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Clonal Analysis of the Late Stages of Erythroleukemia Induced by Two Distinct Strains of Friend Leukemia Virus

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We observed striking differences between the tumorigenic colony-forming cells present in the spleens of mice late after infection with the anemia-inducing strain of Friend leukemia virus (strain FV-A) and those present after infection with the polycythemia-inducing strain (strain FV-P). Cells within primary colonies derived from FV-A- and FV-P-transformed cells (CFU-FV-A and CFU-FV-P, respectively) contained hemoglobin and spectrin, indicating that the CFU-FV-A and CFU-FV-P were transformed erythroid progenitor cells. The proportion of cells containing hemoglobin was relatively high (>25%) in newly isolated cell lines derived from CFU-FV-P colonies, whereas cell lines derived from CFU-FV-A colonies had only low levels (0 to 2%) of hemoglobin-containing cells. A high proportion of the cell lines derived from CFU-FV-A colonies responded to pure erythropoietin and accumulated spectrin and hemoglobin, whereas the cell lines derived from CFU-FV-P colonies did not. A cytogenetic analysis indicated that primary CFU-FV-P colony cells were diploid, whereas chromosomal aberrations were observed in the immediate progeny of CFU-FV-A. The presence of unique chromosomal markers in the majority of the cells within individual colonies derived from CFU-FV-A suggested that these colonies originated from single cells. Finally, leukemic progenitor cells transformed by strain FV-A appeared to have an extensive capacity to self-renew (i.e., form secondary colonies in methylcellulose), whereas a significant proportion of the corresponding cells transformed by strain FV-P did not. In addition, the self-renewal capacity of both CFU-FV-A and CFU-FV-P increased as the disease progressed. From these observations, we propose a model for the multistage nature of Friend disease; this model involves clonal evolution and expansion from a differentiating population with limited proliferative capacity to a population with a high capacity for self-renewal and proliferation.

Two distinct isolates of Friend leukemia virus have been described, and both of these isolates induce rapid splenomegaly and erythroleukemia in susceptible adult mice. The original isolate (strain FV-A) (11) induces anemia, whereas the later isolate (strain FV-P), which was derived from stocks of FV-A (38), induces polycythemia. In addition to these opposite effects on the levels of erythrocytes in the peripheral blood, the diseases induced by FV-A and FV-P can be distinguished by the different responses of erythroid progenitor cells to the hormone erythropoietin (Epo). Erythropoiesis in FV-A-infected mice appears to remain under the control of Epo in vivo (49), whereas erythropoiesis in FV-P-infected mice is Epo independent (39). This difference in Epo requirements is reflected in the properties of relatively mature erythroid colony-forming cells in cell cultures; erythroid progenitor cells from FV-P-infected mice can proliferate and differentiate to form small colonies in vitro in the absence of added Epo (21, 26), whereas erythroid progenitors from FV-A-infected mice, although greatly increased in numbers, remain Epo dependent in vitro (8, 29). This difference in Epo responsiveness has also been observed in erythroid bursts formed after in vitro infection of bone marrow cells with FV-P or FV-A (18).

Although infection with either FV-P or FV-A can result in these rapid changes in erythroid progenitor cells, cells in this early phase (<3 weeks) are not tumorigenic and cannot be established as permanent cell lines (13, 48). Later in infection, spleen fragments from Friend virus-infected mice can give rise to tumors in vivo (3,

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4, 13, 48) and cell lines in vitro (14, 25, 43). Based on these observations, it has been suggested that the erythroleukemia induced by Friend leukemia virus is a multistage disease (25, 49). The relationship between the hemopoietic cell populations early and late after infection with Friend virus is unknown. These populations may be related by a process of clonal evolution and expansion, or, alternatively, malignant Friend virus-transformed cells may arise de novo from a population of target cells that are different from the population early after infection with virus. In addition, although differences in the erythroid progenitors early after infection with FV-A and FV-P have been described, little is known about the late malignant stages of the erythroleukemias induced by these two viruses.

To answer these and other questions, we recently developed two colony assays that detect cells capable of extensive proliferation in the late stages of Friend virus-induced erythroleukemia; one of these is an in vivo method in which genetically anemic *S1/S1*^{d} mice are used (31), and the other is an in vitro procedure in methylcellulose (32). The in vitro colony assay detects cells in the spleens of infected animals that are capable of forming macroscopic colonies in methylcellulose 3 weeks after infection with FV-P and 8 weeks after infection with FV-A. These cells are tumorigenic and can be established as permanent cell lines. In this study, we used this colony method to analyze and compare the tumorigenic colony-forming cells that appear late after infection with FV-A and FV-P. We observed significant differences in the properties of these cells and the cell lines established from them. We also suggest several possible models for the origin of the leukemic colony-forming cells in Friend disease.

