ERYTHROLEUKEMIA INDUCTION BY REPLICATION-COMPETENT TYPE C VIRUSES CLONED FROM THE ANEMIA- AND POLYCYTHEMIA-INDUCING ISOLATES OF FRIEND LEUKEMIA VIRUS*

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In the preceding paper (1), we reported the clonal isolation and characterization of two replication-defective spleen focus-forming viruses: $SFFV_A$, present in stocks of the anemia-inducing isolate of Friend leukemia virus complex (FV-A) and $SFFV_P$, present in the polycythemia-inducing isolate of Friend leukemia virus complex (FV-P). These studies indicated that $SFFV_A$ and $SFFV_P$ can be distinguished by biological and molecular criteria and that, at least in adult mice, the type of erythroleukemic transformation induced by these defective SFFV appears to be independent of whether the helper virus was derived from FV-A or FV-P. These findings, however, do not rule out the possibility that the replication-competent type C viruses, F-$MuLV_A$ and F-$MuLV_P$, present in FV-A and FV-P, respectively, are leukemogenic under certain circumstances. Indeed, other reports have demonstrated that infection of fetal liver cells in culture with an anemia-inducing strain of Friend leukemia virus leads to the generation of permanent cell lines (2), and that injection of newborn BALB/c or NIH Swiss mice with F-$MuLV_A$ is characterized by a splenic leukemia and anemia (3, 4). We report here characterization of the cell populations observed in the leukemic spleens of newborn BALB/c mice infected with either cloned F-$MuLV_A$ or F-$MuLV_P$. The results suggest that both of these replication-competent viruses induce an anemic form of erythroleukemic transformation when administered to newborn, but not adult, mice.

Materials and Methods

Mice. Male and female BALB/c mice, obtained from The Jackson Laboratory (Bar Harbor, Maine) were bred in the animal facility at the Ontario Cancer Institute, Toronto, Canada.

Cells. NIH/3T3 cells (5) cultured in minimum essential medium (α-MEM) (6) without

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Abbreviations used in this paper: EPO, erythropoietin; FFU, spleen focus-forming unit; F-$MuLV_A$, replication-competent virus present in stocks of the anemia-inducing isolate of Friend leukemia virus complex; F-$MuLV_P$, replication competent-virus present in the stocks of the polycythemia-inducing isolate of Friend leukemia virus complex; FV-A, the anemia-inducing isolate of Friend leukemia virus complex; FV-P, the polycythemia-inducing isolate of Friend leukemia virus complex; PBS, phosphate-buffered saline; PFU, plaque-forming unit(s); SFFV, spleen focus-forming virus; $SFFV_A$, the SFFV present in stocks of FV-A; $SFFV_P$, the SFFV present in stocks of FV-P.

nucleosides, supplemented with 10% fetal calf serum (KC Biological, Inc., Lenexa, Kans.) were routinely passaged every 3–4 d by trypsinization.

Virus. An NIH/3T3 fibroblast cell clone productively infected with the FV-A complex ($SFFV_A$ and F-MuLV) is described in the accompanying paper (1). The isolation of NIH/3T3 fibroblast cell clones productively infected with the FV-P complex ($SFFV_P$ and $F\text{-}MuLV_P$) has been described previously (7).

Titers of $F\text{-}MuLV_A$ and $F\text{-}MuLV_P$ in these cell-culture derived stocks were determined using the XC plaque assay as previously described (8) and are expressed as XC-plaque-forming units (PFU) per ml. Titers of $SFFV_A$ and $SFFV_P$ were determined in adult BALB/c mice using the spleen focus assay described previously (9) and are expressed as focus-forming units (FFU) per ml of filtered culture fluid.

Clones of NIH/3T3 fibroblast cells producing replication-competent virus ($F\text{-}MuLV_A$ or $F\text{-}MuLV_P$), free of SFFV, were obtained as follows: after infection at 0.1 PFU/cell with endpoint-diluted FV-A or FV-P, NIH/3T3 cells were immediately cloned at 0.25 cells per microtiter well. The virus released by clones producing XC-plaque-forming virus, but not SFFV activity, was again endpoint-diluted and used to infect NIH/3T3 cells that were cloned as above. Virus stocks produced by clones of NIH-3T3 cells stably releasing high titers of replication-competent virus isolated by this procedure have been designated $F\text{-}MuLV_A$ and $F\text{-}MuLV_P$.

