Proc. Natl. Acad. Sci. USA
Vol. 76, No. 3, pp. 1464–1468, March 1979
Medical Sciences

In vitro transformation of mouse bone marrow cells by the polycythemic strain of Friend leukemia virus

(spleen focus-forming virus)

R. REVOLTELLA*, L. BERTOLINI*, AND C. FRIEND†

*Department of Tumor Immunobiology, Laboratory of Cell Biology, Consiglio Nazionale delle Ricerche Rome, Italy; and †The Mollie B. Roth Laboratory, Center for Experimental Cell Biology, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029

Contributed by Charlotte Friend, December 4, 1978

ABSTRACT **Strains of Friend leukemia virus (FLV) that are associated with polycythemia contain the defective spleen focus-forming virus (SFFV). To determine whether the transforming ability of FLV was affected by the presence of this second agent, DBA/2J mouse bone marrow cells were infected *in vitro*. Criteria for transformation were the establishment of permanent lines, growth on semisolid agarose, and the production of tumors at the site of inoculation in syngeneic hosts. Two lines of immature hematopoietic cells that grow in suspension originated from the infected cultures. Each has an almost diploid karyotype (38–39 chromosomes) and 3–4 metacentric chromosomes. These transformed cells express gp71 viral envelope glycoprotein and p30 viral core protein antigens. Virus production was measured by reverse transcriptase (RNA-dependent DNA polymerase) activity of the virions released into the medium. The virus, assayed *in vivo* for infectivity, has SFFV activity but is attenuated for leukemogenicity. The stimulation of hemoglobin synthesis in the cells grown in medium supplemented with dimethyl sulfoxide or hexamethylene bisacetamide indicates that the cells are erythroid in origin. SFFV may have a function analogous to erythropoietin in influencing the process of transformation by FLV.**

Friend leukemia virus (FLV) causes erythroleukemia in susceptible strains of adult mice after a short latent period (1, 2). This model resembles Di Guglielmo disease in humans in that it is characterized by a rapid proliferation of primitive hematopoietic cells and is associated with an erythropoietic response. Although FLV has been shown to influence the activity of other types of blood cells (3–6), the primary target for FLV *in vivo* is an erythroid precursor (7–9).

While the prototype virus (FLV-A) induces erythroleukemia accompanied by anemia (1, 2), some strains, designated FLV-P, induce the same type of erythroleukemia but associated with polycythemia (10). Antiserum to either strain neutralizes the infectivity of the other, although FLV-P is thought to contain a complex of viruses, among which is the defective spleen focus-forming virus (SFFV) (11). The polycythemia is attributed to its activity. Removal of SFFV from FLV-P preparations eliminates the polycythemia-inducing factor without altering the leukemogenic activity (12, 13). Thus, the role of SFFV in the pathogenesis of FLV-induced erythroleukemia *in vivo* is not clear. SFFV is expressed only in the presence of a "helper," which may be supplied by a variety of viruses and nonviral agents (14–18).

We have compared the ability of the two FLV strains to transform hematopoietic cells *in vitro* in an effort to clarify the role that SFFV may play in the pathogenesis of the leukemia *in vivo*. The preceding report (19) described the transformation of mouse fetal liver cells infected *in vitro* with FLV-A, which lacks SFFV activity. The results of parallel studies on the transformation of mouse bone marrow cells infected with FLV-P *in vitro* is the subject of this communication.

The criteria for transformation were altered morphology, establishment of cells in continuous culture, growth on soft agar, and tumorigenicity in syngeneic hosts. Two permanent lines of malignant hematopoietic cells originated from cultures of DBA/2 mouse bone marrow cells infected *in vitro* with FLV-P. Each grows in suspension, can be cloned on soft agar, and produces tumors in syngeneic mice. The erythroid nature of the transformed cells was verified by stimulation of differentiation after treatment with dimethyl sulfoxide (Me_2SO) (9) and hexamethylene bisacetamide (HMBA) (20).