MATERIALS AND METHODS

Mice and virus. Female strain DBA/2J mice (age, 6 to 10 weeks) were obtained from the Jackson Laboratory, Bar Harbor, Maine. NB-tropic FV-P complex (spleen focus-forming virus strain P [$SFFV_P$] and Friend murine leukemia virus strain P) and NB-tropic FV-A complex ($SFFV_A$ and Friend murine leukemia virus strain A) were obtained from NIH/3T3 fibroblast cell clones productively infected with these viruses (2, 29). The titers of $SFFV_P$ and $SFFV_A$ (in focus-forming units per milliliter) were determined in DBA/2J mice as described previously (1). For all experiments, mice were injected intravenously with either FV-P containing 10^3 focus-forming units of $SFFV_P$ per ml or FV-A containing 10^3 focus-forming units of $SFFV_A$ per ml.

Cell cultures. The assay for Friend virus-transformed colony-forming cells (CFU-FV) was performed as described previously (32). Briefly, single-cell suspensions of individual spleens were made in Iscove modified Dulbecco medium (catalog no. 430-2200; GIBCO Laboratories) containing 15% heat-inactivated fetal calf serum (Flow Laboratories, Inc.). Viable nucleated cells (final concentration, 2×10^5 to 10^6 cells per ml) were plated into 60-mm dishes containing 2% methylcellulose made in Iscove modified Dulbecco medium, 2×10^{-5} M 2-mercaptoethanol, and 30% heat-inactivated fetal calf serum. These dishes were incubated at 37°C for 14 to 16 days, after which large colonies (containing 10^4 to 10^5 cells) were scored by eye.

For the self-renewal studies, individual colonies were picked with a Pasteur pipette and placed into 100 μl of Iscove modified Dulbecco medium containing 15% fetal calf serum. Single-cell suspensions were made by disrupting the colonies with gentle pipetting with a Pipetman. The cell suspensions were then plated onto Iscove modified Dulbecco medium containing methylcellulose, 2×10^{-5} M 2-mercaptoethanol, and 30% heat-inactivated fetal calf serum in 35-mm dishes. Macroscopic colonies were counted 12 to 16 days later.

To establish cell lines, individual colonies were placed in tissue culture wells containing 1 ml of α medium supplemented with 15% heat-inactivated fetal calf serum. As the cells grew, they were diluted with fresh medium every few days. Liquid benzidine staining was performed as described previously (42). The induction studies were performed in wells containing α medium supplemented with 15% fetal calf serum. Purified human Epo (40) was a gift from E. Goldwasser.

Immunofluorescence assay for spectrin. Indirect immunofluorescence assays were performed on single-cell suspensions of individual colonies by a previously described method (28). Briefly, cells were cytocentrifuged onto slides, fixed wtih 3% *para*-formaldehyde, and treated wtih 1% Triton X-100; then 20 μl of rabbit anti-mouse spectrin serum (100 μg of protein per ml) was dropped onto the cells. This antiserum was a gift from H. Eisen, Institut Pasteur, Paris, France, and has been shown to be specific for the protein spectrin (7). After 20 min, the slides were rinsed with phosphate-buffered saline, and 50 μl of a 1/10 dilution of fluorescein isothiocyanate-conjugated $F(ab')_2$ sheep anti-rabbit immunoglobulin $F(ab')_2$ (N.L. Cappel Laboratories Inc.) was dropped onto the cells. After 20 min the slides were rinsed with phosphate-buffered saline at 4°C for 24 to 36 hours. Cells were viewed with a Zeiss microscope equipped with epi-illumination and a filter combination for the detection of fluorescein.

Karyotypic analysis. Individual CFU-FV colonies were picked and placed in 100 μl of Iscove modified Dulbecco medium containing 15% fetal calf serum. The colonies were dispersed, 10 μl of a 1-μg/ml solution of colcemid was added to each preparation, and the cells were incubated at 37°C for 3 h. The cells were then pelleted and suspended in 25 μl of phosphate-buffered saline. To swell the cells, 25 μl of distilled water was added, and the cells were blended in a Vortex mixer periodically for 2 h. Then the cells were fixed in methanol-acetic acid (3:1, vol/vol) for 30 min at room temperature. After two changes of fixative, the cells were dropped onto cold, dry slides. The slides were dried and stained for 5 min with Giemsa solution.