BALB/c mice infected 1 d after birth were inoculated intraperitoneally with 0.1 ml of virus stock. Mice infected as adults (6 wk of age) were injected in a tail vein with 0.5 ml of virus preparation.

Assays for Hemopoietic Cells

MORPHOLOGY. Morphological examination of spleen touch preparations was done using a light microscope (magnification: × 80) after staining the slides with Wright-Geimsa stain.

IMMUNOFLUORESCENCE ASSAYS FOR Thy-1, Ig, AND SPECTRIN. Indirect immunofluorescence assays were done using single-cell suspensions of spleen cells that had been cytocentrifuged onto microscope slides and fixed with 3% paraformaldehyde using a previously described procedure (10). Thy-1 glycoprotein was detected with a rabbit anti-mouse brain Thy-1 glycoprotein serum obtained from Dr. M. Letarte, Ontario Cancer Institute. This serum has been shown to react specifically with Thy-1 glycoprotein (11). Surface immunoglobulin was detected with a rabbit anti-mouse Ig serum. This serum did not react with erythroid cells or thymocytes (data not shown). The membrane-associated protein spectrin is specific for erythroid cells (12) and was detected in fixed cells that had been treated with 0.1% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) (10) followed by rabbit anti-mouse spectrin serum obtained from Dr. H. Eisen, Institut Pasteur, Paris (13). 50 μl of these antisera at 100 μg protein/ml was dropped onto the cells. After 20 min, the sera were removed, the slides rinsed in phosphate-buffered saline (PBS) and 50 μl of a 1/10 dilution of fluorescein isothiocyanate-conjugated $F(ab')_2$ sheep-anti-rabbit Ig $F(ab')_2$ (N. L. Cappel Laboratories Inc., Cochranville, Pa.) was dropped on the cells. After 20 min at room temperature, the slides were rinsed with PBS at 4°C for 24–36 h. Cells were viewed with a Zeiss microscope equipped with epi-illumination and filter combination for the detection of fluorescein (magnification: × 120) (Carl Zeiss, Inc., New York). The proportion of positively staining cells was determined by scoring at least 500 cells in duplicate preparations.

ERYTHROID PROGENITOR CELLS. Erythroid colony-forming cells in single-cell suspensions of spleen or bone marrow were quantitated by plating in 0.8% methylcellulose in Iscove's modified Dulbecco's medium (catalog No. 430-2200; Grand Island Biological Co., Grand Island, N. Y.) using a modification of a procedure described previously (14). Each 1-ml culture contained 1×10^5 viable nucleated cells, 7.5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum (lot No. 4055899; Flow Laboratories, Rockville, Md.). Duplicate cultures contained 0.5 U/ml of Step III sheep erythropoietin (lot No. 3025-1; Connaught Medical Research Laboratories, Willowdale, Ontario, Canada).

After incubation for 2 d at 37°C, erythroid colonies were scored using a light microscope (magnification: × 100). Each culture was done in duplicate and values of erythroid colony formation shown are the average of the values determined in four separate experiments.

Results

Infection of BALB/c Mice with F-MuLV Derived from NIH/3T3 Fibroblast Clones. To determine whether clonal isolates of the replication-competent viruses, $F\text{-}MuLV_A$

