin; FLV, Friend leukemia virus; FLV-A, anemic strain of FLV; FLV-P, polycythemic strain of FLV; SFFV, spleen focus-forming virus.

Table 2. Effect of FLV-A on total nucleated erythrocytes and CFU-E recovered at day 5

Marbrook source	Nucleated erythrocytes	CFU-E	
		No EP	EP added
Control	100,000 ± 40,000	25 ± 10	119 ± 25
Normal spleen	60,000 ± 20,000	44 ± 12	135 ± 31
Virus	270,000 ± 30,000	107 ± 22	294 ± 65
EP	500,000 ± 50,000	297 ± 50	1354 ± 122
EP + virus	760,000 ± 200,000	601 ± 222	1966 ± 428

Uncertainties are expressed as ±SEM.

Cancer Institute Office of Resources and Logistics). The absence of SFFV in Friend murine leukemia virus 201, a clone of FLV-A that induces erythroleukemia accompanied by anemia as does the parent virus, has been reported by Troxler and Scolnick (12).

Cell Cultures. Fetal liver cells were obtained from DBA/2J and C57BL/6 mice (The Jackson Laboratory). Timed pregnancies were followed in order to obtain fetuses at 12.5 to 13.5 days of gestation, because at this time the liver is largely an erythropoietic organ. The fetal livers were dissected out under sterile conditions and single cell suspensions were prepared by mincing the liver tissue and passing the material through progressively smaller bore needles until the cells passed easily through a 25-gauge needle. The Marbrook diffusion chamber technique was used to grow the cells in suspension (13). Three million viable cells were suspended in alpha medium (Flow Laboratories, Rockville, MD) containing 20% fetal calf serum, 0.1 mM α-thioglycerol, and antibiotics. To selected cultures, 50 μl of 1:20 dilution of either normal spleen filtrate or virus was added, with or without 0.5 unit of EP (sheep plasma, step III, Connaught Labs, Toronto, Canada). Untreated cultures were kept as controls. All cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air.

After 5 days, the cells were transferred from the Marbrook cultures to T-25 flasks (Lux) with 10 ml of medium from the outer Marbrook chamber and fresh EP was added at 0.5 unit/ml. These cultures were incubated for 1 week, after which fresh medium was added without any further addition of EP. The cultures were then fed at weekly intervals. The number of viable cells in replicate flasks was determined by the trypan blue exclusion test and the number of erythroid colony-forming cells was assayed by using a modification of the technique of Iscove *et al.* (14, 15). Cells were plated with or without 0.5 unit of EP in 0.8% methyl cellulose with alpha medium, α-thioglycerol, and 30% fetal calf serum.

Assays on Transformed Cell Lines. Reverse transcriptase (RNA-dependent DNA polymerase) activity of the virions in the supernatant culture fluid was determined as described (16). The filtered (0.45 μm, Millipore) culture fluid was assayed for leukemogenicity in DBA/2 and Swiss mice (2) and for spleen focus formation in BALB/c mice (4). The cells were prepared for electron microscope examination as described (17). Tumorigenicity was determined by inoculating 10^6 cells subcutaneously into syngeneic DBA/2 mice (18). Stimulation of erythroid differentiation, after growth in medium containing 2% (vol/vol) Me_2SO (11), was assayed by determining the number of benzidine-positive cells (19, 20).