RESULTS

Cell types within colonies. As noted above, we recently described an in vitro assay method that detects FV-P-transformed colony-forming cells (CFU-FV-A) and FV-A-transformed colony-forming cells (CFU-FV-A) in the spleens of DBA/2J mice late after Friend virus infection (32). These cells form large (10^4 to 10^5 cells) morphologically erythroid colonies in methylcellulose cultures. We examined the erythroid nature of the progeny of these leukemic colony-forming cells more directly in this study by using indirect immunofluorescence to detect the presence of spectrin, an inner membrane-bound protein specific to erythroid cells (33, 44). When we examined 15 colonies that were derived from CFU-FV-P and were obtained from four different spleens 4 weeks after infection, we found that all of the colonies contained more than 90% spectrin-positive cells. Figure 1A shows cells from one such CFU-FV-P colony stained for spectrin. Although some cell debris was observed, most of the intact cells stained brightly for spectrin (Fig. 1A). The cells from six colonies derived from CFU-FV-A were also examined for spectrin. All of these colonies also contained more than 90% spectrin-positive cells. However, the majority of the cells derived from CFU-FV-A appeared to stain less brightly with antibody to spectrin than the cells derived from FV-P-induced colonies (Fig. 1A and B). These results demonstrated that both types of colonies contained erythroid cells. Although a high proportion of cells within CFU-FV colonies stained positively with anti-spectrin serum, only 1% (range, 0 to 4%) of the cells in both CFU-FV-P and CFU-FV-A colonies were hemoglobin positive, as detected by liquid benzidine staining. Because the induction of spectrin synthesis appeared to occur before the induction of hemoglobin synthesis in Friend cell erythroid differentiation (7), the majority of the cells in CFU-FV colonies may be at stages of differentiation before the onset of detectable hemoglobin synthesis.

Karyotypic analysis of CFU-FV. A striking karyotypic aberration that appears in apparently all permanent erythroid cell lines derived from the leukemic spleens of Friend virus-infected mice is the presence of one or more large metacentric chromosomes (37, 43, 45). To determine whether the progeny of CFU-FV also contained these biarmed chromosomes or had other chromosomal changes, we analyzed the karyotypes of primary CFU-FV colonies. An analysis of 20 colonies from three different FV-P-infected mice failed to detect metacentric chromosomes in the progeny of CFU-FV-P. We also observed that the cells within CFU-FV-P colonies appeared to be diploid (40 chromosomes). In contrast, most of the progeny of CFU-FV-A derived from three different mice had acquired additional chromosomes (Table 1). It is interesting that, for two of the three mice, the same abnormal number of chromosomes was found in almost every colony derived from the same mouse. A metacentric chromosome was also found in one of the colonies derived from an FV-A-infected mouse (Table 1). Thus, several karyotypic abnormalities appear to be present in the progeny of CFU-FV-A. The observation that the progeny of most CFU-FV-A have a hyperdiploid karyotype is in agreement with previous findings (51) which indicated an increase in the frequency of cells with 41 chromosomes in the leukemic spleens of mice late after infection with FV-A.

The results of a karyotypic analysis of CFU-FV-A colonies were also useful in examining the clonality of individual colonies. We observed that the variation in chromosome number among the cells within a colony was small. Figure 2 shows this variation for nine CFU-FV-A colonies derived from three mice. Unique chromosomal markers were also detected in the progeny of individual CFU-FV-A derived from mouse C (Table 1 and Fig. 2). The cells in one colony contained 41 chromosomes, whereas the cells in a second colony contained 42 chromosomes, 1 of which was metacentric (Table 1). The narrow spread in chromosome number within a colony and the presence of different unique chromosomal markers in colonies derived from the same mouse suggest that each colony was derived from a single cell.

Self-renewal of CFU-FV-A and CFU-FV-P. An expected attribute of tumorigenic transformed cells is the ability to self-renew (that is, the ability to give rise to progeny identical to the original colony-forming cells). To determine quantitatively the self-renewal capacities of CFU-FV-A and CFU-FV-P, colonies were picked and dispersed, and the cells were replated in methylcellulose under conditions identical to those used in the primary colony assay. The number of secondary colonies was then determined 14 to 16 days later. Using pooled primary CFU-FV-P or CFU-FV-A colonies, we found that the number of secondary colonies obtained was a linear function of the number of cells plated (data not shown). Because this linearity was observed for cell numbers ranging from 10^3 to 10^5 cells plated, the replating efficiencies of individual primary colonies (containing 5×10^4 to 10^5 cells) could be compared directly. Thus, individual colonies were replated, and the numbers of secondary colonies were determined. Figure 3 contains a series of frequency histograms