and F-MuLV$_P$, obtained from our stocks of FV-A and FV-P, respectively, were leukemogenic in newborn animals, BALB/c mice were infected 1 d after birth with culture fluids from NIH/3T3 fibroblasts productively infected with either F-MuLV$_A$ or F-MuLV$_P$. The spleens were removed at day 35 and examined for pathological changes. As shown in Fig. 1, the enlarged spleens of mice infected with either F-MuLV$_A$ or F-MuLV$_P$ contained large numbers of immature cells of the erythroid series. Furthermore, at day 35 the mice infected with either F-MuLV$_A$ or F-MuLV$_P$ were anemic (20% hematocrit) and histological examination of spleen tissue indicated alterations of splenic architecture similar to that described previously for another helper-independent isolate of F-MuLV (3). To determine the kinetics of F-MuLV$_A$- or F-MuLV$_P$-induced splenic enlargement, BALB/c mice were infected 1 d after birth and spleen weights were determined at various times. Adult, 6-wk-old BALB/c mice were also infected with F-MuLV$_A$ or F-MuLV$_P$. The data shown in Fig. 2 demonstrate that infection of newborn BALB/c mice with either F-MuLV$_A$ or F-MuLV$_P$ resulted in progressive splenomegaly beginning at ~30 d. In contrast, no increase in spleen weight was observed when mice were infected at 6 wk of age with the replication-competent viruses (Fig. 2).

Erythroid Progenitor Cells in BALB/c Mice Infected as Newborns with F-MuLV$_A$ or F-MuLV$_P$. The anemic and polycythemic transformation induced by FV-A and FV-P, respectively, in susceptible mice is associated with alterations in the spleen and marrow erythroid progenitor cell populations (1, 15–17). To determine whether the replication-competent Friend viruses also induced alterations in erythropoiesis, the hematocrit and number of erythroid colony-forming cells was measured 40 d after infection of newborn BALB/c mice with either F-MuLV$_A$ or F-MuLV$_P$. As shown in Table I, mice infected with these viruses were severely anemic and their spleens contained increased numbers of erythropoietin (EPO)-dependent erythroid colony-forming cells.

The kinetics of increase in the numbers of erythroid progenitor cells in the hemopoietic tissue was determined by plating marrow and spleen cells in methylcellulose culture at various times after 1-d-old BALB/c mice were infected with either F-MuLV$_A$ or F-MuLV$_P$. Newborn BALB/c mice were also infected with FV-A or FV-P to provide a comparison of the hematologic effects of these isolates of Friend virus complex. The data shown in Fig. 3 indicate that infection of newborn animals with either F-MuLV$_A$ or F-MuLV$_P$ induced an increase in the numbers of EPO-dependent erythroid colony-forming cells relatively late during infection (day 30) in the spleen, whereas the frequency of erythroid progenitors in the marrow of F-MuLV-infected mice was not significantly altered. Furthermore, as shown in Fig. 3, infection of newborn BALB/c mice with either FV-A or FV-P complex induced the rapid appearance of erythroid colony-forming cells from spleen in methylcellulose culture. As described previously for adult mice (16), infection of newborn animals with FV-P led to the rapid appearance of erythroid progenitor cells capable of forming EPO-independent erythroid colonies in methylcellulose culture although no erythroid colonies were observed in the absence of added EPO during infection with FV-A, F-MuLV$_A$, or F-MuLV$_P$.

Cellular Composition of the Spleens of BALB/c Mice Infected as Newborns with F-MuLV. Alterations in hemopoietic cell populations induced by infection of newborn BALB/c mice with either F-MuLV$_A$ or F-MuLV$_P$ were analyzed further by a

