

## FRIEND DISEASE IN VITRO\*

BY T. M. DEXTER,‡ T. D. ALLEN, N. G. TESTA, AND E. SCOLNICK

*From the Departments of Experimental Haematology and Ultrastructure, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Withington, Manchester M20 9BX, England; and the Laboratory of Tumor Virus Genetics, National Institutes of Health, Bethesda, Maryland 20205*

The polycythemia- and anemia-inducing strains (1, 2) of Friend murine erythroleukemia virus (FLV-P and FLV-A, respectively)<sup>1</sup> have been the subject of intensive studies for several years. Virus preparations contain a mixture of a helper-independent virus (F-MuLV) and a replication-defective spleen focus-forming virus (SFFV; 3, 4). At one level, attention has been focused upon the biology of the disease (5, 6); the nature and molecular biology of the helper-independent (F-MuLV) and replication-defective (SFFV<sub>A</sub> and SFFV<sub>P</sub>) transforming viruses (7–10); the target cells and genetic restriction for replication and transformation (6, 11–15); and the response of these cells to the hormone erythropoietin (16, 17). At a second level, cell lines have been established from the leukemic tissue of viral-infected mice (18, 19) and these lines have been very useful in analyzing the molecular events associated with hemoglobin production after treatment with a variety of inducing agents (20).

However, the relationship between the Friend tumor cell lines and the polycythemia or anemia seen after *in vivo* infection with SFFV<sub>P</sub> (F-MuLV) or SFFV<sub>A</sub> (F-MuLV) is still uncertain. For example, shortly after infection of susceptible mice with these viral complexes, marked alterations are seen in the levels of myeloid progenitor cells occurring concomitantly with an increased sensitivity (maybe true independence) of the erythroid progenitor cells to erythropoietin (6, 21). This is followed in the terminal stages of the disease by the emergence of transplantable, malignant cells (6, 22, 23). Whether these two phases represent a manifestation of infection and consecutive development of different cell populations, or merely reflect the sequential expression of transformation in the same cell population, has not been determined.

An *in vitro* system that reproduced all aspects of Friend disease would have obvious advantages in the analysis of the role of the respective viruses and the molecular and cell-biological events following viral infection. Short-term *in vitro* cultures have had some success in determining the developmental stage at which the erythroid progenitor cells become susceptible to SFFV<sub>A</sub> or SFFV<sub>P</sub> in terms of their ability to undergo clonal expansion and maturation in the absence of detectable erythropoietin; and

\* Supported by the Medical Research Council and the Cancer Research Campaign.

‡ Fellow of the Cancer Research Campaign.

<sup>1</sup> *Abbreviations used in this paper:* AMS, anemic mouse serum; BFU-E, erythroid burst-forming unit; CFU-E, erythroid colony-forming unit; CFU-S, hemopoietic stem cells; FLV-A, anemia-inducing strain of Friend leukemia virus; FLV-P, polycythemia-inducing strain of Friend leukemia virus; F-MuLV, helper-independent Friend murine leukemia virus; GM-CFC, granulocyte-macrophage colony-forming cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; Meg-CFC, megakaryocyte colony-forming cells; SFFV<sub>A</sub>, anemia-inducing spleen focus-forming virus; SFFV<sub>P</sub>, polycythemia-inducing spleen focus-forming virus.

using such assays, differences have been detected in the capacity of pseudotyped SFFV<sub>A</sub> and SFFV<sub>P</sub> virus-infected cells to undergo hemoglobinization in the absence of erythropoietin (17). In another system, after infection of hemopoietic cells in vitro with FLV-P, cell lines have been established that have characteristics in common with Friend tumor cell lines derived from in vivo infections (24). So far, however, no in vitro system has been described that faithfully duplicates Friend disease.

In this respect, long-term bone marrow cultures would appear to offer a suitable model system. In these cultures, hemopoietic stem cell proliferation and self-renewal can occur for several months (25) but only in the presence of an appropriate population of marrow-derived adherent cells that provide the inductive environment necessary for hemopoiesis to occur (26). Differentiation of stem cells leads to the continual production of progenitor cells of the different myeloid lineages: the granulocyte-macrophage colony-forming cells (GM-CFC), megakaryocyte colony-forming cells (Meg-CFC), and erythroid burst-forming units (BFU-E; 25-28). However, whereas the GM-CFC and Meg-CFC subsequently undergo maturation to produce mature granulocytes, macrophages, and megakaryocytes, the majority of BFU-E in these cultures are normally "blocked" at the most primitive level of development (28). However, addition of either erythropoietin in combination with mechanical agitation (28) or treatment with anemic mouse serum (29) leads to sequential development of the BFU-E to produce progressively more mature progeny (day 4 BFU-E and day 2 erythroid colony-forming units [CFU-E]) leading ultimately to the production of mature non-nucleated erythrocytes. This ability to induce erythropoiesis in a more or less synchronous fashion offered exciting possibilities in the viral erythroleukemia systems. In this communication we present data on the results of SFFV<sub>P</sub> (F-MuLV) infection and show that all aspects of the SFFV<sub>P</sub> (F-MuLV) disease can now be reproduced in vitro.

### Materials and Methods

*Long-Term Cultures.* These were established as previously described (25) with some modifications (28). The cellular contents of single femora from 12-wk-old, virgin DBA/2 female mice, were flushed into screw-capped Falcon TC flasks (25-cm<sup>2</sup> growing area; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) containing 10 ml of Fischers' medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% horse serum (Flow Laboratories, Hamden, Conn.), antibiotics, and 10<sup>-6</sup> M hydrocortisone sodium succinate. After gassing with 5% CO<sub>2</sub> in air, the screw-caps were tightly closed, and the cultures were incubated at 33°C. The cultures were fed weekly by the removal of half (5 ml) of the growth medium and the addition of an equal volume of fresh medium. In these cultures, residual erythropoiesis disappears within 1-2 wk (28).

After 2 wk growth, when many primitive but few late erythroid progenitors were present, (28) the cultures were demi-depopulated and fed with alpha medium supplemented with (final concentrations) 400 µg/ml transferrin; 10<sup>-4</sup> M 2-mercaptoethanol; 15% horse serum; 5% fetal calf serum (Sera Labs, Crawley Down, England); 10<sup>-6</sup> M hydrocortisone sodium succinate (Sigma Chemical Co., St. Louis, Mo.), and antibiotics. The cultures were gassed as before and transferred to 37°C (29). Some cultures were further supplemented with anemic mouse serum (AMS) at a final concentration of 5% vol:vol. All cultures were subsequently demi-depopulated and re-fed at weekly intervals with growth medium containing the required supplements. AMS addition was maintained only for three weekly consecutive feedings.