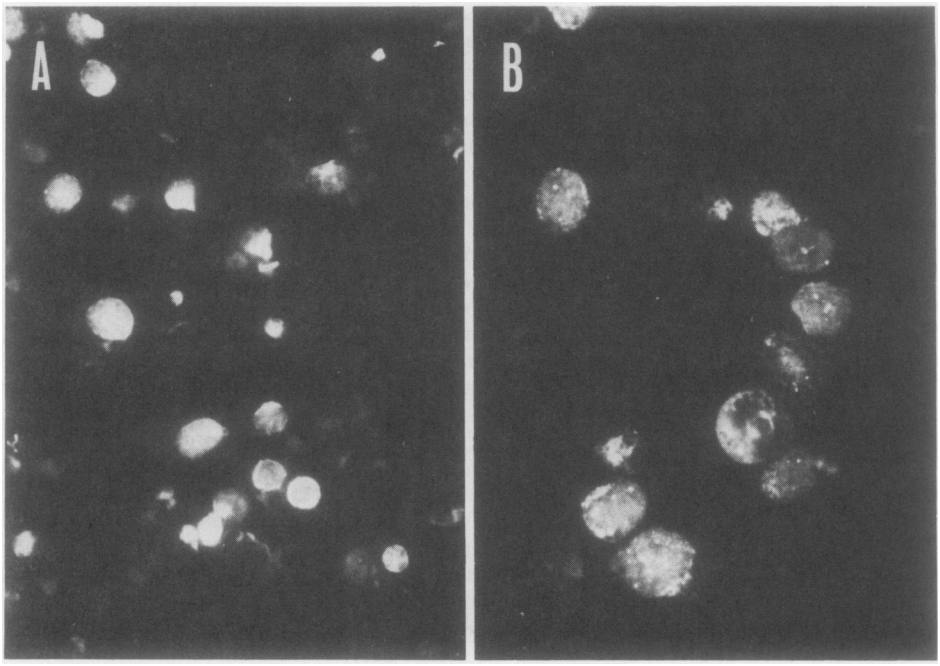

FIG. 1. *Detection of spectrin by immunofluorescence on the surfaces of progeny of CFU-FV. Individual CFU-FV-P (A) and CFU-FV-A (B) colonies were stained for spectrin as described in the text. Photographs were taken at the same magnification. No detectable fluorescence was observed on mouse thymocytes or mouse NIH/3T3 cells stained for spectrin by an identical procedure.*

showing the frequencies at which primary colonies gave rise to a specified number of secondary colonies. This analysis was performed for sets of primary colonies arising from cultures of spleen cells plated at different times after infection with either FV-A or FV-P.

Several observations emerged from this analysis. First, the self-renewal capacities (or the number of secondary colonies per primary colony) of both CFU-FV-A and CFU-FV-P increased with time after infection (Fig. 3). These increases in self-renewal capacity did not correlate with either the size or the appearance of the primary colonies, as no difference was observed in these parameters in colonies obtained at different times after infection. Second, a striking difference between the self-renewal capacities of CFU-FV-P and CFU-FV-A was observed. Although CFU-FV-A are not detected until much later than CFU-FV-P (32), the self-renewal capacity of CFU-FV-A was much higher than the self-renewal capacity of CFU-FV-P (Fig. 3a and b). Whereas all colonies derived from CFU-FV-A gave rise to secondary colonies (Fig. 3b), a significant proportion of colonies derived from CFU-FV-P did not (Fig. 3a). Although not evident from Fig. 3, there was a marked heterogeneity in the self-renewal capacity of CFU-FV-P derived from an individual leukemic mouse 4 to 5 weeks after infection. Colonies that appeared to be identical in the primary dishes gave rise to numbers of secondary colonies ranging from zero to more than 10^3. In contrast, there was much less heterogeneity in the colony-to-colony variation in the self-renewal capacity of CFU-FV-A colonies.

Consistent with the difference in the self-renewal capacities of the colonies derived from CFU-FV-P and CFU-FV-A was the observation that more than 90% of the CFU-FV-A colonies could be placed in liquid culture and established as permanent cell lines. In contrast, only 10 to 20% of the colonies derived from CFU-FV-P could be established as permanent cell lines. Interestingly, if CFU-FV-P colonies were replated and any secondary colonies obtained were placed in liquid culture, approximately 70% of these grew as permanent lines.

Spontaneous hemoglobin synthesis in cell lines derived from CFU-FV. As noted above, permanent cell lines could be established directly from 10 to 20% of the colonies derived from CFU-FV-P and from essentially all of the colonies derived from CFU-FV-A. We have now established over 40 cell lines from CFU-FV-P and approximately 60 cell lines from CFU-FV-A. All of these cell lines grew in suspension, with doubling times varying between 12 and 24 h. In

TABLE 1. *Karyotypes of primary CFU-FV-A colonies*

Mouse	Colony	No. of chromosomes[a]		Presence of metacentric chromosome
		Mode	Range	
A	A1	43	42–43 (39)[b]	–
	A2	43	42–43 (23)	–
	A3	40	39–41 (27)	–
	A4	43	42–45 (7)	–
	A5	43	42–43 (7)	–
	A6	43	41–44 (31)	–
	A7	43	41–43 (11)	–
	A8	43	42–43 (7)	–
B	B1	41	39–42 (31)	–
	B2	41	40–42 (16)	–
	B3	41	40–42 (14)	–
	B4	41	40–41 (9)	–
	B5	41	39–41 (7)	–
C	C1	41	39–41 (33)	–
	C2	42	41–43 (24)	+[c]
	C3	40	39–40 (12)	–
	C4	40	39–40 (10)	–
	C5	40	39–40 (8)	–

[a] The spleen cells of all three mice were plated for CFU-FV-A 10 weeks after infection with FV-A. Karyotyping was done on individual colonies as described in the text. The modal numbers of chromosomes and the ranges in the numbers of chromosomes among the cells within a colony are shown.