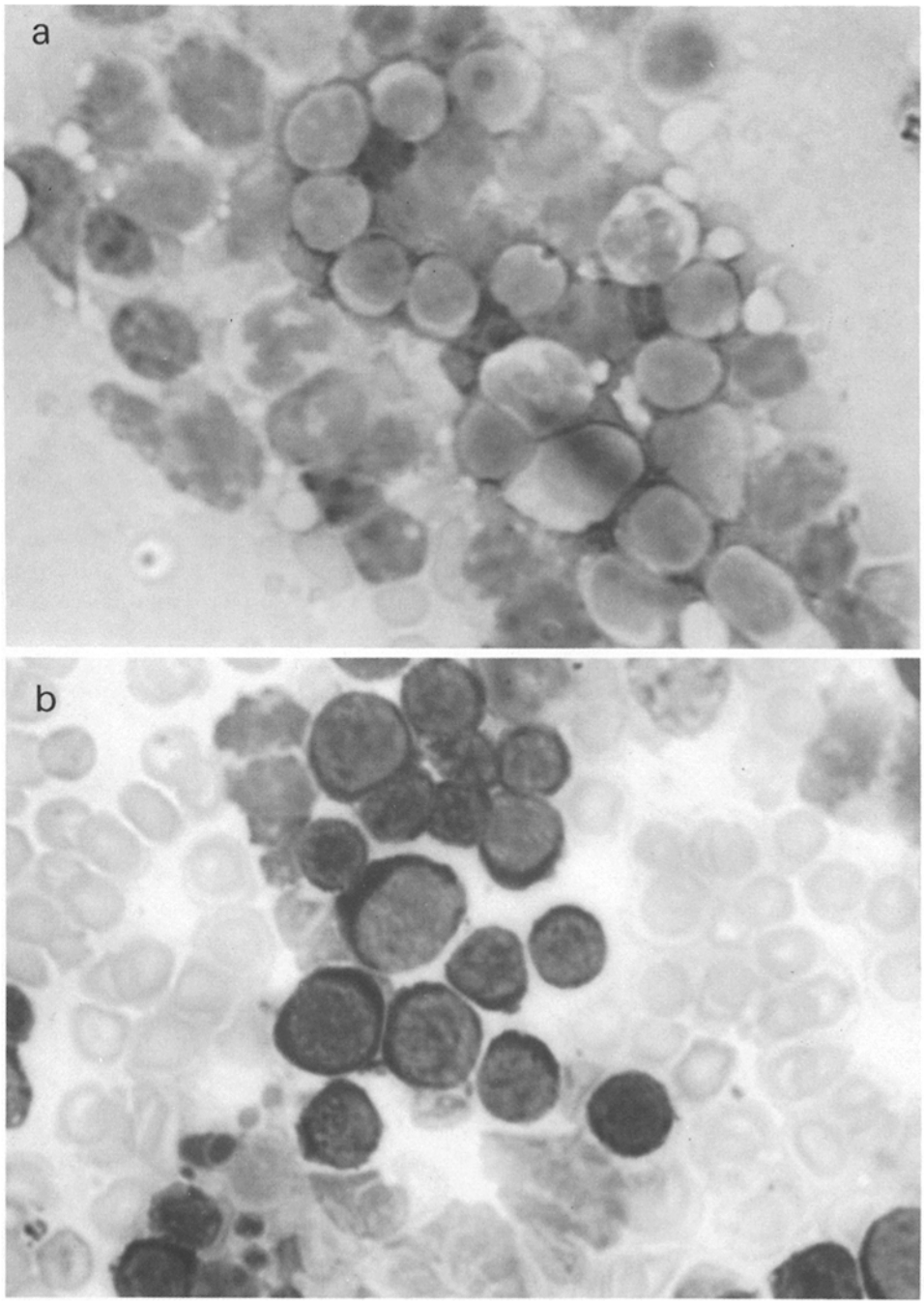

Fig. 1 A and B

FIG. 1. Spleen touch preparations from BALB/c mice infected as newborns with FV-A, F-MuLV$_A$, and F-MuLV$_P$. BALB/c mice were injected intraperitoneally at 1 d of age with (A) 0.1 ml of filtered culture fluid from uninfected NIH/3T3 fibroblast cells, (B) 3×10^3 FFU of SFFV$_A$ in a stock of FV-A, or (C) 5×10^5 PFU of F-MuLV$_P$. Spleens from F-MuLV- and FV-A-infected mice were taken at day 45 and day 25, respectively, for the preparation of touch prints as described in Materials and Methods. × 80.