We have found that using this protocol, AMS will consistently induce high levels of erythropoiesis in the long-term cultures. Either four or five cultures were established for each experimental group and the results represent the data from pooled cell suspensions of these cultures.

For morphological analysis, smear preparations were stained with benzidine, followed by May-Grunwald-Giemsa.

**AMS.** AMS was used as the erythropoietic stimulus to the long-term cultures and was prepared as previously described (29, 30). The batch of AMS used contained 10 U/ml erythropoietin when assayed in hypertransfused mice.

**Hemopoietic Progenitor Cell Assays.** The cells present in the growth medium removed during feeding were counted directly on a hemacytometer. After centrifugation, the cells were resuspended in Fischers' medium at an appropriate dilution, counted again, and assayed for the presence of hemopoietic stem cells (CFU-S), GM-CFC, BFU-E, and CFU-E progenitor cells (25, 28).

Medium conditioned by the growth of WEHI-3 myelomonocytic leukemia cells was used as a source of GM colony-stimulating factor (CSF) and  $10^4$ - $10^5$  cells were plated in 1 ml of Fischers' medium supplemented with 20% horse serum and 0.3% agar in 35-mm plastic Petri dishes (Sterilin, Teddington, England). For GM-CFC assays, triplicate plates were established in the presence and the absence of GM-CSF. Colonies were scored after 7 d at a magnification of 25. Note that in virus-treated cultures, there was a sequential development of GM-CSF-independent colonies. Cells producing these colonies have been termed agar CFC.

CFU-S were assayed by the method of Till and McCulloch (31).  $10^4$ - $10^5$  cells (occasionally more) were injected intravenously into groups of at least five syngeneic mice that had received 8 Gy (a few hours previously) from a linear accelerator producing 12 MeV, dose rate 1.67 Gy/s. This dose of radiation was sufficient to ablate endogenous hemopoiesis.

Assays for BFU-E and CFU-E have been described in detail elsewhere (28, 29). For BFU-E assays, the culture medium was supplemented with 2 U/ml erythropoietin (Connaught Laboratories, Ontario, Canada; step III) and  $10^5$  irradiated bone marrow cells as a source of burst-forming activity (32). In CFU-E assays, 0.5 U/ml of erythropoietin was added before plating. BFU-E were scored on day 10 and CFU-E were scored on day 2. At least two plates were established per point, in the presence and in the absence of erythropoietin.

**Virus Treatment and Assays.** 2 wk after the last treatment with AMS, the cultures were demipopulated and fed with fresh medium. 24 h later, 0.5 ml of SFFV<sub>P</sub> (F-MuLV) was added to the AMS-induced and control (noninduced) cultures. This SFFV<sub>P</sub> (F-MuLV) virus complex (hereafter called FLV-P) was obtained by superinfection of SFFV<sub>P</sub>-infected 3T3 nonproducer cells (33) with clone 57 of F-MuLV (7). This preparation had an activity of  $5 \times 10^6$  XC plaque-forming units/ml and  $2 \times 10^4$  focus-forming units/ml when measured by the spleen focus-forming assay (4).

**Electron Microscopy.** Cell pellets or whole cultures were fixed in 3% glutaraldehyde in Sorenson's phosphate buffer, pH 7.3, for 30 min, followed by three buffer washes and postfixation in 1% OsO<sub>4</sub> in the same buffer for 30 min. After ethanol dehydration, the pellets were infiltrated and embedded using hydroxypropylmethacrylate and Lufts Epon (Taab Laboratories, Reading, England). After sectioning and staining with uranyl acetate and lead citrate, the sections were examined in an AE1 EM801A electron microscope (Kratos Ltd., Manchester, England). For scanning electron microscopy, whole cultures were fixed and dehydrated as above, critical point dried from CO<sub>2</sub>, sputter-coated with 200 nm gold, and viewed in a Cambridge S4-10 scanning electron microscope (Cambridge Instrument Co., Inc., Ossining, N. Y.) at an accelerating voltage of 20 kV.

## Results

**Effects of FLV-P in Non-AMS-induced Cultures.** The results are shown in Tables I and II. In control cultures, not treated with AMS or FLV-P, cell proliferation occurred for 28 wk. During the first 20 wk, the majority of cells produced consisted of granulocytes (all maturation stages), but macrophages subsequently predominated. No morphologically recognizable erythroid cells were produced throughout this period of culture. CFU-S were maintained for at least 14 wk, then declined; GM-CFC were present (albeit at reducing levels) until the cultures were terminated. At 6 and 7 wk, significant numbers of BFU-E were present that were absolutely dependent upon the presence of

TABLE I  
Effect of FLV-P Infection on Total Cellularity and Morphology in Long-Term Marrow Cultures\*

Group	Weeks cultured	Total cells ( $\times 10^5$ )	U	EG	LG	M $\phi$	EBL	BZN	EC
Control	6	29.0	3	18	78	0	0	0	0
Control	7	72.0	12	15	70	0	0	0	0
FLV-P	7 (1)	53.0	14	10	75	0	<1	<1	0
Control	10	43.1	22	9	67	1	0	0	0
FLV-P	10 (4)	37.2	19	12	60	1	6	3	++
Control	14	28.2	3	1	85	9	0	0	0
FLV-P	14 (8)	19.8	2	12	71	10	2	2	1
Control	18	27.1	40	6	52	2	0	0	0
FLV-P	18 (12)	22.2	36	14	36	4	1	0	0
Control	28	14.1	3	1	8	88	0	0	0
FLV-PP	28 (22)	9.8	6	13	9	71	1	0	0

\* U, undifferentiated cells; EG, early granulocytes (promyelocytes and myelocytes); LG, late granulocytes (metamyelocytes and polymorphonuclear cells); M $\phi$ , macrophages; EBL, erythroblasts; BZN, benzidine-positive nucleated cells; EC, mature, nonnucleated erythrocytes. Cultures were terminated after 28 wk. Numbers in parenthesis represent weeks cultured after FLV-P infection. ++, clumps of mature erythrocytes

TABLE II  
Effects of FLV-P Infection on Progenitor Cell Production in Long-Term Marrow Cultures

Group	Weeks cultured	CFU-S*	GM-CFC*		BFU-E*		CFU-E*	
			+	-	+	-	+	-
Control	6	ND $\ddagger$	13,141	0	1,740	0	10	0
Control	7	730	4,795	0	678	0	70	0
FLV-P	7 (1)	410	6,570	0	662	0	23,685	13,912
Control	10	300	2,040	0	ND	ND	ND	ND
FLV-P	10 (4)	175	2,951	0	ND	ND	ND	ND
Control	14	205	790	0	ND	ND	ND	ND
FLV-P	14 (8)	177	872	0	ND	ND	ND	ND
Control	18	0	580	0	0	0	0	0
FLV-P	18 (12)	54	1,509	0	0	0	0	0
Control	28	ND	100	0	0	0	0	0
FLV-P	28 (22)	ND	750	0	0	0	0	0

\* Total number present in the nonadherent cells of the long-term cultures on the weeks indicated. GM-CFC plated with (+) or without (-) GM-CSF. BFU-E and CFU-E plated with (+) and without (-) erythropoietin. Numbers in parentheses represent weeks cultured after FLV-P infection.