[b] The numbers in parentheses are the numbers of chromosomal spreads counted.

[c] For colony C2, one metacentric chromosome was observed in 21 of the 24 spreads counted.

the first few weeks of growth, cell lines derived from the progeny of CFU-FV-P contained a high proportion of cells which were hemoglobin positive. As the cells were passaged in culture, the proportion of cells that spontaneously synthesized hemoglobin declined to 0 to 2%. The vast majority of cell lines derived from CFU-FV-P exhibited this initial high level of hemoglobin-positive cells; examples of three such cell lines are shown in Fig. 4. In contrast to the lines derived from CFU-FV-P, cultures established from colonies derived from CFU-FV-A had very low proportions of hemoglobin-containing cells, even early after the cultures were initiated (Fig. 4). The frequency of hemoglobin-positive cells, as detected by benzidine staining, in CFU-FV-P cell lines declined with time to levels similar to the levels in CFU-FV-A cell lines (Fig. 4). However, the levels of spectrin in the cellular membranes of lines derived from CFU-FV-P were consistently higher than the levels found in CFU-FV-A cell lines even after 16 weeks of culture. These results, which are in agreement with the spectrin studies on primary colonies discussed above, suggest that the primary progeny of CFU-FV-P are more differentiated than the progeny of CFU-FV-A. This difference appears to persist in culture for at least 4 months.

Induction of erythroid differentiation in lines derived from CFU-FV. Most Friend cell lines which have been derived from spleen fragments of Friend virus-infected mice can be induced to synthesize hemoglobin in culture by a variety of chemical agents, including dimethyl sulfoxide (15, 34). However, these cell lines do not respond to Epo (17, 24; unpublished data), the regulator of normal erythropoiesis. We tested several of our newly established cell lines to determine their responses to dimethyl sulfoxide and Epo. Individual cell lines derived by picking individual primary CFU-FV-A or CFU-FV-P colonies 4 to 10 weeks previously were grown for 5 days in 1% (vol/vol) dimethyl sulfoxide or for 4 days in 0.3 U of pure human Epo per ml. The number of hemoglobin-containing cells was then determined by the liquid benzidine assay. We examined a total of 20 cell lines derived from four different FV-P-infected mice and 30 cell lines derived from four different FV-A-infected mice. Figure 5 shows the extents of inducibility of these 50 individual cell lines with dimethyl sulfoxide and Epo. These studies revealed differences between cell lines derived

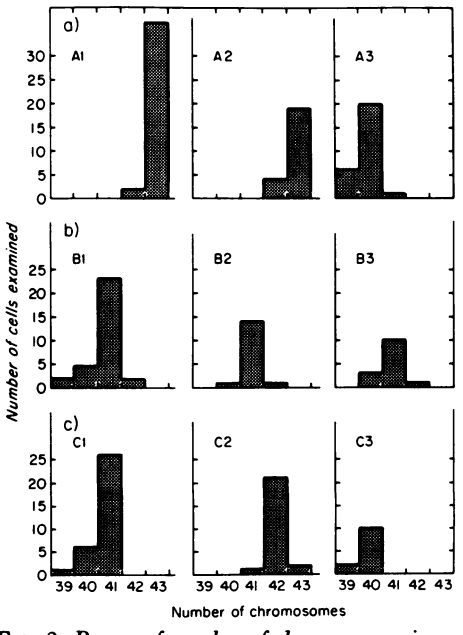

FIG. 2. *Range of number of chromosomes in progeny of individual CFU-FV-A. Each histogram represents the chromosomal counts for cells in a colony listed in Table 1. (a) Three colonies from mouse A. (b) Three colonies from mouse B. (c) Three colonies from mouse C.*

from CFU-FV-P colonies and cell lines derived from CFU-FV-A colonies. Whereas several of the cell lines derived from CFU-FV-P were induced by dimethyl sulfoxide to produce hemoglobin (Fig. 5a), very few of the cell lines derived from CFU-FV-A were induced significantly by this agent (Fig. 5b). Studies on the responses of these cell lines to Epo revealed the opposite result. Cultures of many of the cell lines derived from CFU-FV-A colonies contained high levels of hemoglobin-positive cells after 4 days of growth in 0.3 U of Epo per ml (Fig. 5b), although the sensitivity of some of these lines to Epo tended to decline with time in culture. High levels of spectrin were also induced by Epo in many of these CFU-FV-A cell lines. In contrast, few, if any, of the cell lines derived from CFU-FV-P colonies responded significantly to Epo (Fig. 5a).

