Loss of Pathogenicity of Spleen Focus-Forming Virus after Pseudotyping with Akv

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Friend virus complex (FV), which comprises replication-competent Friend murine leukemia virus (FMuLV) plus replication-defective spleen focus-forming virus (SFFV), induces a multistage erythroleukemia. We have examined the role of replication-competent helper virus in the early and late stages of FV disease by replacing FMuLV, the native helper, with Akv, the endogenous ecotropic MuLV of AKR mice. SFFV_p/FRE, an established fibroblast line nonproductively infected with the polycythemic strain of SFFV, was superinfected with FMuLV or with Akv. Although supernatants from these cells showed similar titers in the XC plaque assay, supernatants from Akv-infected SFFV_p/FRE cells showed 100- to 5,000-fold less activity than did those from FMuLV-infected cells with respect to spleen focus induction in vivo. Since virions isolated from these two supernatants contained similar ratios of SFFV to helper virus genomic RNA, it did not appear that the difference was due to a relative inability of Akv to package SFFV. Although FMuLV- and Akv-rescued SFFV are equally infectious in a mouse fibroblast cell line (NIH 3T3), FMuLV-rescued SFFV was far more efficient in inducing erythroid bursts in cultured primary bone marrow cells. Adding Akv to preparations of FMuLV-rescued SFFV did not significantly interfere with burst induction. Helper-free SFFV induced 50- to 500-fold more spleen foci when coinjected with FMuLV than it did with Akv. Helper virus also affected mortality rates that reflect the late stage of the disease. When FMuLV- or Akv-rescued SFFV was injected into NIH Swiss mice at dosage levels adjusted to give equal numbers of spleen foci, all mice receiving FMuLV-rescued SFFV developed splenomegaly and died, whereas no mice receiving Akv-rescued SFFV died or developed detectable splenomegaly. When FMuLV was coinjected with Akv-rescued SFFV, the mortality rate rose from 0 to 100%. Injection of helper-free SFFV alone did not induce mortality, but coinjection of helper-free SFFV with FMuLV resulted in 100% mortality. Thus, the helper virus used to rescue SFFV plays at least a quantitatively important role in the early stage of FV disease and a crucial role in the late stage of the disease in vivo.

Friend virus complex (FV) induces a rapid erythroleukemia in susceptible adult mice (11). In this multistage disease (25, 42, 43), the early stage is characterized by a rapid, polyclonal proliferation of cells of the early erythroid lineage that have a finite life span. The late stage, beginning about 3 weeks after infection, is characterized by the emergence of truly malignant leukemia cells, massive hepatosplenomegaly, and death at 3 to 8 weeks. Analysis of retroviral integration sites in the genome of erythroleukemic cells strongly suggests that this malignant transformation is a mono- or oligoclonal event (20, 30, 31). This pattern of hyperplasia of certain cell types, followed by immortalization of a subset of those cells, is seen in many retrovirus-induced neoplasms, as well as in other types of cancers. In FV disease, as in many multistage cancers, the relationship between the hyperplasia and the malignancy which follows is not well understood.

FV stocks contain at least two viral components: a replication-competent helper, Friend murine leukemia virus (FMuLV), and a replication-defective spleen focus-forming virus (SFFV). SFFV is related to FMuLV but lacks a major portion of the basic retroviral genome, and it requires the presence of helper virus within the same cell to replicate. A number of studies have demonstrated that SFFV is the component responsible for the capacity of FV to induce

erythroleukemia in adults (7, 8, 27, 46, 47). Molecular and mutational analyses have further mapped this pathogenic character to the SFFV envelope gene, which encodes glycoprotein gp52 (6, 26, 39).

Recent studies using the retroviral packaging line ψ -2 (29) to generate infectious, helper-free SFFV particles (2, 47) have demonstrated that SFFV alone can induce an erythropoietin-independent phenotype in vitro in BFU-E cells, the erythroid target cells of FV (22). Helper-free SFFV can also induce some changes in vivo, indicative of the early stage of FV disease, but only in phenylhydrazine-pretreated mice, which show greatly increased numbers of BFU-E cells, and not in normal mice.

