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Isolation and induction of erythroleukemic cell lines with properties of erythroid progenitor burst-forming cell (BFU-E) and erythroid precursor cell (CFU-E)

(retrovirus/erythroid differentiation/erythropoietin)

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ABSTRACT **We isolated erythroleukemic cell lines arrested at different levels of the erythroid differentiation pathway. One cell line (CB5), established from mice infected with the helper-independent Friend murine leukemia virus (F-MuLV), exhibited properties similar to those of the normal erythroid progenitor burst-forming cell (BFU-E). Six erythroleukemic cell lines, which were established from the anemia-inducing Friend virus complex (FV-A)-infected mice, formed erythroid colonies similar to the erythroid colony-forming precursor cell (CFU-E) after induction with dimethyl sulfoxide or erythropoietin. Three lines that were established from the polycythemia-inducing Friend virus complex (FV-P)-infected mice also formed low proportions (2–5%) of CFU-E-like colonies after induction by these same inducers. These data, together with the earlier findings that F-MuLV induces an increase in the levels of BFU-E and that FV-A or FV-P stimulates enhancement of CFU-E early after infection, indicate that the erythroleukemic cell lines isolated late in the diseases are at the same levels of differentiation as the leukemic cells in the corresponding initial stages. These cell lines with properties of BFU-E and CFU-E can be induced to differentiate in culture and should add to our understanding of the nature of erythroid progenitor cells and their early differentiation programs.**

The hemopoietic compartment consists of a hierarchy of cells undergoing concerted steps of differentiation. In this process, cells lose their capacity to proliferate and to renew themselves (1, 2). These losses will remove them from the stem-cell populations and deprive them of the capacity to maintain independent clones. Early differentiated cells resulting from these events are each committed to either the myeloid or lymphoid lineages. Further commitment of these clones will restrict them to more specific lineages (1–3). Unfortunately, these early precursor cells cannot be identified on the basis of their biochemical, morphological, or immunological properties as they represent only minor subpopulations of the total cells from the hematopoietic tissues.

However, these multipotential stem cells or committed progenitor cells can be identified functionally on the basis of colony assays *in vivo* or *in vitro*. These include quantitative assays for the pluripotent colony-forming stem cells, CFU-S (4) and CFU-GEM (5, 6); the granulocytic progenitor, CFU-C (7, 8); the erythroid precursor cell, CFU-E (9); the early erythroid progenitor burst-forming cell, BFU-E (10); the progenitors for B lymphocytes (11); and T lymphocytes (12). In addition to these assays, much effort also has been devoted to the establishment of long-term bone marrow cultures (13, 14) and cell lines from different hemopoietic lineages (15–19). In some cases, these cells can be induced to differentiate along a specific lineage, thus aiding the definition of the differentiation pathways (15–19). Of these cell lines, the best studied are the Friend erythroleukemia cell lines in which a variety of chemicals has induced differentiation along the erythroid pathway (17, 18). These Friend erythroleukemia cells, isolated directly from spleens of mice infected with the polycythemia-inducing strain FV-P of Friend leukemia virus complex (FLV) (20) are relatively mature (probably post CFU-E), and insights into the early BFU-E are not available. Recently, it has been demonstrated that FV-P, FV-A (the anemia-inducing strain of FLV), and helper-independent Friend murine leukemia virus (F-MuLV) can each induce a distinct class of erythroleukemia in susceptible mice (21–23). These findings suggest that erythroleukemic cell lines of distinct properties and maturity may be isolated from animals infected with the different viruses.

In this study, we describe, with the use of these different strains of Friend virus, the establishment of erythroleukemic cell lines with properties of cells at different levels of early and late erythroid pathways.

MATERIALS AND METHODS

Viruses. The replication competent NB-tropic F-MuLV was cloned by limiting dilution as described (22). FV-P and FV-A have been described by MacDonald *et al.* (22).

Mice. BALB/c and DBA/2 mice were obtained from The Jackson Laboratory. Newborn mice were obtained from the animal colony facilities of the Ontario Cancer Institute (Toronto).