DISCUSSION

Normal hemopoietic cell populations consist of hierarchies of cells which differ in the capacity to proliferate and differentiate. Mature differentiated cells have little or no proliferative capacity and hence must be replaced continuously by the progeny of relatively undifferentiated progenitor or stem cells which have an extensive capacity to proliferate and self-renew. Clonal analyses of cell populations late after infection with Friend virus suggest that these virally transformed cells retain certain attributes of normal hemopoietic cells; however, these leukemic colony-forming cells also have several properties that distinguish them from their normal counterparts. Like normal progenitor cells, CFU-FV-A and CFU-FV-P can either self-renew or differentiate and lose the capacity to proliferate. Unlike normal erythroid progenitor cells, the proliferation of CFU-FV-A and CFU-FV-P does not appear to depend upon the addition of either Epo or burst-promoting activity, two regulatory molecules that are required for the proliferation of normal erythroid progenitor cells (22, 47). In addition, leukemic colony-forming cells transformed by FV-P can proliferate to form macroscopic spleen colonies in irradiated mice of genotype *S1/S1*^{d} (31). These genetically anemic mice carry two mutations at the *steel* (*S1*) locus, resulting in a defective cellular microenvironment that prevents spleen colony formation by normal hemopoietic stem cells (35). Thus, leukemic colony-forming cells transformed by Friend leukemia virus appear to be partially or totally independent of both humoral and cellular factors that regulate the behavior of normal hemopoietic progenitor cells.

Differentiation of CFU-FV-A and CFU-FV-P. Although CFU-FV-A and CFU-FV-P have extensive proliferative capacities, an examination of the primary progeny of these colony-forming cells morphologically and with anti-spectrin antiserum suggests that CFU-FV-P may have a higher probability of differentiating than CFU-FV-A. Conversely, the self-renewal capacity of CFU-FV-A is significantly greater than that of CFU-FV-P (see below). The high levels of hemoglobin-positive cells in newly isolated permanent cell lines established from CFU-FV-P colonies and the low levels of hemoglobin-containing cells in cell lines derived from CFU-FV-A colonies also suggest that CFU-FV-P have a higher probability of undergoing erythroid differentiation.

Permanent cell lines derived from CFU-FV-A

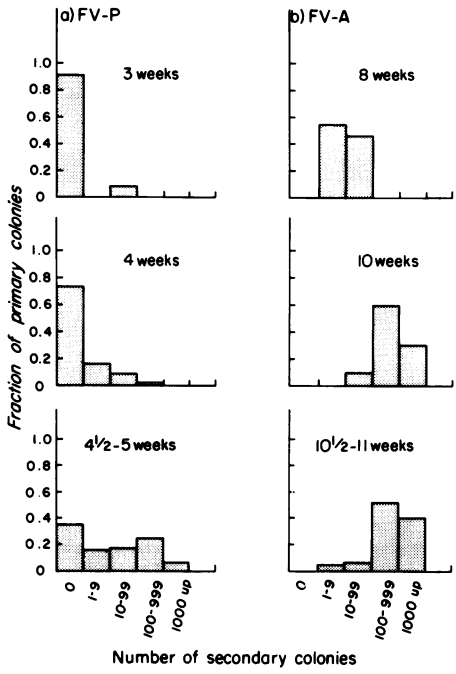

FIG. 3. *Self-renewal capacity of CFU-FV. At different times after infection, spleen cells from individual mice were plated for CFU-FV. Colonies were picked 13 to 16 days later and replated as described in the text. The number of secondary colonies per individual primary colony was determined 14 to 16 days later. For different times after infection, the frequency that primary colonies gave rise to a specified number of secondary colonies is plotted. (a) FV-P infection. The numbers of primary colonies replated were as follows: 3 weeks, 26 colonies from five mice; 4 weeks, 38 colonies from 4 mice; 4.5 to 5 weeks, 93 colonies from six mice. (b) FV-A infection. The numbers of primary colonies replated were as follows: 8 weeks, 20 colonies from two mice; 10 weeks, 24 colonies from three mice; 10.5 to 11 weeks, 33 colonies from three mice. The percentages of viable cells within primary colonies were 30 to 50% for CFU-FV-P colonies and 60 to 80% for CFU-FV-A colonies.*