Although these studies demonstrated the primary role of SFFV in the early stage of the classical FV disease, further studies were needed to elucidate the role of the replication-competent helper, which provides the proteins necessary for the proper packaging of replication-defective SFFV (41). Although SFFV can be rescued by a variety of replication-competent viruses, considerable variation exists in the ability of replication-competent viruses to function efficiently as helpers to SFFV (7, 46). We now report studies in which we have replaced FMuLV with another replication-competent virus, Akv, the endogenous ecotropic virus of AKR mice. We compared the abilities of FMuLV- and Akv-rescued SFFV to infect cells in vitro and to induce the early and late stages of FV disease in vivo. Our findings demonstrate that the helper virus plays a quantitatively important role in the early stages of the disease and a critical role in the mortality

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induced by the later stage of the disease under normal conditions *in vivo*.

MATERIALS AND METHODS

Cells, viruses, and mice. All cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum. The origins of mouse fibroblast cell line (NIH 3T3) (17), Fisher rat embryo cell line (FRE clone 2) (40), FRE cells nonproductively infected with the polycythemic strain of SFFV (SFFV_p/FRE) (45), and the FMuLV_A+SFFV_p/FRE (EY-10) (6) cell line have been previously described (15). ψ -2/SFFV (47) was the generous gift of L. Wolff. The FMuLV/NIH 3T3, Akv/NIH 3T3, and AF226/NIH 3T3 cell lines used in these studies were generated by transfecting NIH 3T3 cells with the molecularly cloned full-length viral DNAs of these viruses by the calcium phosphate method (12). The plasmid 2-lala (pFMuLV₅₇), which consists of a circularly permuted copy of FMuLV_A cloned into pBR322 (32), was used as the FMuLV viral DNA. The Akv viral DNA was pAKR-59, a circularly permuted copy of Akv cloned into pBR322 (24). AF226 is a molecular recombinant in which the *Pst*I-*Kpn*I long terminal repeat (LTR) fragment of Akv has been replaced by the homologous fragment of FMuLV (35). All virus preparations used in these studies were harvested from subconfluent cultures of infected cell lines 24 h after the addition of fresh media. The FMuLV-, Akv-, and AF226-producing SFFV_p/FRE cell lines were generated by either superinfection with these viruses or transfection with the molecularly cloned DNA.

All mice used in this study were male NIH Swiss mice, 4 to 6 weeks old, and were obtained from the National Cancer Institute, Bethesda, Md. Limited studies with females of this strain produced comparable results.

Virus assays. Replication-competent viruses were assayed either by the reverse transcriptase method (37) or, if a virus titer was needed, by the XC plaque induction assay (36). Infectious SFFV was quantitated by the spleen focus assay (1). At 9 days after the mice received diluted virus preparations intravenously, they were sacrificed. Their spleens were weighed and fixed in Bouin solution, and discrete macroscopic focal lesions on the surface of the spleen were counted. For mortality studies, viral complexes containing either equal titers of replication-competent virus (as determined by the XC assay) or equal titers of spleen focus-forming activity were injected intravenously, and the survival of the infected mice was observed for at least 200 days after infection.

Viral RNA isolation and analysis. Genomic RNA was isolated from virus particles by a modification of the method of Pederson and Haseltine (33). Briefly, 1.5 liters of supernatant from each appropriate virus-producing cell line was collected, and the virus was harvested by centrifugation followed by pelleting through a 30% sucrose cushion. This virus pellet was then lysed, treated with proteinase K, and centrifuged on a linear gradient of 15 to 30% sucrose with 0.2% sodium dodecyl sulfate (SDS). RNA-containing fractions, as determined by spectrophotometry, were pooled, ethanol precipitated, and suspended in double-distilled H₂O. RNA was denatured by being heated for 5 min at 65°C, and then denatured RNA was electrophoresed on 1% agarose-MOPS (morpholinepropane sulfonic acid) gels and blotted to nitrocellulose (44). RNA molecular weight standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used to determine size.

Nitrocellulose blots were prehybridized in 50% formamide-0.9 M sodium chloride-0.09 M sodium citrate-0.5%

SDS-0.1% polyvinylpyrrolidone (M_w , 40,000)-0.1% bovine serum albumin-0.2% Ficoll (M_w , 40,000)-denatured salmon sperm DNA (100 μ g/ml) at 37°C for 3 to 16 h with constant agitation. Hybridization was performed overnight under the above-described conditions, with nick-translated plasmid DNA prepared by standard methods (28) as probe. The blots were washed once at room temperature for 5 min in 0.3 M sodium chloride-0.03 M sodium citrate-0.5% SDS and then twice for 2 h at 65°C in 15 mM sodium chloride-1.5 mM sodium citrate-0.5% SDS.