Colony Assay for Friend Virus-Induced Tumorigenic Cells (CFU-FV). The assay for CFU-FV was performed as described (24, 25).

Plating of Erythroleukemic Cell Lines on Methylcellulose. Logarithmically growing Friend erythroleukemic cells (1×10^4) were plated onto 0.8% methylcellulose in Iscove-modified Dulbecco's medium with 30% fetal calf serum. The morphology of cells in colonies was determined by staining the cells with benzidine or for spectrin as described (25, 26).

RESULTS

Isolation of Clonal Cell Lines from Mice Infected with Different Types of Friend Virus. Erythroleukemic cells induced by the different strains of Friend virus were plated for CFU-

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Abbreviations: FLV, Friend leukemia virus complex; FV-A, anemia-inducing FLV; FV-P, polycythemia-inducing FLV; MuLV, murine leukemia virus; F-MuLV, helper-independent Friend MuLV; BFU-E, erythroid progenitor burst-forming cell; CFU-E, erythroid colony-forming precursor cell; Me_2SO, dimethyl sulfoxide; CFU-FV, FLV- or F-MuLV-induced tumorigenic cells; SC medium, spleen cell-conditioned medium.

FV, and cell lines were established as described (24, 25). Eight FV-P-induced (TSP1, TSP2, TSP3, TSP4, TSP5, TSP6, TSP7, and TSP8) and six FV-A-induced (TSA1, TSA2, TSA3, TSA4, TSA6, and TSA7) cell lines were maintained in suspension for >2 yr. All of these remained erythroid in character with nearly 100% of them positive for the erythroid marker spectrin (27). However, benzidine-positive cells were absent in all of the FV-A cell lines but present in four of the eight FV-P lines (≈10% of the total cells).

CFU-FV also were plated from erythroleukemic spleens of BALB/c mice infected at birth with cloned F-MuLV (22, 26). Like the CFU-FV in FV-P- and FV-A-infected mice, the CFU-FV induced by injection of F-MuLV were macroscopic and contained a large number of cells (10,000 per colony). In this study, three additional cell lines designated CB3, CB5, and CB7 were established and have been in culture for >2 yr. These cell lines contain essentially 100% spectrin-positive cells but no detectable benzidine-positive cells.

Comparison of the Different Classes of Friend Erythroleukemic Cell Lines. To examine if the cell lines established from F-MuLV-infected spleens will respond to dimethyl sulfoxide (Me_2SO) or the hormone erythropoietin, we incubated the cell lines CB3, CB5, and CB7 with 1% Me_2SO or 0.5 units of erythropoietin per ml in suspension cultures, and the percentage of benzidine-positive cells was monitored. Benzidine-positive cells were not detected (<0.1%) after 7 days of incubation.

The inability to detect benzidine-positive cells in these F-MuLV-induced lines may be due to the possibility that only a subpopulation of these cells responded. Instead of performing the induction experiments in suspension cultures, we treated cell lines with Me_2SO or erythropoietin and plated onto meth-