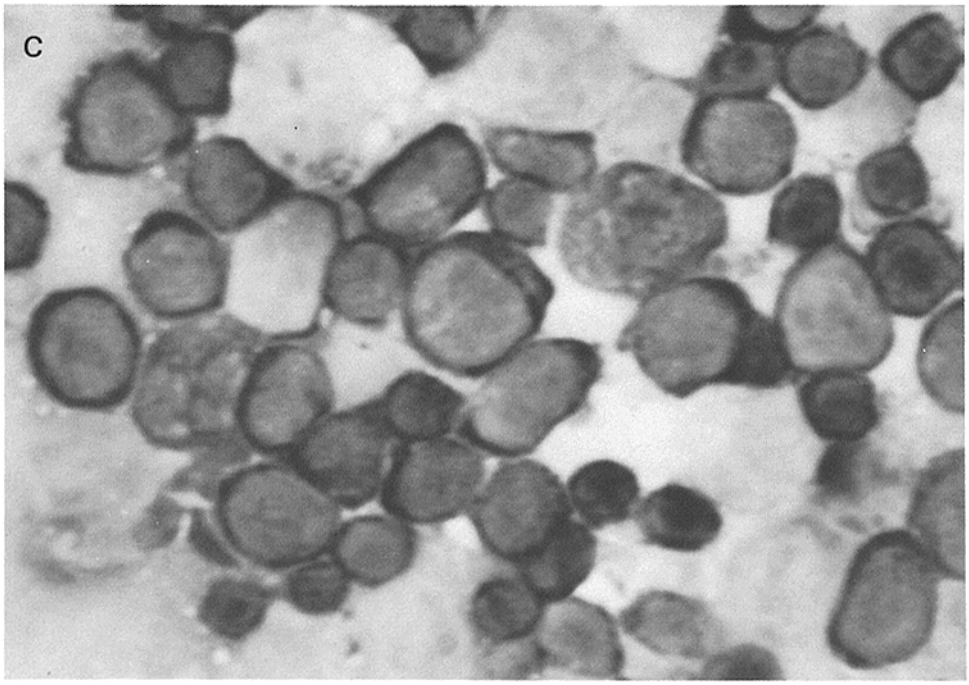

Fig. 1C

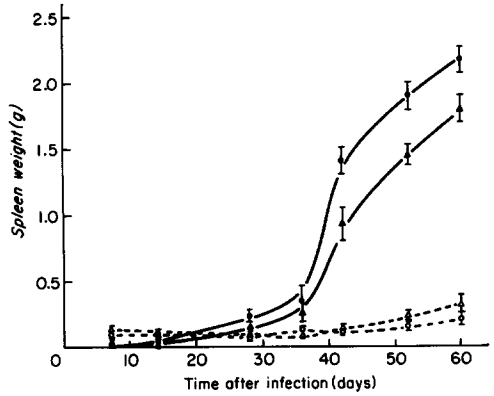

FIG. 2. Effect of F-MuLV$_A$ and F-MuLV$_P$ on spleen weight with time after infection of adult and newborn BALB/c mice. BALB/c mice were injected intraperitoneally 1 d after birth with 5×10^5 PFU of F-MuLV$_A$ (●) or F-MuLV$_P$ (▲) and the spleen weight determined at various times. BALB/c mice 6 wk of age were injected intravenously with 2.5×10^6 PFU of F-MuLV$_A$ (○) or F-MuLV$_P$ (△) and the spleen weights were determined at various times.

determination of the proportion of cells in the enlarged spleens that display Thy-1 glycoprotein, surface immunoglobulin, and spectrin, cell-surface molecules characteristic of differentiated lymphoid, and erythroid cells. The data shown in Table II demonstrate that F-MuLV$_A$ and F-MuLV$_P$, as well as FV-A and FV-P, induce a decrease in the proportion of spleen cells that carry Thy-1 or surface Ig antigens and

TABLE I

*Hematocrit and Erythroid Colony Formation in BALB/c Mice Infected as Newborns with F-MuLV$_A$ or F-MuLV$_P$**

Virus	Hematocrit	Number of erythroid colonies per 10^5 nucleated spleen cells; EPO (U/ml)	
		0	0.5
	%		
Mock‡	49	0	34
F-MuLV$_A$	10	0	758
F-MuLV$_P$	17	0	892

* BALB/c mice 1 d of age were injected intraperitoneally with 5×10^5 PFU of F-MuLV$_A$ or F-MuLV$_P$. Hematocrits on venous blood and erythroid colony formation in methylcellulose culture were determined as described in Materials and Methods 40 d after infection.

‡ Mock-infected mice received 0.1 ml of filtered culture fluid recovered from uninfected NIH/3T3 cells.