Oligonucleotide generation and hybridization. The oligonucleotide used in this study was synthesized on the automated DNA synthesizer (Applied Biosystems) at the Albert Einstein College of Medicine. This SFFV-specific oligonucleotide (FL-1) is homologous to the region near the 5' end of the *env* gene of SFFV that is not present in the *env* gene of FMuLV (21, 48). The sequence of this nucleotide is 5' AACCAGGGGTTGCATCGAC 3', and it was labeled at the 5' end with [γ -³²P]dATP and T4 polynucleotide kinase (28). Prehybridization was carried out in a solution of 0.75 M NaCl-0.075 M sodium citrate-7% SDS-0.2% polyvinylpyrrolidone-0.2% bovine serum albumin-0.2% Ficoll-0.02 M sodium phosphate (pH 6.8)-denatured salmon sperm DNA (100 μ g/ml) at 52°C for 4 to 16 h. Hybridization was carried out for 12 to 18 h under the above-described conditions along with 10% dextran sulfate. Blots were washed in 0.15 M NaCl-0.015 M sodium citrate-0.1% SDS at room temperature for 1 h.

Metabolic labeling and immunoprecipitation. NIH 3T3 cells were infected with virus culture fluid in the presence of Polybrene (4 μ g/ml) for 24 h. After subconfluent cultures of virus-infected cell lines were metabolically labeled with [³H]leucine for 2 h, cell extracts were prepared and incubated with appropriate antisera (15).

The FMuLV-specific gp70^{env} antibody used was a mixture of monoclonal antibodies 55 and 273 (5). Goat antisera to disrupted whole Akv virions and to Rauscher leukemia virus gp70^{env} were obtained through the Office of Program Resources and Logistics, Viral Oncology Division, National Cancer Institute, Bethesda, Md. All antisera were heat inactivated at 56°C for 30 min before use. The resulting immune precipitates were size fractionated by electrophoresis on SDS-7.5% polyacrylamide gels by the Laemmli procedure (23). ¹⁴C-labeled molecular weight markers (Bethesda Research Laboratories) were used as standards. Gels were fixed, fluorographed, and exposed to film (XAR-5; Eastman Kodak Co., Rochester, N.Y.) at -70°C.

In vitro erythroid transformation assay. Bone marrow cells from phenylhydrazine-treated mice were infected with viruses for 2 h at 4°C and cultured in methylcellulose in the presence of 0.1 U of erythropoietin per ml as previously described (14). After 5 days in culture, the cells were clotted, stained with benzidine, and examined microscopically for benzidine-positive bursts.

RESULTS

SFFV rescue. SFFV/FRE is an established line of rat embryo fibroblasts nonproductively infected with replication-defective SFFV_p but containing no replication-competent, or helper, virus. To determine the relative capabilities of different replication-competent viruses to rescue SFFV and to facilitate its pathogenicity, SFFV/FRE cells were superinfected with equipotent inocula of either FMuLV, the native helper of SFFV in FV, or Akv, the endogenous ecotropic MuLV of AKR mice. The superinfected cells

would be expected to produce and release into the culture medium particles of the superinfecting helper (FMuLV or Akv) plus pseudotyped particles containing SFFV RNA packaged with the viral proteins of the helper virus present [SFFV(FMuLV) or SFFV(Akv)]. Supernatants from these two superinfected cell lines were tested periodically for the presence of virus both by the reverse transcriptase assay and by the XC plaque assay, which detects replication-competent virus but not SFFV. These assays revealed that the time course for the appearance of virus activity in Akv-superinfected SFFV/FRE cells lagged slightly behind that of FMuLV-superinfected cells; however, by 3 weeks after infection, virus titers in the two cultures reached similar high levels (data not shown).

Spleen focus-forming activity. The activity of SFFV as rescued by either FMuLV or Akv was determined in the spleen focus assay. Virus-containing supernatants, matched for levels of replication-competent virus, showed large differences in spleen focus-forming activity. Mice injected with Akv-rescued SFFV [Akv+SFFV(Akv)] showed approximately 1,000-fold fewer spleen foci than those injected with FMuLV-rescued SFFV [FMuLV+SFFV(FMuLV)] did (Table 1). In assays with a number of independent virus preparations, Akv+SFFV(Akv) induced between 100 and 5,000 fewer spleen foci than did FMuLV+SFFV(FMuLV). Tests in which the introduction of the helper viruses into SFFV/FRE cells was by transfection with molecularly cloned FMuLV or Akv rather than by infection produced similar results (data not shown). This was also true when the virus preparation used was grown in NIH 3T3, a mouse cell line, rather than in FRE, a rat cell line.