MATERIALS AND METHODS

Virus. Stock FLV-P virus was prepared from filtrates of leukemic spleens of polycythemic DBA/2J male mice (Jackson Laboratory), as described (1), and stored at −180°C. The LD_{50} (mean lethal dose) of the virus titered for leukemogenicity in 6- to 8-week-old DBA/2J mice was 5×10^4 virus per 0.2 ml. SFFV activity was estimated 9 days after the inoculation of virus diluted 1:100 (11). There were approximately 50 foci per spleen.

Cell Culture Methods. Bone marrow cells from femora and tibiae of 2- to 3-month-old adult DBA/2 mice of either sex were collected in cold Dulbecco's modified Eagle's medium GIBCO containing penicillin and streptomycin. The growth medium was Dulbecco's modified Eagle's medium supplemented with 20% heat-inactivated filtered fetal calf serum (Flow Laboratories, Rockville, MD), 2.0 mM glutamine, and 20 μM 2-mercaptoethanol. Cell viability was determined by erythrosin-B dye exclusion. All cultures were incubated at 37°C in a humidified 5% CO_2 environment.

The cells were filtered through sterile gauze and passed several times through a 27-gauge needle. They were then left to settle for 1–2 min to remove cell aggregates. In an effort to obtain the optimal conditions for cell growth, two methods were employed. In the first, the washed cells were resuspended in prewarmed Dulbecco's modified Eagle's medium to give a concentration of 10^7 cells per ml and distributed in 1-ml aliquots. One set of cultures received 0.2 ml of a 1:100 dilution of virus filtrate, and the control cultures received an equal amount of medium. After 30 min at 37°C, the cell suspensions were diluted with 9.0 ml of growth medium and 5-ml aliquots of each were transferred into 60-cm diameter Falcon petri dishes for further incubation. Five days later, the infected cultures were given a second inoculum of 0.2 ml of 1:100 FLV-P in 1.0 ml of fresh medium. Small amounts of fresh growth medium were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Me_2SO, dimethyl sulfoxide; FLV, Friend leukemia virus; FLV-A, anemic strain of FLV; FLV-P, polycythemic strain of FLV; HMBA, hexamethylene bisacetamide; SFFV, spleen focus-forming virus.

Table 1. Nucleated cells in cultures of FLV-P-infected bone marrow

Days	FLV-P	No. of cells × 10^{-3}				
		Total	Primitive	Granulocytes	Monocytes	Lymphoid
0		4,650	240	300	210	3700
2	−	1,760	110 ± 47	6 ± 3	150 ± 31	1500 ± 270
	+	870	318 ± 120	74 ± 10	45 ± 7	450 ± 31
5	−	710	241 ± 18	4 ± 2	118 ± 15	350 ± 52
	+	1,650	646 ± 141	121 ± 7	210 ± 30	658 ± 46
7–8	−	800	270 ± 54	120 ± 18	210 ± 22	210 ± 24
	+	3,640	818 ± 127	810 ± 14	1300 ± 18	660 ± 61
9–12	−	650	141 ± 70	130 ± 14	241 ± 41	140 ± 18
	+	5,260	1241 ± 37	714 ± 36	2715 ± 124	580 ± 37
13–15	−	660	110 ± 34	110 ± 8	300 ± 30	146 ± 18
	+	7,390	2510 ± 124	1200 ± 170	2870 ± 140	710 ± 59
16–20	−	420	80 ± 26	80 ± 7	140 ± 15	120 ± 18
	+	6,870	2417 ± 177	1240 ± 180	2810 ± 205	400 ± 33
21–25	−	260	30 ± 14	20 ± 5	110 ± 27	100 ± 5
	+	11,360	2771 ± 40	840 ± 31	2440 ± 171	306 ± 18

Data from eight experiments. Uncertainties are ±SEM.

added weekly. Twenty to 25 days after initiation, the cultures were divided (1:2) for the first time with fresh growth medium. They were split routinely every 3 days when active growth was evident.

In the second method, bone marrow cells, washed in phosphate-buffered saline, were resuspended in 17% bovine serum albumin and centrifuged through a discontinuous bovine serum albumin gradient (21). Cells at the interface of the gradient were collected and adjusted to 2×10^6 cells per ml. Cell fraction 1 was the layer between 17% and 19%, fraction 2 was between 19% and 21%, etc. After the cells of each fraction had been infected as described above, they were placed in 0.45% (wt/vol) agarose and plated directly on top of 1 ml of 0.9% agarose medium in 2-ml wells of a 24-well Linbro plate. Individual colonies, picked after 15 days of incubation, were pooled and transferred to the wells of Linbro dishes containing growth medium. Subsequently, the cultures were transferred and maintained in 35-mm diameter petri dishes.