$\ddagger$  Not determined.

erythropoietin for their development. Only low levels of CFU-E were present, a result agreeing with our previous observations (28, 29).

In FLV-P-infected cultures, a comparable level of overall cell production was seen. However, although the majority of cells produced consisted of maturing granulocytes, we found that, in contrast to the controls, a low level of erythropoiesis was seen 1-8 wk after infection (Table I). All stages of erythroid maturation were seen from the

proerythroblast through to the mature, enucleated erythrocytes; but the number of erythroid cells produced rarely exceeded 10% of the total cells harvested. In terms of progenitor cell production (Table II), we found that CFU-S and GM-CFC production was equivalent to that seen in control cultures. At no stage was agar colony growth seen that was independent of added GM-CSF. Of considerable interest is that 1 wk after infection, BFU-E numbers were also equivalent to control levels (and showed a similar erythropoietin dependency), but large numbers of CFU-E were also present. Moreover, CFU-E colony development in the absence of exogenous erythropoietin was 50% that seen in the presence of erythropoietin, i.e., late erythroid progenitors that grew independently of added erythropoietin had been generated as a result of FLV-P infection.

Subsequently, there was a gradual conversion of the cultures to macrophage production, erythropoiesis was terminated, and the cultures were discarded.

*Effects of FLV-P in AMS-induced Cultures.* Nonadherent cell production in AMS-treated cultures with or without FLV-P infection is shown in Fig. 1, and the morphology of the cultured cells is shown in Fig. 2. In AMS-treated cultures, cell production was maintained for 14 wk, but subsequently declined. The three consecutive weekly treatments with AMS induced a progression of the normal granulocytic nature of the cultures (Fig. 3a and d) into a fairly well-defined peak of erythropoiesis, commencing 10–14 d after the first treatment and declining within 28 d after the last treatment (Figs. 2 and 3b, f, and g). These results are in agreement with our previous findings (29). It is worth noting that the earliest morphologically recognizable cells (erythroblasts) are out of phase with the later cells, which indicates sequential development.

In cultures treated with AMS and FLV-P, cell production was maintained in excess of 44 wk (Fig. 1), and during this time granulocytic production declined concomitantly with a massive increase in the production of erythroblasts (Figs. 2 and 3c and e). Furthermore, hemoglobinized, nucleated erythrocytes were being produced throughout this period at levels varying from 1 to 10% of total cell production. The major site

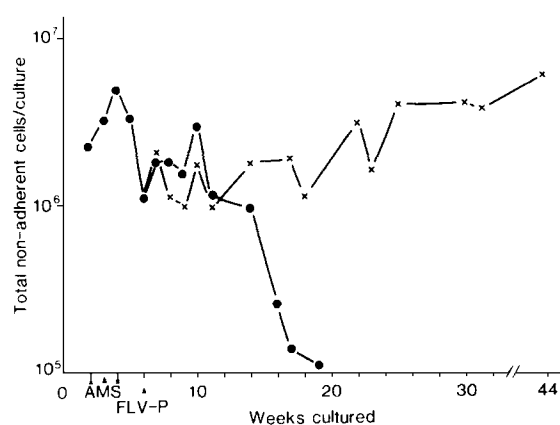


FIG. 1. Production of nonadherent cells in AMS-induced cultures treated with FLV-P. Cultures were treated as indicated with AMS and FLV-P, and were demi-depopulated weekly. Symbols represent the number of cells present in the growth medium at the time of feeding (●), AMS alone; (×), AMS FLV-P.

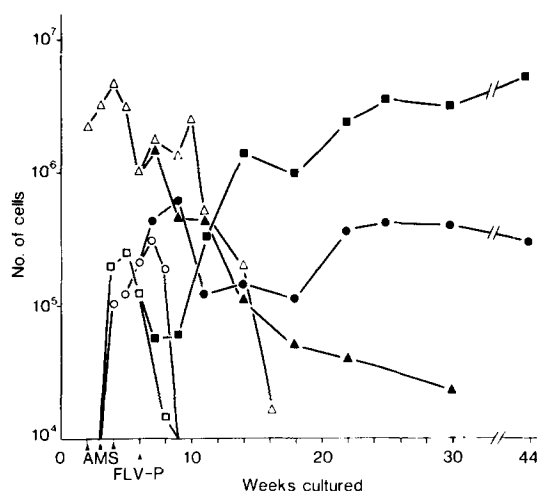


FIG. 2. Morphological analysis of nonadherent cells in AMS-induced and FLV-P-treated cultures. Cultures were treated as indicated with AMS and FLV-P. Open symbols represent cultures treated with AMS alone. Closed symbols represent cultures also treated with FLV-P. ( $\Delta$ ,  $\blacktriangle$ ), granulocytes; ( $\square$ ,  $\blacksquare$ ), erythroblasts; ( $\circ$ ,  $\bullet$ ), hemoglobinized cells. The results are expressed as the total number of cells present in the growth medium at the time of feeding.

of production of these erythroid elements appeared to be the adherent cell layer, which was heavily infiltrated with primitive erythroid cells.

Analysis of hemopoietic progenitor cell populations showed that in the control, AMS-treated cultures, CFU-S and GM-CFC production was maintained for 14 wk (Table III). In these cultures (unlike non-AMS-treated cultures), there was a significant production of CFU-E throughout the period of AMS-induced erythropoiesis. Growth of the majority of these CFU-E was dependent upon added erythropoietin; <10% control level CFU-E formation occurred in the absence of erythropoietin.