Viral RNA packaging. One possible explanation for the reduced capacity of Akv-rescued SFFV to induce splenic foci was that Akv may be far less efficient than FMuLV in its ability to package the SFFV genome. If this was true, Akv-superinfected SFFV/FRE cells would produce relatively little SFFV. To test this hypothesis, virions were harvested from supernatants of SFFV/FRE that had been superinfected with FMuLV or with Akv, and viral genomic RNA was isolated. The RNA was denatured, electrophoresed on MOPS-formaldehyde gels, and blotted to nitrocel-

FIG. 1. Northern blot analysis of genomic RNA. Viral genomic RNA was isolated from virions harvested from the supernatant of FMuLV- and Akv-superinfected SFFV/FRE cells, denatured, and electrophoresed on a 1% agarose-MOPS-formaldehyde gel. The Northern blot of this gel was hybridized under the conditions described in Materials and Methods. (A) Hybridization to an equal mixture of FMuLV and Akv viral probes. (B) Hybridization to an SFFV-specific oligonucleotide. Lanes: 1, FMuLV+SFFV(FMuLV) genomic RNA; 2, Akv+SFFV(Akv) genomic RNA.

TABLE 1. Spleen focus formation by SFFV rescued by different helper viruses

Virus	Dilution ^a	Avg spleen wt (g)	No. of foci/spleen examined
Positive control ^b		0.188	TM, TM, TM, TM ^c
FMuLV+SFFV(FMuLV)	1:50	0.207	TM, TM, TM, TM
	1:250	0.185	10, 15, 15, 5, 6, 4
	1:1,250	0.135	4, 2, 2, 2, 2, 0
Akv+SFFV(Akv)	1:1	0.131	1, 2, 0, 0, 0, 0
	1:10	0.127	0, 0, 0, 0, 0, 0
AF226+SFFV(AF226)	1:1	0.132	1, 1, 1, 0, 0, 0
	1:10	0.142	0, 0, 0, 0, 0, 0
Negative control ^b		0.104	0, 0, 0

^a The 1:1 dilution was adjusted to contain the same titer of replication-competent virus, as determined by the XC assay.

^b Positive control, Complete FV complex in supernatant from EY-10 cells; negative control, supernatant from nonsuperinfected SFFV/FRE cells.

^c TM, Too many to count.

lulose. Since SFFV RNA is significantly shorter than helper RNA (5.8 versus 8.5 kilobases), these two species can easily be resolved after size fractionation. The Northern (RNA) blots were probed with a mixture of ³²P-labeled full-length FMuLV and Akv DNA (Fig. 1A). Although the overall homologies of these viruses with SFFV are not high, these replication-competent viral probes cross-hybridized with SFFV genomic RNA because portions of the genomes of SFFV and the two helper viruses are virtually identical. This same blot was then washed and reprobed with an SFFV-specific synthetic oligonucleotide (Fig. 1B). The amounts of SFFV genomic RNA in FMuLV+SFFV(FMuLV) and in Akv+SFFV(Akv) were similar. Since the amount of SFFV genomic RNA should reflect the number of SFFV genome-containing virions in the supernatants, it appears that the difference between the activities of the two virus preparations with respect to spleen focus formation in vivo was not due to a relative inability of Akv to package the SFFV genome.

Infection of fibroblasts. Since FMuLV+SFFV(FMuLV) and Akv+SFFV(Akv) appeared to contain nearly equal proportions of SFFV-containing virions, we investigated whether SFFV(FMuLV) and SFFV(Akv) were equally infectious in vitro. Supernatants from FMuLV- or Akv-superinfected SFFV/FRE cell lines, containing equal titers of replication-competent virus, were used to infect NIH 3T3 cells. The relative infectivity of helper and SFFV virions was inferred from analysis of the glycoproteins expressed by the