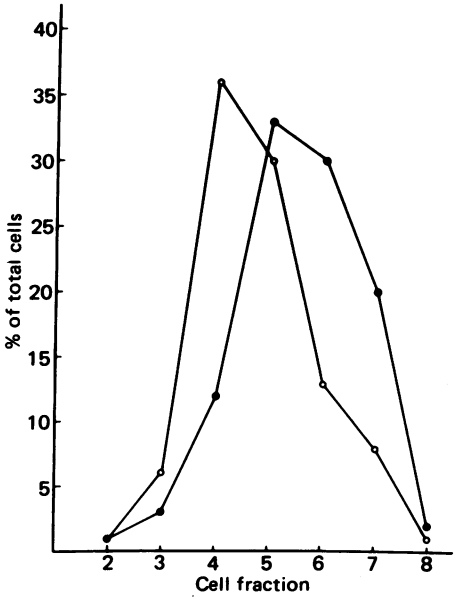

FIG. 1. Bovine serum albumin gradient fractionation. ●, Percent of cells recovered in each fraction of bone marrow cells. Values are the average of four experiments. ○, Percent of cells infected, evaluated by indirect immunofluorescent staining using goat anti-FLV p30 antiserum. The maximum number of positive cells in each assay was taken as 100%.

Line 3BM-77 was isolated by the first method and line 3BM-78 was isolated from a pool of four colonies isolated on agarose by the second method.

Competitive Radioimmunoprecipitation Assay. Purified viral gp71 and p30 from FLV-infected Eveline STU cells and goat anti-FLV gp71 or p30 monospecific antisera were generous gifts of D. Bolognesi. Virus proteins were labeled by the chloramine-T procedure (22). In the procedure for competitive radioimmunoprecipitation, fluid from 3-day-old cultures of FLV-P-transformed cells was used as an inhibitor of the binding reaction of an optimal combination of ^{125}I-labeled FLV antigen (at a specific activity of *ca.* 17,000 cpm/ng) and specific antibody.

Indirect Immunofluorescence Assay. The expression of FLV-associated gp71 or p30 antigens on the cells was determined by the indirect immunofluorescence assay (23), using goat anti-FLV gp71 or p30 antisera obtained from D. Bolognesi. Rabbit anti-goat IgG conjugated to fluorescein isothiocyanate (Cappel Laboratories, Downington, PA) was absorbed before testing for 2 hr at 4°C on 5-86 cells, a subclone of line 745 Friend erythroleukemia cells (9) and on normal DBA/2 liver, kidney, and spleen cells to prevent nonspecific staining.

RESULTS

Origin of Cell Lines. Line 3BM-77: At various intervals after the initiation of the cultures in liquid medium, samples were removed for differential counts. The pooled results obtained from several separate experiments are given in Table 1. Within the first 24–48 hr of culture, a severe decrease in the number of viable cells in both infected and uninfected cultures occurred. There were approximately $1–2 \times 10^6$ viable nucleated cells during the first 5 days of culture. This represented approximately 20% of the original inoculum. The number of viable cells in the infected cultures after the 5th day was usually higher than in the controls and never less than 10% of the original inoculum. The predominant cells were granulocytes, monocytes at various levels of differentiation, as well as large, irregularly shaped adherent cells approximately 60–70 μm in diameter. Many of these latter cells appeared to be multinuclear. A few foci of round cells, 10–20 μm in diameter, were visible. These foci increased in size rapidly 8–10 days after infection. The small cells first tended to gather around on top of the larger cells, then detached and grew in suspension. By 12–25 days after infection, these cells were representative of the majority of viable cells. Fibroblastic or epithelioid elements were not observed after the cells were in serial passage.