In FLV-P-treated cultures, CFU-S and GM-CFC were maintained for 11 wk, during which time the progenitor cells were apparently normal: producing spleen colonies containing all the myeloid lineages (erythroid, granulocytic, and megakaryocytic), and agar colonies containing granulocytes and macrophages. 1 wk after infection with FLV-P, erythropoietin-dependent BFU-E were present. CFU-E were also being produced, but it is of considerable interest that ~50% of these would produce colonies in the absence of added erythropoietin (compare this with control cultures in which <10% erythropoietin-independent CFU-E were present). After 14 wk (8 wk after infection) "normal" clonogenic hemopoietic progenitor cells could not be detected in our assay systems and the morphology of the cultures showed a dominance of primitive erythroid cells. Injection of these cells into potentially lethally irradiated mice produced spleen colonies containing only erythroid cells—mainly erythroblasts, although some maturing cells were present. Furthermore, by 8 wk after infection, large numbers of undifferentiated clonogenic cells were present. These cells would readily clone in soft agar or in methocel in the absence of the appropriate stimulating factors required for normal GM-CFC, BFU-E, or CFU-E development. Morphological analysis of these colonies showed them to consist of undifferentiated cells. Occasional hemoglobinized cells were present. When examined individually, all

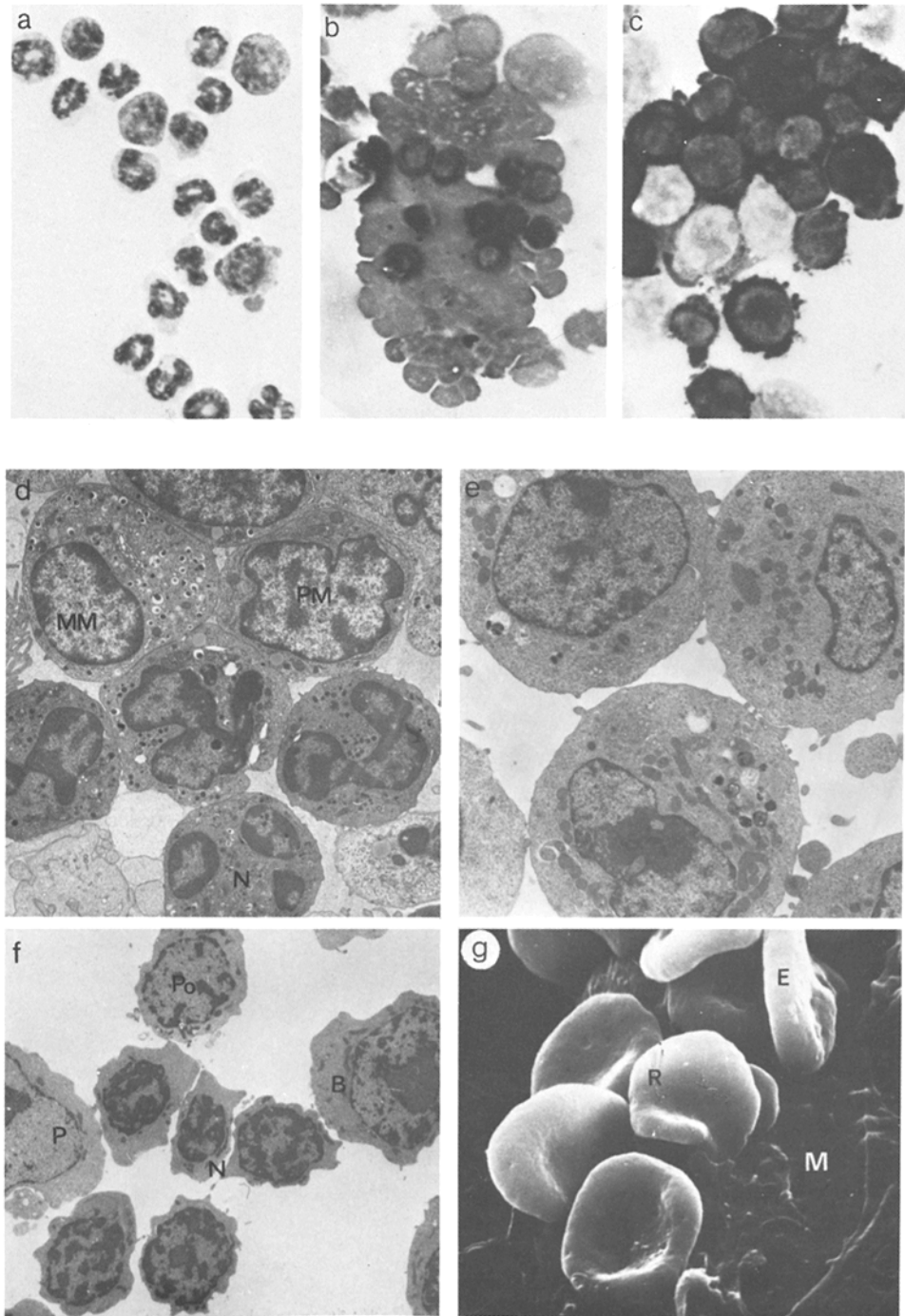


FIG. 3. Light and electron microscopic analysis of control cultures, AMS-induced cultures, and AMS-induced cultures treated with FLV-P. (a) Appearance of nonadherent cells before the addition of AMS. All stages of granulopoiesis are present, including fully mature neutrophils.  $\times 1,000$ . (b) Appearance of cultures at the time of maximal erythropoiesis induced by AMS. This preparation shows an aggregate of mature erythrocytes with a few normoblasts. Such aggregation com-

cultures in the groups documented above showed a similar progression, and the events described are not unique events occurring in occasional cultures.

*Leukemia and Virus Assays.* To check for the production of SFFV, cell-free supernates obtained from AMS-induced cultures 2, 14, and 28 wk after FLV-P infection, and from non-AMS-treated cultures 2 and 14 wk after FLV-P treatment, were injected at limiting dilutions into normal adult DBA/2 mice. The mice were killed 8 d later and the number of spleen foci were enumerated. On these occasions, between  $3 \times 10^3$  and  $3 \times 10^4$  focus-forming units/ml were present in the supernates. Control supernates yielded no spleen foci. Also, no significant differences were seen in the titers of SFFV recovered from noninduced and AMS-induced cultures treated with FLV-P.

Both the cells and the cell-free supernates derived from FLV-P-treated cultures would produce leukemia in nonirradiated adult DBA/2 mice. Gross splenomegaly was observed between 14 and 30 d after intravenous infection and morbid animals had hematocrits between 62 and 71, i.e., the polycythemia-inducing capacity of the original virus had been maintained in the long-term-cultured cells.