FIG. 2. Expression of virally encoded proteins in NIH 3T3 cells after infection with FMuLV+SFFV(FMuLV) or Akv+SFFV(Akv). Lanes: 1 and 2, NIH 3T3 cells superinfected with FMuLV+SFFV(FMuLV); 3 and 4, NIH 3T3 cells superinfected with Akv+SFFV(Akv). The antisera used to immunoprecipitate virally encoded proteins were anti-FMuLV gp70 (lane 1), anti-Rauscher gp70 (lanes 2 and 4), and anti-Akv whole virion (*gag* and *env*) (lane 3). kd, Kilodalton.

viral *env* genes after isolation from metabolically labeled cells, immunoprecipitation with appropriate antisera, and size fractionation by SDS-polyacrylamide gel electrophoresis. Both the FMuLV and Akv *env* gene products have molecular masses of approximately 70 kilodaltons, whereas that of the *env* gene product of SFFV is approximately 52 kilodaltons. The relative amounts of helper and SFFV *env*

products were similar in NIH 3T3 cells that had been infected with either FMuLV+SFFV(FMuLV) or Akv+SFFV(Akv) (Fig. 2). This indicates that SFFV(Akv) was as infectious as SFFV(FMuLV) in NIH 3T3 cells.

Erythroid burst induction. Even though SFFV pseudotyped by different helpers appeared equally infectious in NIH 3T3 cells, a mouse fibroblast line, this might not necessarily be true in the specific target cell for FV disease, the late BFU-E cell. To test this possibility, the abilities of FMuLV+SFFV(FMuLV) and Akv+SFFV(Akv) to induce clusters of erythroid colonies in bone marrow cells in vitro were determined. This assay measures the ability of SFFV to infect BFU-E cells independently of helper virus (13, 47). The results (Table 2, experiment 1) demonstrate that SFFV(Akv) was far less efficient than SFFV(FMuLV) in productively infecting BFU-E cells in vitro. Results from a spleen focus assay performed on the same virus preparations showed that the difference in the erythroid burst-inducing capacities of SFFV(Akv) and SFFV(FMuLV) in culture was of the same magnitude as the difference in their capacities to induce spleen foci in vivo.

The effect of Akv on the ability of FMuLV+SFFV(FMuLV) to induce erythroid bursts in vitro was also determined. The number of bursts was not significantly different when FMuLV, active Akv, or heat-inactivated (65°C, 1 h) Akv was added to FMuLV+SFFV(FMuLV) (Table 2, experiment 2). Thus the inability of Akv+SFFV(Akv) to induce erythroid bursts was not due to a *trans*-acting inhibitory effect of Akv itself.

These results demonstrate that the replication-competent virus used to rescue SFFV is quantitatively important in determining the ability of SFFV to productively infect the target cell of FV disease. From these data, however, it is not

TABLE 2. Erythroid burst induction by SFFV rescued by different helper viruses

Expt. no.	Virus ^a	Dilution	Erythroid bursts/ 10 ⁶ cells	Avg no. of spleen foci ^b
1	Positive control (FV complex)	1:1	>100	ND
		1:100	44	ND
	FMuLV+SFFV- (FMuLV)	1:1	64	TM
		1:100	7	1
	Akv+SFFV(Akv)	1:1	0	2
2		1:100	0	0
	Negative control (SFFV/FRE supernatant)	1:1	0	0
	Positive control	1:1	119	
	FMuLV+SFFV(FMuLV)	1:1	64	
		1:100	8	
	Akv+SFFV(Akv)	1:1	0	
	AF226+SFFV(AF226)	1:1	0	
	FMuLV mixed with FMuLV+SFFV- (FMuLV)	1:1	59	
	Akv mixed with FMuLV+SFFV- (FMuLV)	1:1	61	
	Killed Akv mixed with FMuLV+SFFV(FMuLV)	1:1	51	
	Negative control		0	

^a Positive and negative controls are as described in Table 1, footnote b.