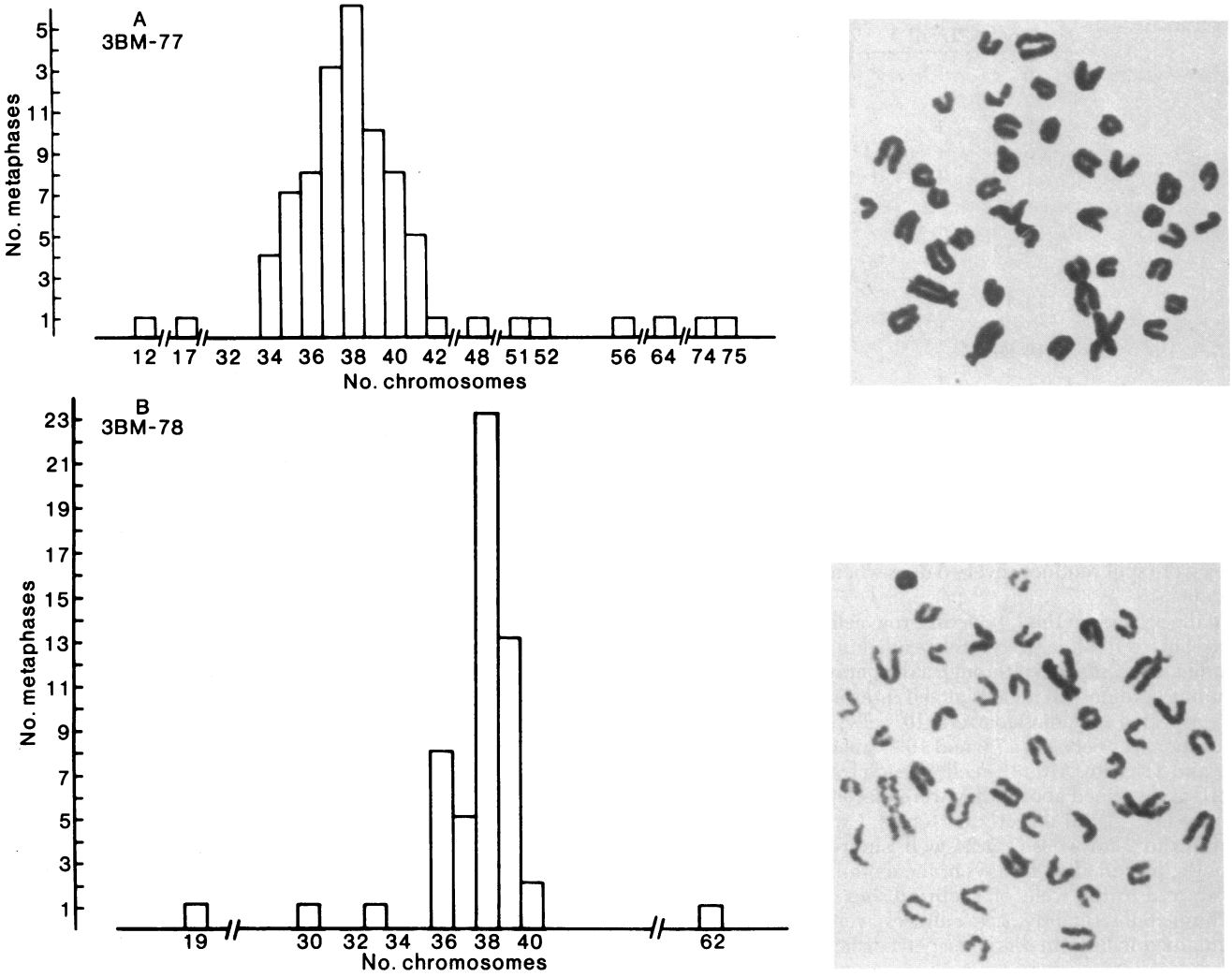

FIG. 2. Karyotype of established cell lines. Metaphases of cells, illustrating the presence of metacentric chromosomes (*Insets*). (*A*) Line 3BM-77; (*B*) line 3BM-78.

Line 3BM-78: In the second method, the distribution of nucleated bone marrow cells in each fraction of the gradient was determined and, after infection, the percent of virus-infected cells plated on agar was evaluated. Most of the virus-positive cells were in fractions 4 and 5, while the majority of cells were in fractions 5 and 6 (Fig. 1). Colonies of the infected cells were visible after 8–10 days and were picked on the 15th day. A continuous cell line was established from a pool of four colonies obtained from fraction 4 and dispersed in fresh growth medium. It was never possible to obtain long-term cultures from small colonies of the uninfected bone marrow cells.