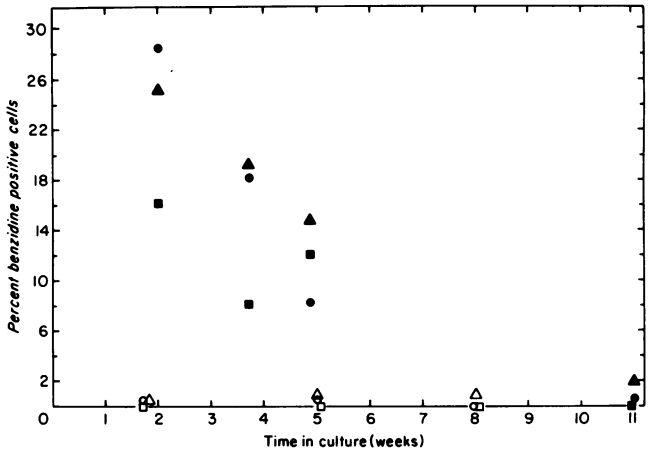

FIG. 4. *Levels of hemoglobin-containing cells in cell lines derived from CFU-FV. The time in culture represents the time from the day on which the primary colony was placed in liquid culture. The solid symbols represent three cell lines established from CFU-FV-P colonies which were derived from the spleen of a mouse infected 5 weeks previously with FV-P. The open symbols represent three cell lines established from CFU-FV-A colonies which were derived from the spleen of a mouse infected 12 weeks previously with FV-A. The assay for hemoglobin-containing (benzidine-positive) cells was performed as described previously (42).*

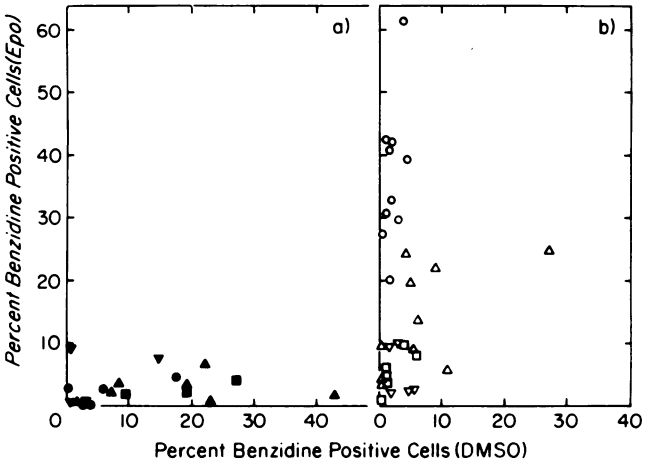

FIG. 5. *Induction of erythroid differentiation in cell lines derived from CFU-FV colonies. Cell lines established from individual CFU-FV-P colonies (a) and cell lines derived from individual CFU-FV-A colonies (b) were stained with benzidine after either 4 days of growth in 0.3 U of pure Epo per ml or 5 days of growth in 1% (vol/vol) dimethyl sulfoxide (DMSO). Each point represents one cell line, and the same symbols represent cell lines established from CFU-FV colonies derived from the same mouse. The percentage of benzidine-positive cells in untreated cultures of the cell lines shown here was less than 3% in all cases. All cell lines had been established from CFU-FV colonies 4 to 10 weeks previously. A minimum of 200 cells were scored for each inducer.*

and CFU-FV-P also differed in the ability to respond to certain inducers of erythroid differentiation. In general, cell lines derived from CFU-FV-P could be induced to synthesize hemoglobin by dimethyl sulfoxide, whereas cell lines derived from CFU-FV-A could not. In contrast, many cell lines derived from CFU-FV-A could be induced to undergo erythroid differentiation by purified human Epo, whereas cell lines derived from CFU-FV-P did not respond to this glycoprotein hormone. These observations are of interest for several reasons. First, other cell lines established from spleen fragments of mice infected with Friend leukemia virus do not respond to Epo (17, 24). However, it has been reported that cell lines obtained from the spleens of mice infected with Rauscher leukemia virus, a virus which induces a disease very similar to that induced by FV-A (20, 49), can be induced by Epo to produce hemoglobin (5). Second, the

availability of clonal cell lines which respond to purified Epo should facilitate studies on the interaction of this hormone with its target cells. And third, the difference in sensitivity to Epo of cell lines derived from CFU-FV-P and CFU-FV-A appears to mimic the difference in Epo responsiveness of erythroid progenitor cells in the early phases of the diseases induced by FV-P and FV-A (29).

Karyotypic analysis. Increases in chromosomal abnormalities are frequently associated with the progression of neoplastic diseases (19, 23, 41). As noted above, all established Friend cell lines have several karyotypic abnormalities, including the presence of one or more metacentric chromosomes (16, 37, 43, 45, 46). Although the cells in most primary CFU-FV-A colonies had a hyperdiploid karyotype, a metacentric chromosome was detected in the progeny of only one CFU-FV-A colony. In addition, no metacentric chromosomes were detected in the primary progeny of CFU-FV-P, which, unlike the progeny of CFU-FV-A, appeared to be strictly diploid. These results suggest that the appearance of metacentric chromosomes may not be an essential event in the initial appearance of tumorigenic CFU-FV.

Two additional conclusions can be derived from the chromosomal analysis of the leukemic colony-forming cells induced by FV-A. First, the observation that the large majority of cells within one colony had the same hyperdiploid chromosome number provides evidence that the CFU-FV-A assay is clonal (that is, that each colony is derived from a single cell). This conclusion is further supported by the observation that the number of CFU-FV-A detected was a linear function of the number of spleen cells plated (32).