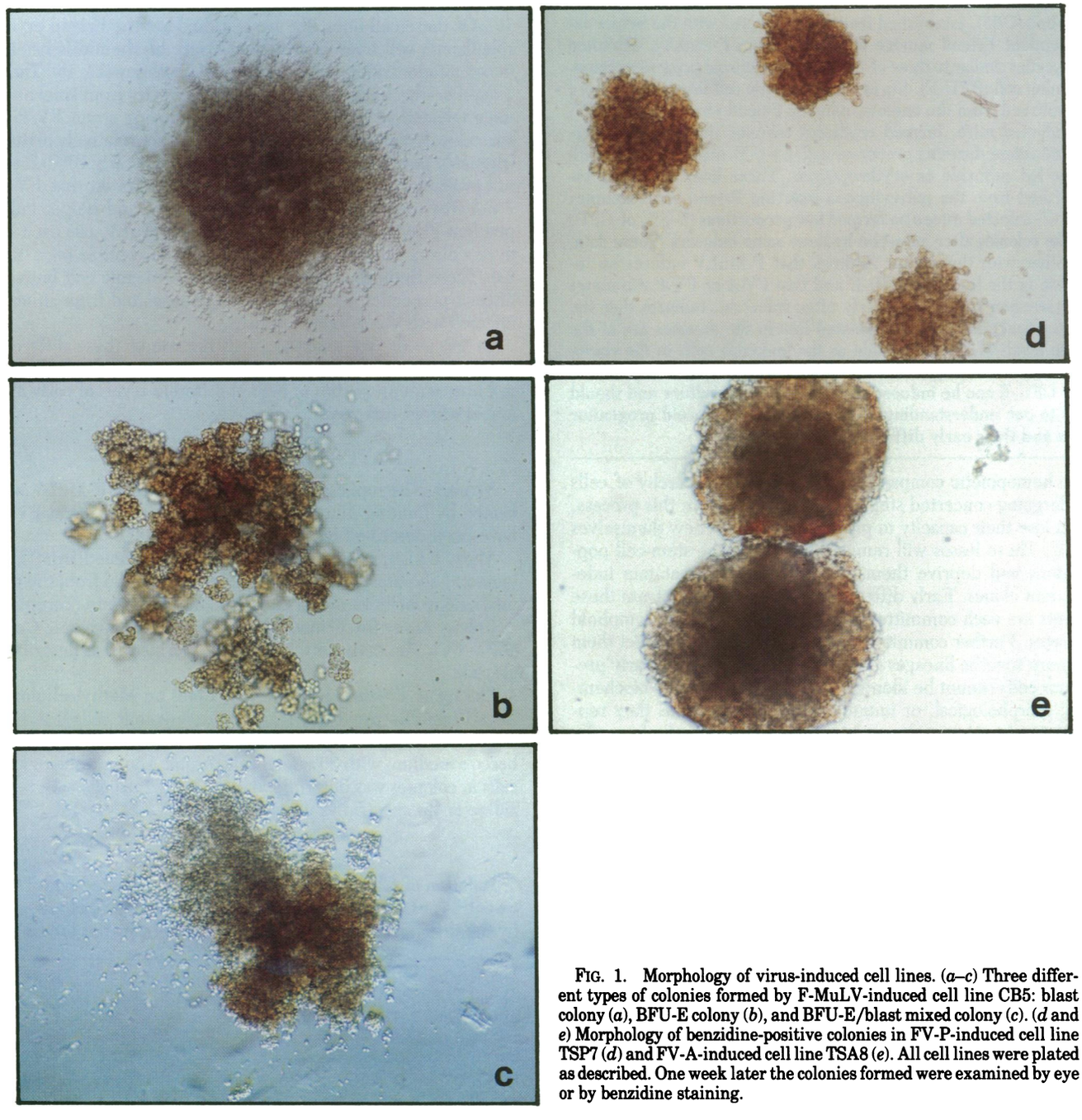

FIG. 1. Morphology of virus-induced cell lines. (*a–c*) Three different types of colonies formed by F-MuLV-induced cell line CB5: blast colony (*a*), BFU-E colony (*b*), and BFU-E/blast mixed colony (*c*). (*d* and *e*) Morphology of benzidine-positive colonies in FV-P-induced cell line TSP7 (*d*) and FV-A-induced cell line TSA8 (*e*). All cell lines were plated as described. One week later the colonies formed were examined by eye or by benzidine staining.

ylcellulose. The number of colonies with benzidine-positive cells was then monitored. Fig. 1 shows two such colonies. They were obtained from one such FV-A-derived line TSA7 (Fig. 1*d*) or FV-P-derived cell line TSP8 (Fig. 1*e*). These colonies contain mainly blast cells, which were not stained by benzidine. Subpopulations of benzidine-positive cells, localized as patches on the colonies, also can be detected (Fig. 1*e*). When the F-MuLV-induced erythroleukemia cell lines CB3, CB5, and CB7 were plated, only blast colonies were detected with CB3 and CB7. With cell line CB5, however, three types of colonies were observed: the blast colonies (Fig. 1*a*) with no benzidine-positive cells, colonies with morphology indistinguishable from the normal BFU-E (Fig. 1*b*), and colonies with mixed BFU-E and blast cells (Fig. 1*c*). These BFU-E-like colonies had the characteristics of clusters of erythroid colonies of about 1,000 cells.