RESULTS

Effect of Virus on Erythroid Colony-Forming Cells. Preliminary experiments indicated that DBA/2 fetal liver cells did not survive well in suspension culture in the absence of EP. In cultures containing 0.5 unit of EP, there were $18 \pm 2 \times 10^5$ nucleated erythrocytes present at 48 hr, compared to a mean of $5 \pm 1 \times 10^5$ in control cultures containing no EP. By day 5, none of the cells in the control, unstimulated cultures incorporated tritiated thymidine, whereas there was a 3% nucleated erythrocyte labeling index in the EP-containing cultures. The effects of FLV-A and EP, alone or in combination, on the total number of nucleated red blood cells recovered from each of the Marbrook cultures on day 5 are shown in Table 2, as are the number of EP-independent and EP-dependent erythroid colony-forming units (CFU-E). There was little difference between untreated control and normal spleen treated cultures. In EP-treated cultures, erythrocyte proliferation was stimulated and the number of EP-independent and EP-dependent CFU-E was increased. Smaller but significant increases were also observed in cultures treated with virus alone. When virus was added together with EP, stimulation was further enhanced. This effect was not observed in virus-infected cultures of fetal liver of C57BL mice, which are highly resistant to the induction of leukemia.

Origin of Cell Lines. On the 5th day after initiation of the cultures, they were transferred from Marbrook into T-flasks. EP was added for the second time to those that had been treated with this compound. Within 48 hr after transfer, almost all of the viable cells were adherent and the EP-treated cells exhibited a pattern of growth in which foci consisting of a central core of tightly packed round cells with a peripheral skirt of elongated epithelial-like cells were observed. The number of such foci was increased in the cultures containing virus, and the foci were particularly prominent in those flasks containing both EP and virus.

Round cells that detached from the foci into the medium did not initially survive when transferred. After several weeks, however, those detaching from the DBA/2 cultures exposed to both virus and EP began to proliferate rapidly on transfer. The three permanent cell lines originating from the detached cells have been in serial passage for 2½ years. None of the other DBA/2 cultures, nor any of the C57BL fetal liver cultures, whether or not they had been treated, survived beyond the third month.

Table 3. Properties of fetal liver cells transformed by FLV-A *in vitro*

Line	Doubling time, hr	Saturation density, days	Karyotype		Malignancy, mice with tumors/mice inoculated	Benzidine-positive, %	
			Modal no. chromosomes	No. metacentric chromosomes		Control	+Me_2SO
G-1	16	4	70	6–15	5/5	14	93
G-2	23	4	58	None	0/5	—	—
G-3	21	4	121	None	0/5	—	—

Doubling time: Cells seeded at 10^5/ml in 60-mm Falcon dishes were counted daily for 5 days. The proportion of living cells was determined by the trypan blue exclusion test. Karyotype: Cells were arrested in metaphase by treatment with Colcemid (10 μM). The chromosomes stained with Giemsa were counted. Erythroid stimulation: At time of seeding (10^5/ml), 2% Me_2SO was added to each culture. The number of benzidine-positive cells was determined after 5 days of treatment.