An analysis of the colonies derived from CFU-FV-A also revealed a striking predominance of the same hyperdiploid karyotype in different colonies derived from the same mouse. This observation suggests that, at least in some mice, the tumorigenic colony-forming cells arising late in the disease may be derived from only one or a few cell clones. The extremely low numbers of these colony-forming cells in the spleen, even months after virus injection, is also consistent with this conclusion.

Self-renewal analysis of CFU-FV. At all times tested, the self-renewal capacity of CFU-FV-A was considerably greater than that of CFU-FV-P. Indeed, CFU-FV-P colonies derived from mice relatively early after infection had a very low or undetectable self-renewal capacity in vitro. The self-renewal capacity of these leukemic colony-forming cells also increased with time after infection. This was particularly true for CFU-FV-P, although an increase in the self-renewal capacity of CFU-FV-A was also observed as the disease progressed. A comparison of the spontaneous differentiation and self-renewal probabilities of CFU-FV-A and CFU-FV-P suggested that there is an inverse relationship between the proliferative and developmental capacities of these CFU-FV. This conclusion is consistent with findings on the relationship between the colony-forming ability and the extent of differentiation of permanent erythroid cell lines transformed by Friend virus (30).

FV-P and FV-A. The results discussed here and in previous studies on the early stages of Friend leukemia (29) indicate that there are significant differences in the properties of the erythroid progenitor cells in the spleens of mice infected with FV-A and FV-P. The clonal isolates of FV-A and FV-P used in this study were both complexes consisting of a defective SFFV ($SFFV_A$ and $SFFV_P$, respectively) and a replication-competent virus (Friend murine leukemia virus) that served as a helper virus for the defective SFFV genome (29). Characterizations of the diseases induced by different pseudotypes of $SFFV_A$ and $SFFV_P$ rescued with different helper viruses from SFFV nonproducer clones have shown that it is the SFFV components of FV-A and FV-P that specify the unique features of the early stages of the diseases induced by these two viruses (8, 27, 29). Given the central role that these defective viruses appear to play in determining the nature of the early stages of Friend leukemia, it is tempting to speculate that differences in the genomes of these two viruses are also responsible for the differences in the properties of the leukemic colony-forming cells described here.

Models for Friend leukemia. The leukemias induced by FV-A and FV-P appear to be multistage diseases (25, 51) that are characterized by rapid increases in the numbers of relatively mature erythroid progenitor cells with very limited proliferative capacities, followed later by the appearance of tumorigenic colony-forming cells capable of some erythroid differentiation as well as extensive proliferation. The following two distinguishable models can be proposed to explain the origin of the tumorigenic cells present late after infection with Friend virus: a two-population model, in which the late tumorigenic cells are derived from cells different from those transformed early in the disease, and a one-population model, in which the leukemic cells arising late in the disease are derived from early virus-infected cells through a process of clonal evolution or progression (36, 41).

One prediction of the clonal evolution model is that it should be possible to detect cells that, with time after infection, have an increased capacity to proliferate and self-renew and a corresponding decreased capacity to differentiate. As discussed above, our analysis of the self-renewal probabilities of both CFU-FV-A and CFU-FV-P is consistent with this prediction. Also consistent with this model is the observation that the sensitivity to Epo of cell lines derived from CFU-FV-A and CFU-FV-P correlates with the difference in Epo dependence of erythroid progenitor cells present early after infection with FV-A and FV-P. This result indicates that the tumorigenic cells and the early erythroid progenitors present in Friend virus-infected mice may share common characteristics and hence may be related.

Alternatively, the two-population model suggests that the truly leukemic cells are not related clonally to the erythroid progenitor cells affected early after Friend virus infection. The identity of these possible second target cells for malignant transformation is unknown. A number of human myeloproliferative disorders have been shown to have a clonal origin in pluripotent hemopoietic stem cells (9), and it has been suggested that Friend virus-transformed tumorigenic cells may also result from the malignant transformation of pluripotent stem cells (50). Although cell lines established from Friend virus-infected spleens have been clearly demonstrated to have only erythroid potential (12, 34), there have been reports that in vitro infection of hemopoietic cells with Friend virus can result in the emergence of permanent cell lines which appear to be bipotential in differentiative capacity (6, 10). Thus, further studies will be necessary to determine the origin of the truly leukemic cells arising during the course of Friend leukemia.

Regardless of the target cell type for transformation in vivo, the differences in the properties of cells malignantly transformed by FV-A and FV-P suggest that the information included on these viral genomes determines the nature of the CFU-FV. However, the time lag between virus infection and the appearance of leukemic colony-forming cells and the heterogeneous properties of these cells both suggest that viral infection per se is not sufficient for this transformation event to occur. Alterations in self-renewal probabilities and concomitant autonomy from normal cellular and humoral regulator factors may also be necessary before truly malignant cell clones emerge.

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