Properties of FLV-P-Transformed Cells. Table 2 summarizes the chief properties of the two transformed bone marrow cell lines. Cells of line 3BM-77 double in 14–16 hr, whereas cells of 3BM-78 double in 18 hr. Both reach saturation density by day 4. The majority of these undifferentiated hematopoietic cells (Fig. 2 *A* and *B*) have an almost diploid karyotype with a mode of 38 or 39 chromosomes (Fig. 3). Both cell lines have 3–4 bi-

Table 2. Properties of bone marrow cell lines transformed by FLV-P *in vitro*

Line	Doubling time, hr	Saturation density, days	Karyotype: Modal no. chromosomes	Karyotype: No. metacentric chromosomes	Malignancy, mice with tumors/ mice inoculated	Benzidine-positive, %: Control	Benzidine-positive, %: +Me_2SO	Benzidine-positive, %: +HMBA
3BM-77	14–16	4	38	3–4	10/10	1	81	89
3BM-78	18	4	30	4	10/10	1	40	77

Doubling time: Cells (10^5/ml) were seeded in duplicate and the total cell number was counted every 2 hr for the first 48 hr after seeding, then daily up to day 6. Karyotyping: Cells were arrested in metaphase with Colcemid (1 μM). Chromosomes of 100 suitable cells stained with Giemsa were counted and examined for gross abnormality compared to normal bone marrow cells cultured under the same conditions. Tumorigenicity: Cells (10^6) resuspended in Dulbecco's modified Eagle's medium were injected subcutaneously into 6- to 8-week-old DBA/2 mice. Erythroid stimulation: The number of benzidine-positive cells in cultures seeded (10^5 cells per ml) into medium containing 1.5% (vol/vol) Me_2SO or 3 mM HMBA was determined on day 6 by the method of Orkin *et al.* (24) with modifications (25). Cell viability of treated 3BM-77 cells was 83% after Me_2SO and 92% after HMBA, and 3BM-78 cells were 87% and 82% viable, respectively.