Fig. 2 *Inset* shows that the number of total colonies (including blast colonies, BFU-E-like colonies, and mixed BFU-E/blast colonies) and BFU-E-like colonies increased with the number of cells plated. Cells from the cell line CB5 also were plated in the absence or presence of 1% Me_2SO, 0.5 unit/ml erythropoietin, or 5% spleen cell-conditioned medium (SC medium) (5). In the presence of erythropoietin, the number of each of the three types of colonies increased ≈2-fold (Fig. 2). With Me_2SO, there was no change in the number of blast cell colonies. Instead, a severe selective suppression of the number of BFU-E-like and BFU-E/blast mixed colonies was observed. Under the influence of SC medium, there was a slight increase (<2-fold) in the level of blast cell colonies and a stimulation of ≈4-fold in the BFU-E-like colonies.

Kinetics of Induction of Cell Lines Established from FV-A-, FV-P-, or F-MuLV-Induced CFU-FV. Results in Fig. 3 show the kinetics of the increase in percentage of colonies with benzidine-positive cells. In the absence of any added inducer other than serum (Fig. 3*a*), a high percentage (≈80%) of the colonies from the FV-P-induced cell lines (TSP7 shown) contained benzidine-positive cells. In contrast, only a low percentage of the FV-A-induced cell lines (TSA8 shown) and F-MuLV-induced cell lines (CB5) formed colonies with benzidine-positive cells. The kinetics of the formation of these benzidine-positive colonies were different. The benzidine-positive colonies from cell lines TSA8 (FV-A-induced) and TSP7 (FV-P-induced) appeared between 3 and 5 days, while those from cell line CB5 (F-MuLV-

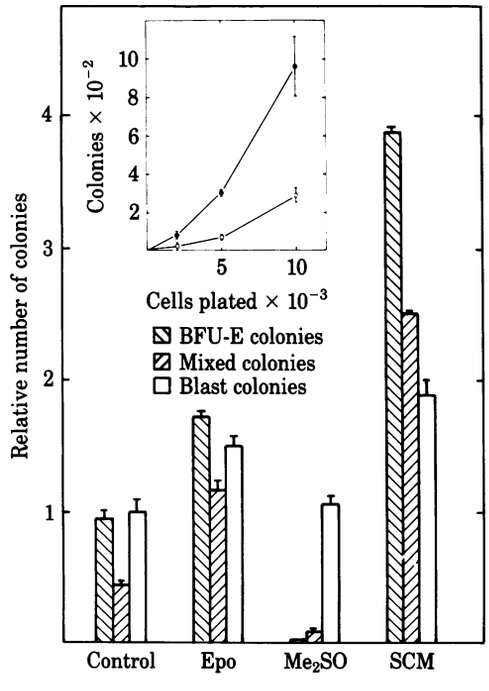

FIG. 2. Relative numbers of blast, BFU-E, or BFU-E/blast mixed colonies in F-MuLV-induced erythroleukemic line CB5 after induction by 0.5 unit/ml of erythropoietin (Epo), 1% Me_2SO, or 5% SC medium (SCM). Cells (1×10^4) from erythroleukemic cell line CB5 were plated as described in the absence or presence of inducer. After 8 days, the number of blast, BFU-E, or BFU-E/blast mixed colonies (see Fig. 1 *a–c*) were counted. The value 1 corresponds to 380 colonies. (*Inset*) Number of total colonies (blast, BFU-E, or BFU-E/blast mixed colonies) (●) and of BFU-E colonies (○) as a function of the number of CB5 cells plated.