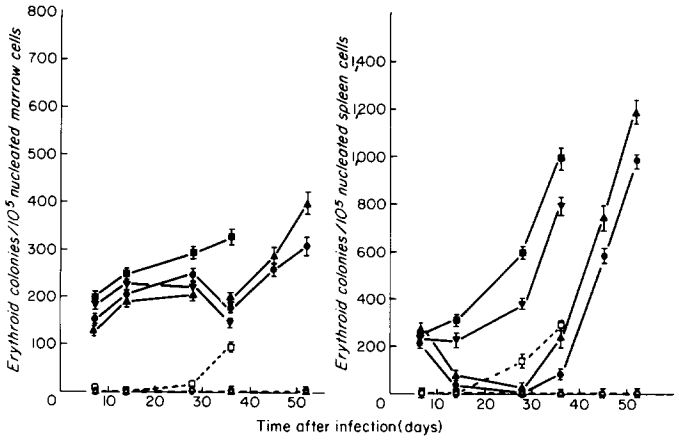

FIG. 3. Effects of FV-A, FV-P, F-MuLV$_A$, and F-MuLV$_P$ on bone marrow and spleen erythroid colony formation with time after infection. BALB/c mice 1 d of age were injected intraperitoneally with 3×10^3 FFU of SFFV$_A$ in FV-A (▼, ▽), 3×10^3 FFU of SFFV$_P$ in FV-P (■, □), 5×10^5 PFU of F-MuLV$_A$ (●, ○), or 5×10^5 PFU of F-MuLV$_P$ (▲, △). The mice were killed at various times, and the marrow and spleen cells plated in methylcellulose culture as described in Materials and Methods without the addition of EPO (open symbols) or in the presence of 0.5 U/ml EPO (closed symbols). The marrow of uninfected BALB/c mice 7, 14, and 35 d of age contained 268, 250, and 256 erythroid colonies in the presence of 0.5 U/ml EPO, respectively. The spleens of uninfected BALB/c mice 7, 14, and 35 d of age contained 257, 68, and 32 erythroid colonies, respectively, in 0.5 U/ml EPO. No erythroid colony formation was observed when spleen or marrow cells were cultured without the addition of exogenous EPO.

an increase in the proportion of cells that contain the erythroid-specific protein spectrin. Thus, infection of newborn BALB/c mice with either replication-competent F-MuLV, or Friend virus complex, results in a marked increase in nucleated erythroid spleen cells.

Cellular Composition of the Spleens of BALB/c Mice Infected as Adults with F-MuLV$_A$, F-

TABLE II

*Cellular Composition of the Spleens of BALB/c Mice Infected as Newborns with F-MuLV$_A$, F-MuLV$_P$, FV-A, or FV-P**

Virus	Spleen weight	Cell-surface antigen		
		Thy-1	Ig	Spectrin
	g		%	
Mock‡	0.10	41	45	5
F-MuLV$_A$	1.21	7	1	35
F-MuLV$_P$	1.00	8	1	31
FV-A	1.15	1	2	43
FV-P	1.40	1	1	76

* BALB/c mice 1 d of age were injected intraperitoneally with 5×10^5 PFU of F-MuLV$_A$ or F-MuLV$_P$ and 3×10^3 FFU of SFFV$_A$ or SFFV$_P$ that was contained in FV-A or FV-P, respectively. Spleens from F-MuLV-infected mice were taken at 45 d and from FV-A- or FV-P-infected mice at 25 d after infection. The proportion of cells in the spleen that was positively stained for Thy-1 glycoprotein, surface immunoglobulin, and spectrin was detected using indirect immunofluorescence as described in Materials and Methods.

‡ Mock-infected mice received 0.1 ml of filtered culture fluid from uninfected NIH/3T3 fibroblasts.