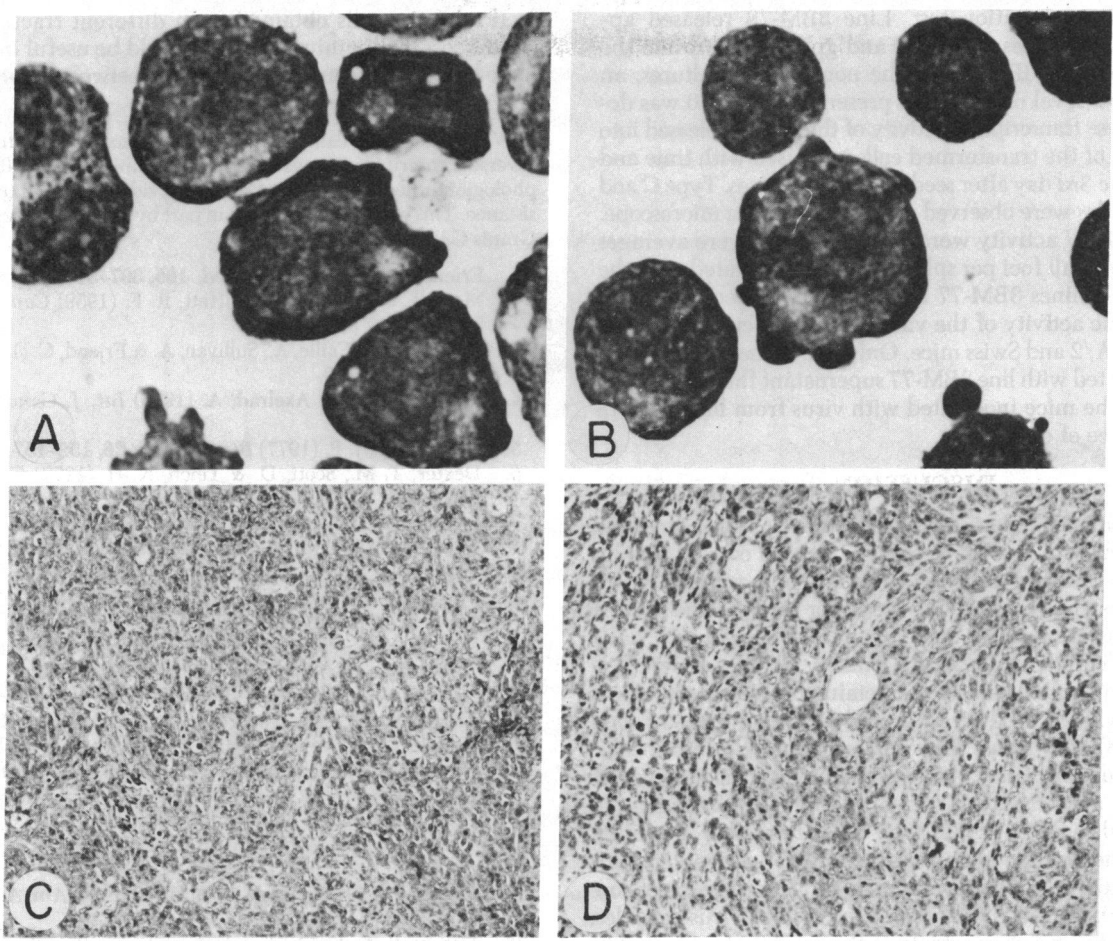

FIG. 3. (*A* and *B*) Undifferentiated hematopoietic cells of the transformed cultures. (*A*) Line 3BM-77. (*B*) Line 3BM-78. (May–Gruenwald–Giemsa stained. ×400.) (*C* and *D*) Tumors, resembling reticulum cell sarcomas, produced in DBA/2 mice after subcutaneous inoculation of 10^6 cells. (*C*) Line 3BM-77; (*D*) line 3BM-78. (Hematoxylin–eosin stained. ×100.)

armed chromosomes, which were detected early after infection (9th passage) and were still present after over 60 continuous passages *in vitro*.

The response of the cells to treatment with Me_2SO (9) or HMBA (20) identified them as being erythroid in origin. When the cells were grown without an inducer, approximately 1% of each line stained benzidine-positive. When grown with 1.5% (vol/vol) Me_2SO for 6 days, 81% of the treated 3BM-77 cells and 40% of the treated 3BM-78 cells were stimulated to differentiate. Greater stimulation was observed when the cells were grown in the presence of 3 mM HMBA for 6 days. Eighty-nine percent of the treated 3BM-77 cells and 77% of the treated 3BM-78 cells were benzidine-positive.

Subcutaneous injection of 10^6 cells of either line produced solid tumors at the site of inoculation in DBA/2 mice, which ultimately led to the death of the hosts. These tumors, which resembled reticulum cell sarcomas, were histologically indistinguishable from those induced by FLV-A malignantly transformed cells (19) and by cells transformed by FLV *in vivo* (27) (Fig. 2 *C* and *D*).

Virus Synthesis in FLV-P Transformed Cells. Several methods were used to study virus production in the transformed cells (Table 3). With monospecific antisera directed against the viral envelope glycoprotein gp71 and core protein p30, viral antigens were detected on the cells by the indirect immunofluorescence technique and in the supernatant fluid by the

Table 3. Viral activity in FLV-P-transformed bone marrow cell lines

Cells	Immunofluorescence*		Radioimmunoprecipitation,† ng protein/10^6 cells		Reverse transcriptase, cpm/10^6 cells	Leukemogenicity§		SFFV¶ in DBA/2
	FLV gp71	FLV p30	FLV gp71	FLV p30		DBA/2	Swiss	
3BM-77	+	+	324 ± 18	204 ± 15	24,618 ± 3.08	1/3	0/5	15 ± 3
3BM-78	+	+	1250 ± 31	648 ± 36	14,216 ± 1.21	0/3	0/5	20 ± 6
Control DBA/2 BM	−	−	18.1 ± 3	0.4 ± 0.2	6.3 ± 3.0			

Assays were carried out on 3-day-old cultures. Uncertainties are ±SEM.