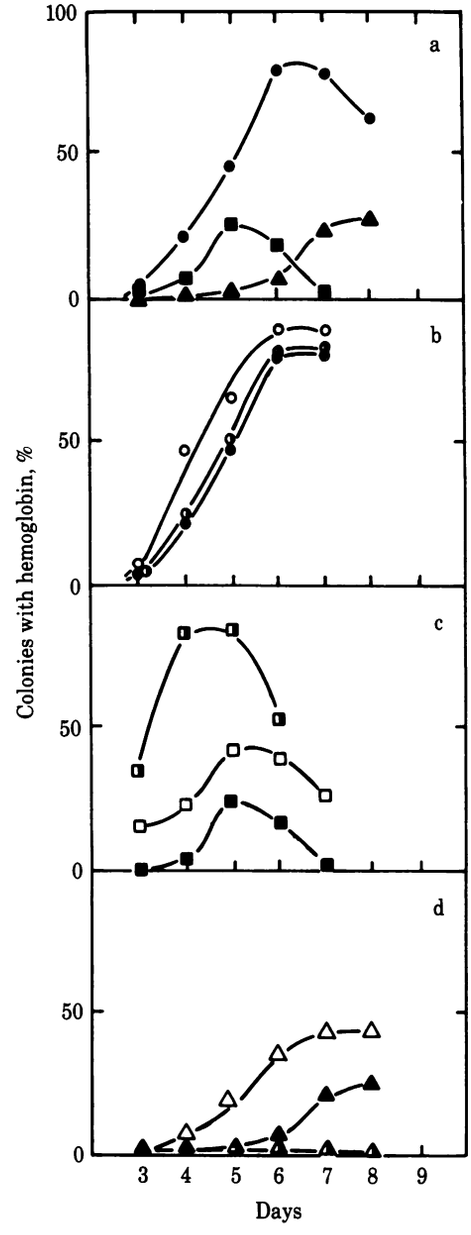

FIG. 3. The kinetics of formation of colonies containing hemoglobin in three distinct classes of erythroleukemic cell lines in the presence or absence of inducers. Three different cell lines established from CFU-FV from (*i*) FV-P-infected DBA/2 mice, TSP7 (●); (*ii*) FV-A-infected DBA/2 mice, TSA8 (■); and (*iii*) F-MuLV-infected BALB/c mice, CB5 (▲) were plated as described in the absence or presence of 0.5 unit/ml of erythropoietin or 1% Me_2SO. At different days after plating, the number and percentages of colonies with hemoglobin were determined by benzidine staining. (*a*) Uninduced cell lines TSP7 (●), TSA8 (■), and CB5 (▲). (*b*) TSP7 in the absence (●) or presence of erythropoietin (○) or Me_2SO (◑). (*c*) TSA8 in the absence (■) or presence of erythropoietin (□) or Me_2SO (◨). (*d*) CB5 in the absence (▲) or presence of erythropoietin (△) or Me_2SO (◭).

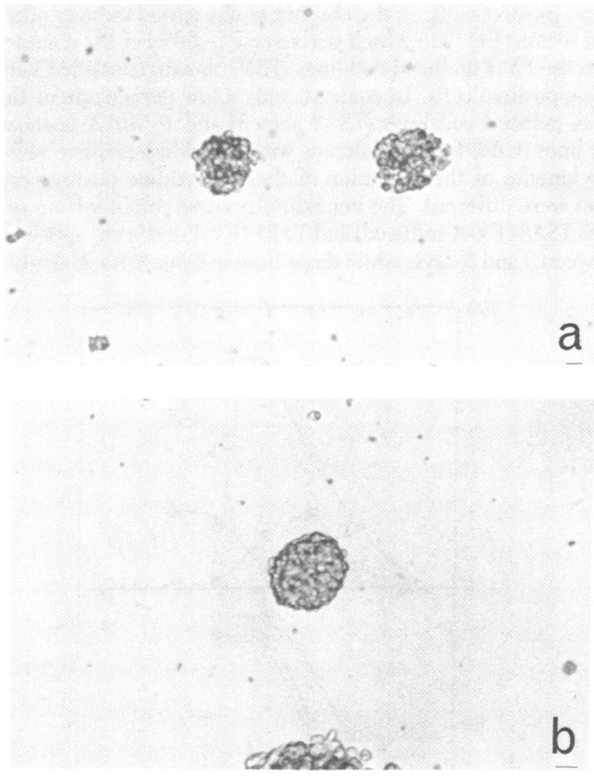

FIG. 4. CFU-E-like colonies from FV-A- and FV-P-induced cell lines after induction with Me_2SO. (*a*) FV-A-induced cell line TSA8. (*b*) FV-P-induced cell line TSP7. Both were plated as described in the presence of 1% Me_2SO. At day 4 after plating, colonies were examined.