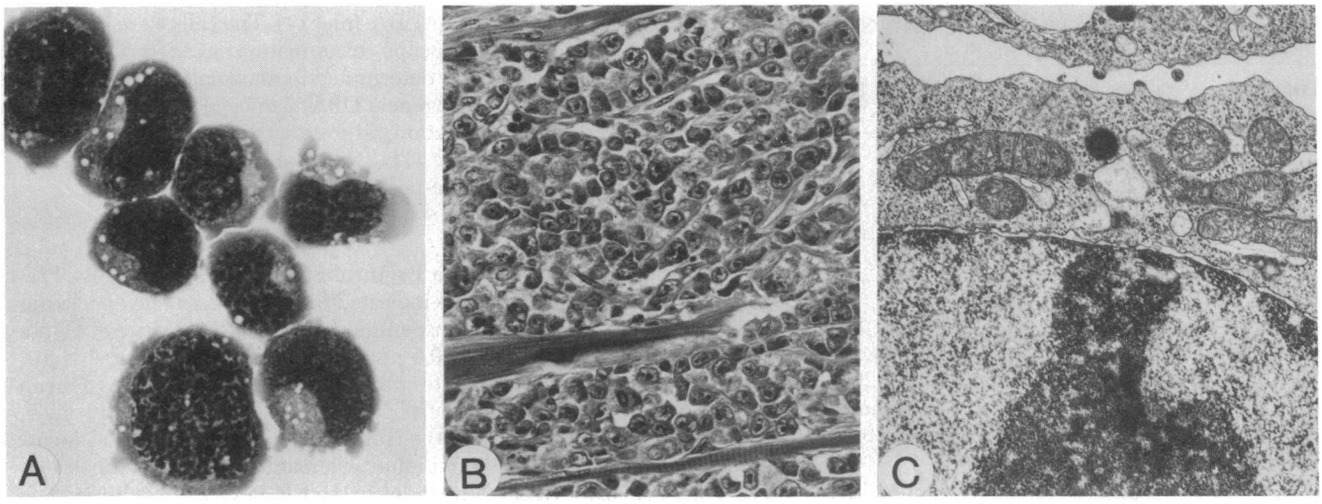

FIG. 1. (*A*) Undifferentiated hematopoietic cells of line G-1. (Giemsa–Wright stain, ×280.) (*B*) Tumor resembling reticulum cell sarcoma produced by subcutaneous inoculation of 10^6 line G-1 cells into DBA/2 mice. Note cutaneous muscle filaments. (Hematoxylin–eosin stain, ×280.) (*C*) Virus budding from cell surface. (×14,800.)

Properties of the Transformed Cell Lines. The characteristics of the three lines are compared in Table 3. Line G-1 grows in suspension and lines G-2 and G-3 are adherent. All are aneuploid, but only line G-1 has metacentric chromosomes. The cells of line G-1 resemble undifferentiated hematopoietic cells (Fig. 1*A*) and have properties comparable to those of the permanent lines derived from the leukemic spleens of the virus-infected mice (11). They grow in suspension, double in 16 hr, and reach saturation density in 4 days. The subcutaneous inoculation of 10^6 cells into syngeneic mice results in rapidly growing tumors (Fig. 1*B*) lethal to the host (18). Erythroid differentiation is stimulated when the cells are grown in medium supplemented with 2% Me_2SO. After 5 days of treatment, the number of benzidine-positive cells increased from a high spontaneous base line of 14% in the untreated controls to 93%. It has been reported that clone D, derived from erythroleukemia cell line 745, is nearly triploid and also has a high spontaneous level of differentiating cells (21).

Although lines G-2 and G-3 also arose from the exfoliated cells, these quickly became adherent and have remained so. Line G-2 appears to have two cell types, one that is fibroblastic and the other epithelioid (Fig. 2*A*). Line G-3 appears predominantly epithelioid (Fig. 2*C*). Unlike line G-1, the adherent lines are nonerythroid and do not respond to Me_2SO stimulation. They may have been derived from nonhematopoietic cells, which are present in fetal liver. No tumor growth was obtained after the inoculation of 10^6 cells of lines G-2 or G-3 into syngeneic mice. Although the nonadherent line G-1 can be readily cloned on soft agar, cloning of the adherent lines has not been successful.

Table 4. Viral activity in FLV-A-transformed cultures

Line	Reverse transcriptase, cpm/10^5 cells	Leukemogenicity,* mice leukemic/ mice inoculated	SFFV†
G-1 (suspension)	46,546	0/10	–
G-2 (adherent)	130,435	0/10	–
G-3 (adherent)	220,868	0/10	–

* Undiluted supernatant fluid (0.5 ml) inoculated intraperitoneally into five 8-week-old DBA/2J and five Swiss mice.

† Undiluted supernatant fluid (0.5 ml) inoculated intravenously into 8-week-old BALB/c female mice (4).

Virus Synthesis in FLV-A-Transformed Cells. All three cell lines synthesize virus (Table 4). There is substantial reverse transcriptase activity released into the medium of all cultures, and type C viruses are present (Figs. 1*C*, 2 *B* and *D*). The virus synthesized *in vitro*, however, is attenuated. None of the DBA/2 or Swiss mice inoculated with the filtered culture fluid developed leukemia during the 3-month observation period. Assays in BALB/c mice for SFFV activity on the same samples were negative.