^b ND, Not done; TM, too many to count.

clear whether the difference in spleen focus induction was solely caused by a difference in the initial round of infection or whether there was an additional effect from the spreading of SFFV during the subsequent spread of infection. To clarify this, helper-free SFFV was obtained from ψ -2 cells which had been stably transfected with a molecular clone of SFFV_P (47). This ψ -2/SFFV cell line, which also contains integrated packaging-defective mutants (pMOV- ψ^-) of Moloney murine leukemia virus (MMuLV), produces helper-free SFFV_P(ψ -2) pseudotyped with MMuLV viral proteins but no infectious MMuLV particles. This helper-free SFFV can infect erythropoietic target cells, as shown by its erythroid burst-inducing activity in vitro, but it cannot induce spleen foci or FV disease in normal adult mice unless FMuLV is coinjected with it, thereby permitting further cell-to-cell spread of SFFV. To determine the relative capacities of FMuLV and Akv to support spleen focus formation after infection of erythropoietic targets with helper-free SFFV, mice were injected with helper-free SFFV(ψ -2) plus either FMuLV or Akv and examined 9 days later for spleen foci. SFFV(ψ -2) mixed with FMuLV was approximately 100-fold more efficient at inducing spleen foci than SFFV(ψ -2) mixed with Akv (Table 3), and in other experiments, the difference ranged from 50- to 500-fold. Although the initial infection by SFFV(ψ -2) virions in these two preparations would proceed similarly, during subsequent rounds of infection, SFFV would presumably be pseudotyped by the coinjected replication-competent virus (FMuLV or Akv). These data indicate that spleen focus formation in vivo by Akv-rescued SFFV is inefficient because of the poor capacity of SFFV(Akv) to productively infect BFU-E cells, both during the initial infection and during the subsequent spread of SFFV.

Viral polymorphism. Another way to determine why FMuLV and Akv differ in their abilities to rescue SFFV is to locate where in the viral genome this difference maps. For this purpose, we studied a molecular recombinant, AF226, consisting of Akv in which the majority of the LTR region (*Pst*I-*Kpn*I fragment) has been replaced by the homologous fragment of FMuLV (35). This region contains well-characterized transcriptional enhancer sequences and is critical in determining the target cell and disease specificity of several different retroviruses (3, 35). When AF226 was used to rescue SFFV and the resulting viral complex was tested for spleen focus-inducing activity, the potency of AF226-rescued SFFV, like that of Akv-rescued SFFV, was 100- to 1,000-fold less than that of FMuLV-rescued SFFV (Table 1). Moreover, SFFV pseudotyped by AF226, like SFFV pseudotyped by Akv, was at least 100-fold less efficient at

FIG. 3. Effect of helper virus used to rescue SFFV on mortality caused by FV disease. Five-week-old NIH Swiss mice were injected intravenously with various helper-rescued SFFV preparations as described in Materials and Methods. The length of survival after infection was observed. Symbols: ■, FV undiluted (positive control; $n = 5$); ●, FMuLV+SFFV(FMuLV) undiluted ($n = 7$); +, FMuLV+SFFV(FMuLV) diluted 1:1,000 ($n = 10$); △, Akv+SFFV(Akv) undiluted ($n = 7$); ★, supernatant from uninfected SFFV/FRE (negative control; $n = 5$).

inducing erythroid bursts in the BFU-E assay (Table 2, experiment 2). These data indicate that the pertinent difference between FMuLV+SFFV(FMuLV) and Akv+SFFV(Akv) does not map to the LTR region.

Late-stage FV disease in vivo. The studies presented thus far have examined the relative effects of two helper viruses, FMuLV and Akv, on the initial stages of FV disease, as measured by spleen focus formation at 9 days postinfection in vivo and the induction of erythroid bursts in vitro. To determine the effect of helper virus on the mortality occurring in later stages of the FV disease, NIH Swiss mice were injected with undiluted Akv+SFFV(Akv), undiluted FMuLV+SFFV(FMuLV), or FMuLV+SFFV(FMuLV) diluted 1:1,250. The data in Table 1 were the internal controls for this study, and they show that the undiluted viral complexes contained similar XC assay titers of replication-competent virus, whereas FMuLV+SFFV(FMuLV) diluted 1:1,250 induced the same number of spleen foci as undiluted Akv+SFFV(Akv). Additional mice receiving these same inocula were observed for mortality. All mice injected with FMuLV+SFFV(FMuLV) diluted 1:1,250 were dead by day 55, whereas none of those injected with undiluted Akv+SFFV(Akv) died (Fig. 3). Mice injected with undiluted AF226+SFFV(AF226) also showed no mortality (Fig. 4), indicating that the relevant difference between FMuLV and Akv does not map in the LTR region of the helper virus.