induced) appeared in 6–7 days. In the presence of the inducers (Me_2SO or erythropoietin), a variety of responses was obtained. With the use of FV-P-induced cell line TSP7, for example, the percentage of benzidine-positive colonies did not change, although the rates of appearance of these colonies may be slightly faster in the presence of erythropoietin (Fig. 3*b*). However, the FV-A-induced cell line TSA8 showed a dramatic increase in the level of benzidine-positive colonies in the presence of Me_2SO; a smaller increase could be detected with erythropoietin as inducer (Fig. 3*c*).

In the presence of Me_2SO, all of the benzidine-positive colonies formed had morphologies similar to CFU-E (Fig. 4*a*). These CFU-like colonies were formed in all six Me_2SO-induced FV-A-derived cell lines. A low proportion (≈2–5%) of the FV-P-induced cell line colonies also had morphologies similar to CFU-E (Fig. 4*b*). With the F-MuLV-induced erythroleukemic cell lines CB3 and CB7 no benzidine-positive colonies were observed before or after induction with Me_2SO or erythropoietin. When cells from the F-MuLV-induced erythroleukemic clone CB5 were plated, benzidine-positive BFU-E-like and mixed colonies were observed at a level of about 25% (Fig. 3*d*). In the presence of erythropoietin, additional benzidine-positive colonies (up to 50%) were found. The rates of appearance of these colonies were faster. In the presence of Me_2SO, however, no benzidine-positive colonies were observed.

DISCUSSION

With the use of mice of defined genotypes (23) and the newly developed colony assay for CFU-FV (23–26), we have isolated 18 Friend erythroleukemic cell lines from mice infected with either one of the two strains of FLV or the helper-independent F-MuLV. The properties of these Friend erythroleukemic lines are distinct and exhibited properties similar to cells arrested at different stages of the erythroid differentiation pathway.

The cell lines with the most immature erythroid properties are those derived from F-MuLV-induced CFU-FV. They did not respond in culture to the inducers Me_2SO or the hormone erythropoietin. However, in semi-solid medium, one of these cell lines, CB5, exhibited signs of erythroid differentiation. In addition to the base cell colonies, a large proportion of the colonies had morphologies indistinguishable from the normal BFU-E.

It is not yet clear what the relationships are between these BFU-E-like colonies from this clonal cell line CB5 and (*i*) the normal BFU-E (10), (*ii*) BFU-E-like colonies in F-MuLV-infected spleen cells (23, 28), or (*iii*) the BFU-E-like colonies induced *in vitro* by FLV (29, 30). Compared to the normal BFU-E, the BFU-E-like colonies in F-MuLV-infected spleens contain fewer benzidine-positive cells per colony (28). The BFU-E-like colonies induced by FLV *in vitro* (29, 30) and those BFU-E-like colonies in CB5 line need less erythropoietin.

In addition to the effect of erythropoietin on the BFU-E-like colonies, the levels and proportions of these colonies are also affected by Me_2SO and SC medium. SC medium favors the formation of benzidine-positive colonies; Me_2SO has a selective inhibitory effect on them. The finding that Me_2SO suppressed these differentiating erythroid colonies further demonstrated the differences between these F-MuLV-induced erythroleukemia lines and those lines derived from FLV-infected animals. As SC medium is known to promote the growth and differentiation of immature hemopoietic cells (5), the stimulating effect of SC medium is consistent with the hypothesis that the F-MuLV-induced erythroleukemic cells may be more immature. It would be of interest to fractionate these factors and to compare the effects of other stimulating factors known to affect the proliferation capacity and other characteristics of mouse and human BFU-E (31–35).