DISCUSSION

The fact that FLV-induced leukemia in mice occurs in at least two separable forms has been generally overlooked (22). Mice infected with the anemic strain (FLV-A) develop erythroleukemia and severe anemia, whereas those infected with the polycythemic strain (FLV-P), which contains SFFV, also develop erythroleukemia but with erythrocytosis (8). In both cases, erythroid precursors appear to be the major target cell (23). Erythropoiesis, whether effective or ineffective, then proceeds without the requirement of erythropoietin (8).

In the present studies, active erythropoiesis of hematopoietic cells from DBA/2 fetal livers, which was sustained in cultures supplemented with EP, was further potentiated when the cells were infected with FLV-A. Within 5 days of the initiation of the cultures, a significant increase in the number of EP-independent and EP-dependent erythroid colony-forming cells in excess of that produced by EP alone was detected. Permanent lines were obtained only from the virus-infected, EP-treated cultures of the virus-susceptible DBA/2 mouse cells. One line, G-1, grows in suspension and is erythroid in origin. It is readily cloned in agar, is tumorigenic in syngeneic hosts, and is stimulated to synthesize hemoglobin by Me_2SO treatment, as are the erythroleukemic lines established from the spleens of FLV-infected leukemic mice (11). The other two lines, G-2 and G-3, are adherent and apparently nonerythroid. They do not clone on soft agar, nor does the inoculation of 10^6 cells into syngeneic hosts produce tumors. The metacentric chromosomes observed in line G-1 may be associated with its malignancy. Other permanent tumorigenic lines of erythroleukemia cells have these chromosomal markers (24), as do the lines transformed by FLV-P *in vitro* (10). The transforming event appears to be virus specific, since there were no alterations in CFU-E in the *in vitro* infected fetal liver cells from C57BL mice, which are genetically resistant to the induction of leukemia by FLV