MuLV$_P$, and FV-P. The results presented above suggest that the replication-competent viruses F-MuLV$_A$ and F-MuLV$_P$, like SFFV$_A$ and SFFV$_P$, affect erythroid differentiation, although the kinetics of the changes induced by these viruses are different. The experiment in Fig. 2 suggested, however, that adult BALB/c mice were resistant to erythroleukemia induction by F-MuLV. To examine this possibility more closely, the hematocrits, the number of erythroid colony-forming cells, and the proportion of differentiated hemopoietic cells in the spleens of BALB/c mice, infected at 6 wk of age with F-MuLV$_A$, F-MuLV$_P$, FV-A, or FV-P were measured. The results shown in Table III demonstrate that adult BALB/c mice were fully susceptible to erythroleukemic transformation induced by FV-A or FV-P. In contrast, as shown in Fig. 2 and Table III, infection of adult BALB/c mice with either F-MuLV$_A$ or F-MuLV$_P$ did not produce splenomegaly or changes in hematocrit, alter the number or EPO-dependence of erythroid colony-forming cells, or change the proportion of differentiated cell types in the spleen. Furthermore, these parameters remained unaltered when examined at 18 d or 120 d after infection of 6-wk-old BALB/c mice (data not shown).

Discussion

The results presented in this study indicate that clonal isolates of the replication-competent type C viruses, F-MuLV$_A$ and F-MuLV$_P$, isolated by endpoint-dilution and cloning in NIH/3T3 fibroblasts, can induce a relatively rapid splenomegaly and anemia in newborn BALB/c mice. The results of three independent types of experiment indicate that the large increase in spleen cellularity in leukemic mice is due to the proliferation of cells of the erythroid pathway of differentiation. First, the cells in these spleens resemble immature nucleated erythroid cells. Second, the leukemic spleens contain a marked increase in the proportion of nucleated cells that contain spectrin and a corresponding decrease in the proportion of cells with the surface

TABLE III

*Cellular Composition of the Spleens of BALB/c Mice Infected as Adults with F-MuLV$_A$, F-MuLV$_P$, FV-A, or FV-P**

Virus	Spleen weight	Hemato-crit	Number of erythroid colony-forming cells/10^5 nucleated spleen cells; EPO (U/ml)		Cell-surface antigen		
			0	0.5	Thy-1	Ig	Spectrin
	g	%				%	
Mock‡	0.12	49	0	46	40	46	7
F-MuLV$_A$	0.15	48	0	34	41	46	6
F-MuLV$_P$	0.14	48	0	37	40	45	5
FV-A	1.72	29	0	4,480	1	1	45
FV-P	1.50	85	6,943	8,203	1	1	90

* BALB/c mice 6 wk of age were injected intravenously with 2.5×10^6 PFU of F-MuLV$_A$ or F-MuLV$_P$ and 1.5×10^4 PFU of SFFV$_A$ or SFFV$_P$ that was contained in FV-A or FV-P, respectively. Hematocrits were determined using venous blood obtained from a tail vein 35 d after infection. Spleens were removed at day 35 and the cells were plated in methylcellulose culture for erythroid colony formation as described in Materials and Methods.

‡ Mock-infected mice received 0.5 ml of undiluted filtered supernatant fluid from uninfected NIH/3T3 fibroblast cells.

markers characteristic of lymphoid differentiation pathways, Thy-1 and Ig. Spectrin is a complex of two polypeptide chains of 240,000 and 220,000 mol wt, found on the cytoplasmic side of the plasma membrane of mature erythrocytes (13). The presence of spectrin is specific for hemopoietic cells of the erythroid lineage (12), and its synthesis is induced in erythroid progenitor cells before the accumulation of hemoglobin (18). Thus, the presence of spectrin is a relatively early and characteristic membrane marker of erythropoiesis. Third, the leukemic spleens of BALB/c mice infected as newborns with either F-MuLV$_A$ or F-MuLV$_P$ contained an increased proportion of cells capable of forming erythroid colonies in culture. Taken together, these results suggest that F-MuLV$_A$ and F-MuLV$_P$ induce an anemic form of erythroleukemia when injected into newborn mice. Because the target-cell populations for these viruses are unknown, it is possible that progenitor cells from other hemopoietic cell lineages may also be affected.