* Indirect immunofluorescence assay on cells. Goat antisera (1:10) were used after absorbance as described in the text.

† Competitive radioimmunoprecipitation assay on antigen released into the medium; goat antisera.

‡ Reverse transcriptase (RNA-dependent DNA polymerase) activity of virions released into the medium, assayed as described (26).

§ Number leukemic/number of mice inoculated intraperitoneally with 0.5 ml of undiluted filtered supernatant fluid.

¶ SFFV assay (11). Average number of foci per spleen in mice inoculated intravenously with 0.5 ml of undiluted filtered supernatant fluid.

radioimmunoprecipitation test. Line 3BM-78 released approximately 3–4 times more p30 and gp71 antigens into the medium than line 3BM-77. In the noninfected cultures, an extremely low level of gp71 was present, but no p30 was detected. Reverse transcriptase activity of the virions released into the medium of the transformed cells increased with time and peaked on the 3rd day after seeding of the cultures. Type C and type A particles were observed under the electron microscope. Assays for SFFV activity were positive. There were averages of 15 and 20 small foci per spleen in mice inoculated with the medium from lines 3BM-77 and 3BM-78, respectively. The leukemogenic activity of the virus from each culture was assayed in DBA/2 and Swiss mice. Only one of the three DBA/2 mice inoculated with line 3BM-77 supernatant fluid developed leukemia. The mice inoculated with virus from line 3BM-78 remained free of disease.

DISCUSSION

The two permanent lines established from DBA/2 bone marrow cells infected with FLV-P *in vitro* have been carried serially for 2 years. Both are erythroid in origin and are induced to differentiate when treated with Me_2SO or HMBA. The *in vitro* FLV-P-transformed cell lines are nearly diploid and have metacentric chromosomes, an abnormality which was identified early after infection and is still retained. Metacentric chromosomes, which have been observed in Friend leukemia cell lines (19, 28), may signal the transformation of infected cells. Subcutaneous inoculation of the cells into syngeneic mice causes tumors that are lethal to the hosts, as do the cells malignantly transformed by FLV-A (19). The cells are readily cloned on semisolid medium and express specific FLV-associated antigens, the major envelope glycoprotein, gp71, and the major core protein, p30. The cells synthesize type C RNA virus particles, and reverse transcriptase activity in the medium is appreciable. The virus produced by the transformed cells has spleen focus-forming activity, but is decreased in leukemogenicity.

Characterization of clones derived from the continuous lines indicates that some of them contain cells that phenotypically express traits of the granulocytic series after 6 days of treatment with Me_2SO (R. P. Revoltella, L. Bertolini, G. Fioritoni, and G. Torlontano, unpublished data). This is in line with evidence that cells other than erythroid are affected by FLV infection (3–6). For example, the survival times of the hematopoietic stem cells of FLV-P-infected murine bone marrow cultures were extended and granulopoiesis was stimulated (6, 29). In addition, FLV affected the proliferative capacity of mouse embryo fibroblasts (30), and continuous lines have been isolated from such infected cultures (31). *In vitro* infection with FLV-P or FLV-A (19) led to the development of immortalized cell lines. Erythropoietin appears to be required for transformation to occur in hematopoietic cells infected with FLV-A. The possibility that SFFV in the FLV-P strains may have an erythropoietin-like function in preparing the cells for the transformation event is suggested by its *in vivo* activity in hypertransfused mice (12) and its *in vitro* activity in inducing erythropoietin-independent erythropoiesis in bone marrow cells from virus-permissive mice (32).

It would appear that there are several pathways open to the virus. Depending on the cell type, selective pressures exerted by the microenvironment, and other as yet little understood factors, infection may trigger a spectrum of biological modifications ranging from transformation to differentiation. An experimental system to study the events that occur after infection of the cells obtained from different fractions in the bovine serum albumin gradients should be useful in shedding some light on the complex relationship between normal growth and malignant transformation.

We thank Drs. Deane Tsuei and C. Carcone for performing the reverse transcriptase assays and Dr. M. Amini for help with the morphologic studies. Mrs. Eliana Vigneti provided excellent technical assistance. This work was supported in part by National Cancer Institute Grants CA 10,000 and CA 13,047.