*Production of Cell Lines from FLV-P-treated Cultures.* At 2, 10, 20, and 40 wk after FLV-P treatment of AMS-induced cultures, we attempted to subculture the nonadherent cells in fresh culture flasks. Various growth conditions were used: Fischers', McCoy's, or alpha media supplemented with various concentrations of horse sera, fetal calf serum, with or without transferrin and hydrocortisone, and cultured at 33 or 37°C. In these circumstances, cell lines that grew independently of the adherent layer of the primary culture could not be established. After an initial growth, the cells died.

However, at 20 and 30 wk after infection, we transferred the floating cells from the FLV-P-infected cultures onto normal, noninfected bone marrow-derived adherent cell layers from DBA/2 mice. These adherent cells had been established in the conventional way as for long-term marrow cultures and had been grown for 3 wk in 20% horse serum supplemented with  $10^{-6}$  M hydrocortisone before inoculation of the FLV-P-infected cells. To ablate endogenous hemopoiesis, occurring within the adherent cell layer, the cultures were irradiated (with 10 Gy from a Cs source) immediately before use. After irradiation, all the growth medium was removed and replaced with 10 ml of fresh medium (alpha medium supplemented with 15% horse serum and 5% fetal calf serum) containing  $\sim 10^6$  pooled cells from the FLV-P-treated cultures. Within a few days, the irradiated adherent layer was seen to be infiltrated with primitive erythroid cells and extensive proliferation was obviously occurring. These cultures have now been maintained for 25 and 15 wk, respectively, and in all respects are similar to the original cultures from which the FLV-P-infected cells were derived,

---

monly occurs around a central macrophage (29).  $\times 1,000$ . (c) Nonadherent cells harvested from cultures 20 wk after AMS induction and treatment with FLV-P. Note the hyperbasophilic nature and homogenous appearance of these cells.  $\times 1,000$ . (d) TEM section through pellet of cells from a granulopoietic culture before addition of AMS. Several stages of granulocyte maturation are apparent. PM, promyelocytes, MM, metamyelocytes; N, mature neutrophil.  $\times 4,575$ . (e) TEM section through pellet of cells from cultures 20 wk after AMS induction and treatment with FLV-P. Both nuclear and cytoplasmic morphology are undifferentiated, indicating a relatively early erythroid type cell. (f) TEM section through *in situ*-prepared-AMS-induced erythrocytic culture. P, proerythroblasts; B, basophilic erythroblast; Po, polychromatophilic erythroblasts; and N, normoblasts, indicate the stages of erythrocyte maturation.  $\times 4,000$ . (g) SEM preparation of erythrocytic culture, showing stages in the maturation of reticulocytes (R) grouped on a central macrophage (M) into typical erythrocytes (E).  $\times 5,000$ .



TABLE III  
*Effects of FLV-P Infection on Progenitor Cell Production in AMS-induced Long-Term Marrow Cultures*

Group	Weeks cultured	CFU-S	Agar CFC*		BFU-E*		CFU-E*	
			+	-	+	-	+	-
AMS	6	180	3,016‡	0	160	0	7,016	680
AMS	8	218	4,080‡	0	83	0	9,661	677
AMS + FLV-P	8 (2)	270	1,280‡	0	78	0	5,804	2,667
AMS	14	70	680‡	0	ND	ND	ND	ND
AMS + FLV-P	14 (8)	10 <sup>3</sup> §	10 <sup>3</sup> §	10 <sup>3</sup> §	ND	ND	ND	ND
AMS + FLV-P	20 (14)	2 × 10 <sup>3</sup> §	2 × 10 <sup>3</sup> §	2 × 10 <sup>3</sup> §	2 × 10 <sup>3</sup> §	2 × 10 <sup>3</sup> §	SC¶	SC
AMS + FLV-P	24 (18)	4 × 10 <sup>3</sup> §	4 × 10 <sup>3</sup> §	4 × 10 <sup>3</sup> §	4 × 10 <sup>3</sup> §	4 × 10 <sup>3</sup> §	SC	SC
AMS + FLV-P	44 (38)	6 × 10 <sup>3</sup> §	8 × 10 <sup>3</sup> §	4 × 10 <sup>3</sup> §	4 × 10 <sup>3</sup> §	6 × 10 <sup>3</sup> §	SC	SC

\* All results are expressed as number of colonies/10<sup>6</sup> cells. Agar CFC were plated with (+) or without (-) GM-CSF. BFU-E and CFU-E were plated with (+) or without (-) erythropoietin.

‡ Normal colonies of granulocytes and macrophages. Control AMS-treated cultures were discarded after 20 wk. Numbers in parenthesis (week cultured) represent number of weeks after FLV-P infection).

§ Colonies of undifferentiated erythroblasts with occasional hemoglobinized cells.

|| Not determined.

¶ Small clusters of undifferentiated cells.

TABLE IV  
*Karyotype of Cell Lines Derived from FLV-P-infected Cultures*

Cell line	Chromosome number (centromeres)*					Polyploid	38 + 1 mc‡	38 + 2 mc	39 + 1 mc	40 + 1 mc
	38	39	40	41	42					
	%									
AV-1	0	21	28	2	0	15	2	1	15	16
AV-2	1	1	80	1	1	13	0	0	0	3

\* 100 metaphases were scored for each cell line. Chromosome analysis was performed as previously described (35, 36).

‡ Metacentric chromosomes.

i.e., they are producing SFFV; they show proliferation of erythroblasts with production of 1-5% hemoglobinized cells; and cells can be cloned in soft gels and produce spleen colonies in mice.

Of major interest is that from these secondary cultures, permanently growing autonomous cell lines could readily be established, i.e., when the nonadherent cells were removed from the secondary cultures (even after only 2 wk) and diluted in growth medium, proliferation occurred and the cells could be routinely subcultured. This growth occurred autonomously of added regulators such as erythropoietin. Furthermore, these cells have become completely independent for growth on the marrow-adherent cells. Of four such cell lines so far examined, the majority of cells consist of primitive erythroid cells (similar to those shown in Fig. 3c and e), show low levels of spontaneous hemoglobinization (1-5% of cells), and can be cloned in soft agar with a plating efficiency of 10-35% and show a population doubling time of 12-18 h. Karyotype analysis of two cell lines is shown in Table IV. Both cell lines show the development of aneuploidy. Of some interest is that the addition of dimethyl sulfoxide at final concentration of 1.5, 2, 3, or 4% did not lead to enhanced production of hemoglobinized cells over a 7-d incubation period.

### Discussion

In the erythroid lineage, a complex series of events occurs after FLV-P infection *in vivo*, including an increase in the number of hyperbasophilic erythroid cells, the production of erythropoietin-independent erythroid progenitor cells, and a marked polycythemia leading ultimately to the generation of Friend tumor cells, which are able to grow autonomously *in vitro* and "spontaneously" produce hemoglobinized cells (6). We have now shown that all these aspects of Friend disease can be reproduced *in vitro*.