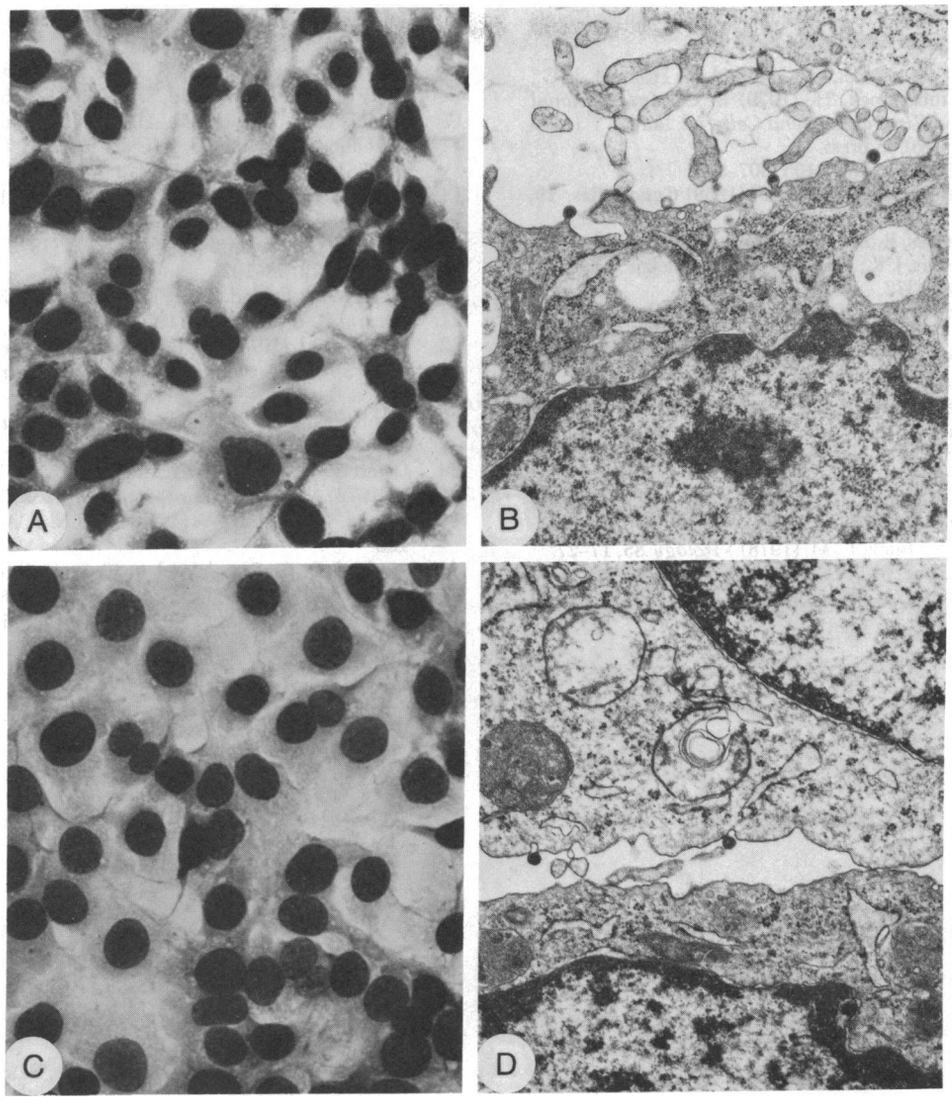

FIG. 2. (*A* and *C*) Cells of the adherent cultures. (Giemsa–Wright stain, ×300.) (*A*) The spindle-shaped and epithelioid cells of line G-2. (*C*) The predominantly epitheloid cells of line G-3. (*B* and *D*) Electron micrographs of transformed cells of adherent cultures, showing budding viruses on the cell surfaces. (×16,100.) (*B*) Line G-2; (*D*) line G-3.

(9), or in DBA/2 cultures treated with normal spleen filtrates. No continuous lines were obtained from these cultures.

While substantial viral activity can be demonstrated by reverse trancriptase assays and electron microscopy, the virus synthesized by each of the permanent transformed lines is attenuated. A loss in leukemogenic potential of the virus synthesized by our permanent lines established from leukemic cells of FLV-infected mice was also noted (25).

Thus, while infection may not always result in the malignant changes observed in line G-1, transformation may be evident by changes in morphology and by the immortalization of the infected cells, as observed in the adherent lines G-2 and G-3. In view of the report that the round, nonadherent cell hybrids between FLV erythroleukemia cells and fibroblasts—but not the flat adherent cells—are induced by Me_2SO to synthesize hemoglobin (26), it would be of interest to determine whether the correlation between morphology and hemoglobin inducibility is applicable to the round cells that float off the adherent cell lines.

The observations that the leukemogenicity of FLV-P is not altered by the elimination of SFFV (8) and that FLV-A clone 201 acquired SFFV sequences only after replication in newborn Swiss mice (5) suggest that this defective virus may not be a prerequisite for the induction of leukemia by FLV. SFFV may act by potentiating erythropoiesis *in vitro* as it does *in vivo* (8). Until the role of SFFV is satisfactorily determined, the analogy that has been proposed between the defective murine sarcoma virus and its helper murine leukemia virus, and SFFV and FLV, is open to question (27). The oncogenicity of the defective murine sarcoma virus and its helper murine leukemia virus are easily distinguished by various assays and by the different neoplasms they produce, but the defective SFFV and its helper FLV cannot be so readily identified. Both have target cells that are erythroid precursors that may be at different levels of maturation. The fact that SFFV is not expressed in splenectomized mice that develop leukemia after inoculation with FLV-P (1, 8) suggests that SFFV may stimulate a splenic EP-sensitive erythroid precursor, causing the onset of polycythemia. FLV-A may induce leukemia by transforming an earlier erythroid progenitor cell. Further study of the conditions favoring *in vitro* transformation may shed new light on the mechanisms involved in leukemogenesis and more clearly define the role SFFV may have in the evolution of erythroleukemia.

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