1. Friend, C. (1947) *J. Exp. Med.* **105,** 307–318.
2. Metcalf, D., Furth, J. & Buffett, R. F. (1959) *Cancer Res.* **19,** 52–58.
3. Golde, D. W., Faille, A., Sullivan, A. & Friend, C. (1976) *Cancer Res.* **36,** 115–119.
4. Brown, W. M. & Axelrad, A. (1976) *Int. J. Cancer* **18,** 766–773.
5. Okunewick, J. P. (1977) *Biomedicine* **26,** 152–157.
6. Dexter, T. M., Scott, D. & Teich, N. H. (1977) *Cell* **12,** 355–364.
7. Zajdela, F. (1962) *Cancer (Brussels)* **59,** 351–373.
8. Tambourin, P. E. & Wendling, F. (1975) *Nature (London)* **256,** 320–322.
9. Friend, C., Scher, W., Holland, J. G. & Sato, T. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 378–382.
10. Mirand, E. A., Prentice, T. C., Hoffman, J. G. & Grace, J. T., Jr. (1961) *Proc. Soc. Exp. Biol. Med.* **106,** 423–426.
11. Axelrad, A. A. & Steeves, R. A. (1966) *Virology* **24,** 513–518.
12. Mirand, E. A. (1966) *Natl. Cancer Inst. Monogr.* **22,** 483–489.
13. Friend, C., Scher, W., Tsuei, D., Haddad, J., Holland, J. G., Szrajer, N. & Haubenstock, H. (1978) in *Oji International Seminar on Friend Virus and Friend Cells,* ed. Ikawa, Y. (Academic, New York), in press.
14. Flickinger, J. T. & Gentile, J. M. (1971) *Can. J. Microbiol.* **17,** 481–486.
15. Riley, V. & Fitzmaurice, M. A. (1973) *Proc. Am. Assoc. Cancer Res.* **14,** 112–123.
16. Steeves, R. A., Fjelde, A. & Mirand, E. A. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 2391–2395.
17. Steeves, R. A. & Minowada, J. (1973) *Proc. Am. Assoc. Cancer Res.* **14,** 71.
18. Steeves, R. A., Mirand, E. A., Thomson, S. & Avila, L. (1969) *Cancer Res.* **29,** 1111–1116.
19. Golde, D., Bersch, N., Friend, C., Tsuei, D. & Marovitz, W. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 962–966.
20. Fibach, E., Reuben, R. C., Rifkind, R. A. & Marks, P. A. (1977) *Cancer Res.* **37,** 440–444.
21. Rosenberg, N. & Baltimore, D. (1976) *J. Exp. Med.* **143,** 1453–1463.
22. Bilello, J. A., Strand, M. & August, J. T. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3234–3238.
23. Cloyd, M. W., Bolognesi, D. & Bigner, D. D. (1977) *Cancer Res.* **37,** 931–938.
24. Orkin, S., Harosi, F. I. & Leder, P. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 98–102.
25. Scher, W. & Friend, C. (1978) *Cancer Res.* **38,** 841–849.
26. Friend, C., Marovitz, W., Henle, G., Henle, W., Tsuei, D., Hirschhorn, K., Holland, J. G. & Cuttner, J. (1978) *Cancer Res.* **38,** 2581–2591.
27. Friend, C., Patuleia, M. C. & de Harven, E. (1966) *Natl. Cancer Inst. Monogr.* **22,** 505–522.
28. Ostertag, W., Melderis, H., Steinheider, G., Kluge, N. & Dube, S. (1972) *Nature (London)* **239,** 231–234.
29. Moore, M. A. S. (1979) in *Hematopoietic Cell Differentiation,* eds. Golde, D. W., Cline, M., Metcalf, D. & Fox, C. F. (Academic, New York), in press.
30. Gableman, N., Scher, W. & Friend, C. (1974) *Int. J. Cancer* **13,** 343–352.
31. Eckner, R. J. (1975) *J. Exp. Med.* **162,** 935–968.
32. Clark, B. J., Axelrad, A. A., Shreeve, M. M. & McLeod, D. L. (1975) *Proc. Natl. Acad. Sci. USA* **73,** 3556–3560.