The disease induced by F-MuLV$_A$ or F-MuLV$_P$ is similar to, but can be distinguished from, the erythroleukemias induced by FV-A or FV-P. Newborn BALB/c mice appear to be susceptible to F-MuLV$_A$ and F-MuLV$_P$, wheras BALB/c mice 6 wk of age or older are resistant to erythroleukemic transformation by these replication-competent viruses. In contrast, FV-A and FV-P induce the anemic and polycythemic form of Friend disease in newborn and adult mice, suggesting that neither SFFV$_A$ nor SFFV$_P$ show the age dependence exhibited by F-MuLV$_A$ and F-MuLV$_P$. In addition, the erythroleukemia induced by F-MuLV$_A$ or F-MuLV$_P$ appears after a longer latent period than the erythroleukemias induced by FV-A and FV-P. The diseases induced by these viruses can also be distinguished by their effects on erythroid progenitor cells. Infection of newborn BALB/c mice with FV-P rapidly results in the appearance of increased numbers of erythroid colony-forming cells that can be detected, both in the presence and absence of exogenously added EPO, whereas only EPO-dependent

erythroid progenitor cells were observed in the spleens of mice infected as newborns with either FV-A, F-MuLV$_A$, or F-MuLV$_P$.

The results presented in this and the accompanying study (1) indicate that the various isolates of Friend leukemia virus, each of which appears to have erythroleukemic potential, can be distinguished by both biological and molecular criteria. As shown in the accompanying study (1), the two isolates of Friend SFFV differ both with respect to their effects on erythroid progenitor cells, and the translational products encoded by their *gag* and *env* genes. The anemia- and polycythemia-inducing isolates of SFFV can also be distinguished from their associated helper viruses: Both SFFV$_A$ and SFFV$_P$ are replication-defective, whereas F-MuLV$_A$ and F-MuLV$_P$ are not. In addition, the spleen focus-forming viruses appear to be the major determinants of erythroleukemia induction in both newborn and adult BALB/c mice, whereas the replication-competent F-MuLV only appear to induce an anemic erythroleukemia in newborn animals. The replication-competent and replication-defective Friend virus isolates can also be distinguished by an examination of their genome structure and translational products. The SFFV$_P$ genome has been shown to include specific sequences that are not shared with its helper, F-MuLV$_P$ (7, 19, 20). In addition, as shown previously (21, 22) and in the accompanying study (1), both SFFV isolates encode a 55,000-mol wt protein related to the *env* gene product of murine xenotropic leukemia viruses, whereas Friend MuLV does not. Given the biological and molecular properties of the related Friend virus isolates, it will be of interest to determine whether the erythroleukemias associated with each of these viruses develop as the result of common or distinct molecular mechanisms.

Summary

In this study, the biological properties of the replication-competent viruses, F-MuLV$_A$, present in the anemia-inducing isolate of Friend leukemia virus complex (FV-A); and F-MuLV$_P$, present in the polycythemia-inducing isolate of Friend leukemia virus complex (FV-P) have been examined. BALB/c mice infected as newborns with clonal isolates of F-MuLV$_A$ or F-MuLV$_P$ become anemic and show splenic enlargement characterized by an increased proportion of cells that resemble immature nucleated erythroid cells. In addition, the spleens of these F-MuLV$_A$- or F-MuLV$_P$-infected mice contain a markedly increased proportion of both erythropoietin-dependent erythroid progenitor cells and spectrin-containing erythroid cells. These results suggest that Friend murine leukemia virus (F-MuLV) by itself can induce an erythroleukemic transformation in newborn BALB/c mice similar to that induced by the anemia-inducing spleen focus-forming virus (SFFV$_A$) in newborn or adult mice. Kinetic studies indicated that the alterations in hemopoietic cell populations induced by F-MuLV$_A$ or F-MuLV$_P$ in newborn BALB/c mice occurred more slowly than the rapid changes observed after infection with FV-A. In addition, adult BALB/c mice were fully susceptible to the erythroleukemic transformation induced by either SFFV$_A$ or SFFV$_P$, whereas only newborn mice were susceptible to F-MuLV. Taken together, these results suggest that, although the replication-defective Friend spleen focus-forming viruses appear to be the major determinant of erythroleukemia induction in adults, the replication-competent helper F-MuLV also have erythroleukemic potential when assayed in newborn animals.

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