The experimental data are best considered in two parts: (a) FLV-P infection of normal long-term cultures, and (b) FLV-P infection of AMS-induced erythroid cultures.

(a) In the first case, we have consistently found that in the conditions used, FLV-P regularly induces the production of low but significant levels of morphologically recognizable erythroid cells as well as the production (1 wk after infection) of a large number of CFU-E (at least 50% of which will grow independently of added erythropoietin). However, hemoglobinized cells are present only for 8–9 wk after infection, and erythroid progenitor cells cannot be detected by 12 wk after infection. Because long-term cultures normally contain relatively high levels of primitive BFU-E (forming colonies after 10 or 14 d incubation), and progressively fewer late BFU-E (forming colonies after 4 or 7 d incubation) and CFU-E (28, 29), it is not possible to determine whether the massive increase in CFU-E observed 7 d after FLV-P infection is due to a feed-in from the early BFU-E compartment, and/or amplification from a later progenitor cell (the day 4 BFU-E or the CFU-E). Nonetheless, infection with FLV-P is certainly having a greater effect on the generation of CFU-E rather than the primitive BFU-E, which are retained at control levels. Furthermore, the erythropoietin-independent production of mature erythrocytes in the long-term cultures, and in methocel, suggests that the sensitivity of the CFU-E to this hormone has been markedly altered. This agrees with the effects of FLV-P observed by others (16, 17). However, the major feature to emerge from this work is that although FLV-P infection generated CFU-E formation and erythropoietin-independent erythropoiesis, this was not sufficient by itself to give a sustained erythropoiesis or to lead to the production of tumor cell lines.

Another point is worthy of comment: infection of noninduced or AMS-induced cultures with FLV-P does not lead to a marked increase in the CFU-S or committed granulocyte progenitor cells, the GM-CFC. This contrasts with the effects of FLV-P *in vivo* (34) and is in direct contrast to our earlier *in vitro* studies, using Friend erythroleukemia virus (35), where marked increases in CFU-S and GM-CFC were seen. However, in these earlier studies, the virus preparations had not been cloned and the growth conditions *in vitro* were different from those described herein, i.e., the growth medium used did not facilitate erythropoiesis. Consequently, it is difficult to ascribe the effects observed in our previous cultures to FLV-P. The increase in the numbers and maintenance of CFU-S and GM-CFC (35) may have been only indirectly related to viral infection (a leukemoid reaction?) and the generation of permanently growing cell lines (36) may be related to infection with the Friend helper component, the F-MuLV. However, recent work by Greenberger et al. (37), who also found that FLV-P infection of long-term marrow cultures had a marked effect upon granulopoiesis and GM-CFC production, suggests that the SFFV<sub>P</sub> may also be

implicated in the generation of "promyelocytic" leukemia cell lines. Nonetheless, the data presented here clearly show that infection with cloned FLV-P per se is not sufficient to induce hyperplasia of the stem cell or granulocytic progenitor cell compartments in circumstances where one of the biological activities of FLV-P infection (erythropoietin-independent erythropoiesis) is induced. In other words, in non-AMS-induced FLV-P-infected cultures, the multiparametric nature of Friend disease in vivo has been restricted to only one cell lineage.

(b) Infection of AMS-induced (erythroid) cultures with FLV-P gives a biological response consistent with the effects observed in vivo, i.e., erythropoietin-independent erythropoiesis occurring concomitantly with a massive production of primitive erythroid cells. Unlike non-AMS-induced cultures, this erythroid hyperplasia is maintained for a prolonged period without the necessity of further addition of an erythropoietic stimulus (AMS). This raises several intriguing questions.

First, by what mechanism are these erythroid cells being generated? The simplest explanation would be to assume that the late erythroid cells are continually being generated from the BFU-E, a progression that is mediated by FLV-P infection. This seems unlikely, however, because a sustained erythropoiesis does not occur in non-AMS-induced cultures that are productively infected with FLV-P and where a transient erythropoietin independent erythropoiesis is seen. Therefore, a more likely explanation is that relatively late (post-BFU-E) erythroid cells are undergoing extensive amplification and/or self renewal as a result of FLV-P infection. If so, the obvious difference between noninduced and AMS-induced cultures would be the initial number of target cells that were present at the time of viral inoculation. Noninduced cultures show only low levels of CFU-E. On the other hand, AMS-induced cultures, at the time of virus treatment, were undergoing extensive erythropoiesis and showed significant production of the whole range of clonogenic progenitor cells (from the day 10 BFU-E to the day 2 CFU-E). In this respect, it is significant that the target cells for erythropoietin-independent "bursts," seen after in vitro infection of marrow cells with FLV-P, are BFU-E that attain maximal size 4-5 d after infection and probably represent a cell midway between day 10 BFU-E and day 2 CFU-E (38). This leads us to suggest that the marked effects of FLV-P infection on AMS-induced cultures is a reflection of the initial number of target cells present. Accepting that there is no continuous generation of these target cells from the primitive BFU-E, the sustained erythropoiesis over many months indicates that at least some of these target cells are capable of self-renewal. In fact, this is supported by our data (Table III), which show that 8 wk after FLV-P infection, there were no detectable normal progenitor cells (CFU-S or day 14 BFU-E) in the cultures, although large scale production of erythroid cells (some of them "spontaneously" hemoglobinizing) was still occurring.

Therefore, at least two components have so far been recognized, perhaps not mutually exclusive: the generation of erythropoietin independency, and the self-renewal of target cells.

Finally, there is the question of autonomous growth: the production of Friend tumor cell lines. Thus far, we have been unable to produce permanently growing cell lines by subculturing cells directly from the primary, FLV-P-infected cultures. This is somewhat surprising in view of the finding that 8 wk after FLV-P infection, the primary cultured cells will readily grow in soft-gel media in the absence of added regulatory factors. But, when the cells from the primary cultures were subcultured in

liquid media, no sustained growth was seen. However, when the cells were reseeded onto a fresh marrow-adherent layer, extensive proliferation was seen and autonomously growing cell lines could routinely be established from such secondary cultures. These lines are erythroid in character, are aneuploid and are (so far) not inducible with dimethyl sulfoxide (19). These data are reminiscent of the description of Friend disease in vivo. The early stages of the disease are notoriously difficult to transplant (6, 22), and cell lines are difficult to produce in vitro. In fact, the majority of Friend tumor cell lines in vitro have been produced from subcutaneously transplanted Friend tumor cells (18, 39), i.e., passage in vivo somehow enhances the capacity of cells to grow independently of host environmental influences and be propagated as in vitro cell lines. That the host environment is indeed important in the development of Friend tumor cells is indicated by a recent study (23) that only in the late stages of Friend disease (10 wk after infection) were the cells able to colonize the spleens of environmentally defective Steel (*Sl/Sl<sup>d</sup>*) mice, i.e., to proliferate independently of the environment. Also, studies using F-MuLV have demonstrated that only the late stages of the leukemia are transplantable and that from these secondary recipients, permanently growing cell lines can be established.<sup>2</sup> This initial host dependency of developing leukemic cells is not unique to the Friend-associated viruses. Leukemias induced by chemicals (40) or by certain strains of Rad-LV (radiation-induced viruses) (41) also demonstrate the intimate interactions between the host and the developing leukemic cell.