The erythroleukemic cell lines established from FV-A- or FV-P-infected spleens are more mature than those derived from F-MuLV-induced CFU-FV. First, the FLV lines, especially the FV-P-induced lines, contained low percentages of benzidine-positive cells. Second, the rates of formation of benzidine-positive colonies after induction, a measure of the rate of reaching erythroid maturity, are faster with the FLV lines. And, third, whereas some of the colonies from the FV-P-induced lines and all the FV-A-induced lines had morphologies resembling the more mature CFU-E, those of the F-MuLV-induced line CB5 formed colonies resembling BFU-E.

The relative maturities of the FV-A- and FV-P-induced lines to each other is not clear. In view of the findings that the FV-P-induced lines contain more benzidine-positive cells (25, 36) and are independent of the effect of the hormone erythropoietin, it is tempting to speculate that the FV-P-derived lines may be more mature than those from the FV-A-induced lines.

The exact mechanisms by which the different viruses interact with the hosts to generate those cell lines that apparently are arrested at different stages of erythroid differentiation are not known. It is clear that the Friend diseases induced by the FLV complexes or by F-MuLV are all multistage diseases (23–26, 36, 37). In FLV-induced infection, the spleen focus-forming virus components of FV-A or FV-P are responsible for the main effects of the initial stages of the disease (22, 38). In the case of the F-MuLV infection, however, the agent(s) responsible for the early proliferation stage is not known. It has been proposed that the effects correlate with the appearance of mink cell focus-forming-like viruses (39), which thus may be important. This postulation is interesting as it suggests a similar mechanism to that proposed for other MuLV (40, 41). On the other hand, this hypothesis will be difficult to prove as F-MuLV is also present

in these animals, and this latter agent alone may be capable of inducing rapid erythropoietic proliferation given the appropriate host hemopoietic environment (28, 42).

The evidence that the host hemopoietic environment governs the susceptibility is well established. In FLV infection, susceptibility is controlled by the *Fv-2* locus (43, 44), whereas the susceptibility to early erythroleukemia induction by F-MuLV is governed by the *Fv-6* locus (23). In addition, the F-MuLV-induced erythroleukemia is also governed by developmental controls, as only newborn mice of $Fv\text{-}6^{ss}$ genotype are susceptible (23, 26, 28, 39). Developmental controls can affect susceptibility by modulating the hemopoietic composition as adult mice of both $Fv\text{-}6^{ss}$ or $Fv\text{-}6^{rr}$ genotypes can be rendered partially susceptible by external agents known to modulate the hemopoietic compartment (28, 42, 44, 45).

Several well-defined genes that affect susceptibility and the types of hemopoietic modulation by these viruses have been identified (44, 46, 47), and some of these gene loci (e.g. *Steel*, *w*, and *H-2*) are known to affect normal development as well (44, 46, 47), suggesting that there is a close relationship between the controls of normal and viral-induced hemopoiesis. More recently, it has been proposed that two gene loci, *Fv-2* (43, 45, 48) and *Fv-5* (49, 50), which were discovered originally as determinants affecting viral-induced hemopoiesis, may have roles in normal hemopoietic development (30, 44, 45, 48). This type of mechanism may play an important role in the control of other normal or viral-induced hemopoietic developments.

Finally, there are distinct differences between these early and late leukemic cells. For example, although the early cells are capable of actively dividing *in vivo*, they are incapable of autonomous proliferation in culture (23, 24, 28, 36). In addition, at least for the FV-P-induced erythroleukemia, the early and late leukemic cells are under distinct genetic controls (51). However, in spite of these differences and other possible genetic and epigenetic changes that may occur during the progression, the tumorigenic cells that emerge appear to be, at first approximation, arrested at the same level of differentiation as those found in the early phases. This is illustrated by the cells from the F-MuLV-induced CB5 line which, like those in early F-MuLV-infected spleens, are arrested at the BFU-E level (23, 28), whereas those from FLV-induced cell lines and their early infected spleens are arrested at the CFU-E level (21, 22). Furthermore, the early and late FV-P-induced erythroleukemic cells are independent of the influence of erythropoietin, whereas those from FV-A-induced erythroleukemic cell lines and those in the early spleens are still under its control (21, 22).

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