Our finding that permanently growing cell lines can only be established after a further passage of the cells onto a marrow environment (the adherent layer) seems analogous with transplantation of tumor cells in vivo, and represents a third component of the Friend disease in vitro. The lack of dimethyl sulfoxide inducibility of these cells allows them to be tentatively placed in the stage III category of Friend leukemic cells discussed by Levy et al. (39). In this scheme, a later stage of in vitro transformation, manifested by dimethyl sulfoxide inducibility (stage IV), is described. It remains to be seen whether our cell lines will undergo this further progression.

In conclusion, we have shown that in appropriate conditions Friend disease can now be reproduced in vitro. This system allows sequential analysis of events occurring at all developmental levels of the hemopoietic system; and by using the wide variety of clonogenic and molecular biological techniques now available, the system should be very useful for studying events occurring in so-called tumor progression to malignancy. At the present, we are investigating the role of the helper-independent and replication-defective transforming viruses (the anemia and the polycythemia strains) in the generation of the different stages of Friend disease in vitro and examining the importance of the Fv-2 gene locus (42) in marrow chimeras in vitro.

### Summary

In long-term marrow cultures, hemopoiesis can be maintained for several months, although erythropoiesis is normally suppressed at the most primitive level of development (the erythroid colony-forming cells). Infection of these cultures with a viral complex combining helper-independent murine leukemia virus (F-MuLV) and a

<sup>2</sup> Oliff, A., S. Ruscetti, E. C. Douglass, and E. Scolnick. Isolation of transplantable erythroleukemia cells from mice infected with helper-independent Friend murine leukemia virus. Manuscript submitted for publication.

spleen focus-forming virus (SFFV<sub>P</sub>) results in a productive infection of both the replication defective SFFV<sub>P</sub> and the F-MuLV. After infection, the cultures show a dramatic elevation in the numbers of late erythroid progenitor cells (CFU-E), many of which will grow in the absence of added erythropoietin, and a transient erythropoietin, independent erythropoiesis, including the production of mature, enucleated erythrocytes. Hemopoiesis eventually declines, with no evidence for the generation of Friend tumor cells. When erythropoiesis is induced in the long-term cultures by addition of anemic mouse serum before infection by polycythemia-inducing Friend virus, the generation of erythropoietin-independent CFU-E and erythrocyte formation is followed by the sustained production (>40 wk) of primitive erythroid cells with low spontaneous levels (<5%) of hemoglobinization. Although these cells will produce spleen colonies in irradiated mice and can be cloned in soft-gel media, they do not produce autonomous, permanently growing cell lines in vitro, i.e., they retain a dependency upon the marrow-adherent layer for their continued growth. However, following a further passage on a "virgin" marrow environment, permanent cell lines can be established that are able to grow independently of environmental influences. Thus, this system is the first description of a complete in vitro system for the reproducible production and isolation of Friend virus-induced erythroid cell lines.

We would like to thank Dr. David Scott for allowing us to publish the karyotype data and Ms. J. Trent and Mr. G. R. Bennion for excellent technical assistance.

*Received for publication 4 May 1981.*

### References

1. Friend, C. 1957. Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. *J. Exp. Med.* **105**:307.
2. Mirand, E. A., T. C. Prentice, J. G. Hoffman, and J. T. Grace. 1961. Effect of Friend virus in Swiss and DBA/1 mice on <sup>59</sup>Fe uptake. *Proc. Soc. Exp. Biol. Med.* **106**:423.
3. Steeves, R. A., R. J. Eckner, M. Bennett, E. A. Mirand, and P. J. Trudel. 1971. Isolation and characterization of a lymphatic leukemia virus in the Friend leukemia virus complex. *J. Natl. Cancer Inst.* **46**:1209.
4. Axelrad, A. A., and R. A. Steeves. 1964. Assay for Friend leukemia virus: rapid quantitative method based on enumeration of macroscopic spleen foci in mice. *Virology.* **24**:513.
5. Metcalf, D., J. Furth, and R. F. Buffet. 1959. Pathogenesis of mouse leukemia caused by Friend virus. *Cancer Res.* **19**:52.
6. Tambourin, P. E., F. Wendling, C. Jasmin, and F. Smadja-Jaffe. 1979. The pathophysiology of Friend leukemia. *Leukemia Res.* **3**:117.
7. Troxler, D. H., and E. M. Scolnick. 1978. Rapid leukemia induced by cloned Friend strain of replicating murine type C virus. *Virology.* **85**:17.
8. Ruscetti, S., D. H. Troxler, D. L. Linemeyer, and E. M. Scolnick. 1979. Three distinct strains of spleen focus forming virus: comparison of their genomes and translational products. *J. Virol.* **33**:140.
9. MacDonald, M. E., F. A. Reynolds, W. J. M. Van de Ven, J. R. Stephenson, T. W. Mak, and A. Bernstein. 1980. Anemia- and polycythemia-inducing isolates of Friend spleen focus-forming virus. Biological and molecular evidence for two distinct viral genomes. *J. Exp. Med.* **151**:1477.
10. Troxler, D. H., J. K. Boyars, W. P. Parks, and E. M. Scolnick. 1977. Friend strain of spleen focus-forming virus: a recombinant between mouse ecotropic viral sequences related to xenotropic virus. *J. Virol.* **22**:361.

11. Sveda, M. M., and R. Soeiro. 1976. Host restriction of Friend leukemia virus: synthesis and integration of the provirus. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2356.
12. Jolicoeur, P., and D. Baltimore. 1976. Effect of Fv-1 gene product on proviral DNA formation and integration in cells infected with murine leukemia virus. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2236.
13. Yoosok, C., R. Steeves, and F. Lilly. 1980. Fv-2<sup>r</sup>-mediated resistance of mouse bone marrow cells to Friend spleen focus-forming virus infection. *Int. J. Cancer.* **26**:101.
14. Mak, T. W., A. A. Axelrad, and A. Bernstein. 1979. Fv-2 locus controls expression of Friend spleen focus-forming virus specific sequences in normal and infected mice. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5809.
15. Blank, K. J., R. A. Steeves, and F. Lilly. 1976. The Fv-2<sup>r</sup> resistance gene in mice: its effect on spleen colony formation by Friend virus-transformed cells. *J. Natl. Cancer Inst.* **57**:925.
16. Clarke, B. J., A. A. Axelrad, M. M. Shreeve, and D. L. McLeod. 1975. Erythroid colony induction without erythropoietin by Friend leukemia virus in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **72**:3550.
17. Hankins, W. D., and D. Toxler. 1980. Polycythemia- and anemia-inducing erythroleukemia viruses exhibit differential erythroid transforming effects in vitro. *Cell.* **22**:693.
18. Friend, C., M. C. Patuleia, and E. de Harven. 1966. Erythrocytic maturation in vitro of murine (Friend) virus induced leukemic cells. *Natl. Cancer Inst. Monogr.* **22**:505.
19. Friend, C., W. Scher, J. G. Holland, and T. Sato. 1971. Hemoglobin synthesis in murine virus-induced leukemia cells in vitro. Stimulation of erythroid differentiation by dimethyl sulfoxide. *Proc. Natl. Acad. Sci. U. S. A.* **68**:378.
20. Marks, P. A., and R. A. Rifkind. 1978. Erythroleukemic differentiation. *Ann. Rev. Biochem.* **47**:419.
21. Liao, S. K., and A. A. Axelrad. 1975. Erythropoietin-independent erythroid colony formation in vitro by hemopoietic cells of mice infected with Friend virus. *Int. J. Cancer.* **15**:467.
22. Friend, C., and J. R. Haddad. 1959. Local tumor formation with transplants of spleen or liver of mice with a virus-induced leukemia. *Proc. Am. Assoc. Cancer Res.* **3**:21.
23. Mager, D., T. W. Mak, and A. Bernstein. 1980. Friend leukemia virus-transformed cells, unlike normal stem cells, form spleen colonies in Sl/SI<sup>d</sup> mice. *Nature (Lond.)*. **288**:592.
24. Revoltella, R., L. Bertolini, and C. Friend. 1979. In vitro transformation of mouse bone marrow cells by the polycythemia strain of Friend leukemia virus. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1464.
25. Dexter, T. M., T. D. Allen, and L. G. Lajtha. 1977. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J. Cell Physiol.* **91**:335.
26. Dexter, T. M. 1979. Cell interactions in vitro. In *Clinics in Haematology*. L. G. Lajtha, editor. W. B. Saunders Co. **8**:413.
27. Williams, N., H. Jackson, A. P. C. Sheridan, M. J. Murphy, A. Elste, and M. A. S. Moore. 1978. Regulation of megakaryopoiesis in long-term murine bone marrow cultures. *Blood.* **51**:245.
28. Eliason, J. F., N. G. Testa, and T. M. Dexter. 1979. Erythropoietin-stimulated erythropoiesis in long-term bone marrow cultures. *Nature (Lond.)*. **281**:382.
29. Scolnick, E. M., A. Oliff, D. Linemeyer, S. Ruscetti, N. G. Testa, and T. M. Dexter. 1980. Helper-independent and replication-defective components of the Friend virus complex. In *ICN-UCLA Symposium on Molecular and Cellular Biology: animal virus genetics*. B. H. Fields, R. Jaenisch, and C. F. Fox, editors. Academic Press, Inc., New York. **18**:425.
30. Tambourin, P. E., F. Wendling, O. Gallien-Lartigue, and D. Huaulme. 1973. Production of high levels of erythropoietin in mice. *Biomedicine (Paris)*. **19**:112.
31. Till, J. E., and E. A. McCulloch. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* **14**:213.

32. Wagemaker, G. 1978. Cellular and soluble factors influencing the differentiation of primitive erythroid progenitor cells (BFU-E) in vitro. *In In Vitro Aspects of Erythropoiesis*. M. J. Murphy, editor. Springer-Verlag, New York. 44.
33. Troxler, D. H., S. Ruscetti, D. L. Linemeyer, and E. M. Scolnick. 1980. Helper-independent and replication-defective erythroblastosis inducing viruses contained within anemia-inducing Friend virus complex (FV-A). *Virology*. **102**:28.
34. Wendling, F., P. E. Tambourin, O. Gallien-Lartigue, and M. Charon. 1974. Comparative differentiation and numeration of CFU-S from mice infected either by the anemia or polycythemia-inducing strains of Friend virus. *Int. J. Cancer*. **13**:454.
35. Dexter, T. M., D. Scott, and N. M. Teich. 1977. Infection of bone marrow cells in vitro with FLV: effect on stem cell proliferation, differentiation and leukemogenic capacity. *Cell*. **12**:355.
36. Dexter, T. M., T. D. Allen, D. Scott, and N. M. Teich. 1979. Isolation and characterisation of a bipotential haemopoietic cell line. *Nature (Lond.)*. **277**:471.
37. Greenberger, J. S., R. J. Eckner, W. Ostertag, G. Colletta, S. Boschetti, H. Nagasawa, A. Karpas, R. R. Wichselbaum, and W. C. Maloney. 1980. Release of spleen focus-forming virus (SFFV) from differentiation inducible promyelocytic leukemia cell lines transformed in vitro by Friend leukemia virus. *Virology*. **105**:425.
38. Kost, T. A., M. J. Koury, W. D. Hankins, and S. B. Krantz. 1979. Target cells for Friend virus-induced erythroid bursts in vitro. *Cell*. **18**:145.
39. Levy, S. B., L. A. Blankenstein, E. C. Vinton, and J. J. Chambers. 1979. Biologic and biochemical characteristics of Friend leukemic cells representing different stages of malignant disease. *In Oncogenic Viruses and Host Cell Genes*. Y. Ikawa and T. Odaka, editors. Academic Press, Inc., New York. 409.
40. Haran-Ghera, N. 1973. Relationship between tumor cell and host in chemical leukemogenesis. *Nature (Lond.)*. **246**:84.
41. Haran-Ghera, N. 1980. Pathogenesis of murine leukemia. *In Viral Oncology*. G. Klein, editor. Raven Press, New York. 161.
42. Suzuki, S., and A. A. Axelrad. 1980. Fv-2 locus controls the proportion of erythropoietic progenitor cells (BFU-E) synthesizing DNA in normal mice. *Cell*. **19**:225.