To elucidate further the role of FMuLV in FV mortality, FMuLV alone was coinjected with undiluted Akv+SFFV(Akv) or AF226+SFFV(AF226). Mortality in these mice was 100%, in contrast to 0% in those injected with Akv+SFFV(Akv) or AF226+SFFV(AF226) alone (Fig. 4).

DISCUSSION

Our results indicate that the pathogenicity associated with SFFV is drastically reduced when the genome of the virus has been rescued and pseudotyped by Akv rather than by its native helper, FMuLV. The proteins encoded by Akv are competent to package SFFV RNA, and SFFV(Akv) appears

TABLE 3. Spleen focus formation by coinjection of helper-free SFFV(ψ -2) and FMuLV or Akv

Helper virus with SFFV(ψ -2) ^a	Dilution	No. of foci/spleen examined	Avg spleen wt (g)
FMuLV	1:1	TM, TM ^b	0.290
	1:100	2, 4	0.118
	1:1,000	0	0.143
Akv	1:1	0, 2	0.126
Controls ^c		0, 0	0.126

^a FMuLV and Akv injected at a titer of 5×10^4 (determined by the XC plaque assay).

^b TM, Too many to count.

^c SFFV(ψ -2) injected alone.

FIG. 4. Effect of coinjection of FMuLV with Akv- and AF226-rescued SFFV on mortality in FV disease. Akv+SFFV(Akv) or AF226+SFFV(AF226), with or without FMuLV, was injected intravenously into 5-week-old NIH Swiss mice as described in Materials and Methods. The length of survival after infection was observed. Symbols: ●, FMuLV+SFFV(FMuLV) undiluted ($n = 5$); +, FMuLV+SFFV(FMuLV) diluted 1:100 ($n = 5$); △, Akv+SFFV(Akv) undiluted ($n = 4$); ▲, FMuLV with Akv+SFFV(Akv) undiluted ($n = 7$); □, AF226+SFFV(AF226) undiluted ($n = 4$); ■, FMuLV with AF226+SFFV(AF226) undiluted ($n = 4$); ☆, supernatant from uninfected SFFV_pFRE (negative control; $n = 4$).

to be fully infectious in mouse fibroblasts. However, comparison of Akv+SFFV(Akv) with FMuLV+SFFV(FMuLV) showed that the former is at least 100-fold less capable than the latter of inducing erythroid bursts in cultured bone marrow cells (by infecting BFU-E cells), spleen foci in vivo, or the multistage FV erythroleukemia syndrome in vivo.

Taken together, the data indicate that viruses pseudotyped by Akv, although capable of replicating in fibroblasts, have strongly diminished abilities to infect or carry out their reproductive cycles in cells of the erythroid lineage that are primary targets for SFFV pathogenesis. The step of the reproductive cycle of SFFV(Akv) at which this blockage occurs remains to be identified. *Prima facie*, the most likely step appears to be the interaction with a target cell receptor or the chromosomal integration of a proviral reverse transcript.

It is clear that different classes of murine retroviruses (e.g., ecotropic, xenotropic, mink cell focus-forming viruses) use different murine cell surface molecules as receptors in the initial stage of infection. In no case has this receptor molecule been identified, but a gene governing the receptor for ecotropic viruses has been mapped to chromosome 5 (38). Viral interference studies suggest that all ecotropic viruses, including FMuLV and Akv, use this receptor, and the receptor function of this molecule is not known to be polymorphic in mice of different strains. Recently, however, exceptions to the categories of receptors have been observed in cell lines derived from different strains and species of mice (4). Since nearly all of the interference studies defining these groups were done with fibroblast cell lines, one might speculate that the molecule that recognizes ecotropic viruses on fibroblasts may not be expressed on erythroid cells, which express a completely different molecule that readily recognizes the *env* gene product of FMuLV but recognizes poorly or not at all the *env* product of Akv.

Once inside the target cell, viral RNA must be reverse transcribed into double-stranded DNA before integration into a host chromosome. Blockage of the viral replication cycle during or after viral DNA synthesis but before integration can be effected by the host *Fv-1* gene in certain

combinations of ecotropic virus and mouse strains, apparently as a consequence of an interaction between the p30 region of the viral *gag* gene and the cellular *Fv-1* gene product (9, 10, 16, 18, 19, 34). By analogy, it is possible that a gene product of Akv functions efficiently for virus replication in fibroblasts but, by comparison with the homologous product of FMuLV, very poorly in erythroid cells. Although our experiments suggest that the addition of Akv does not inhibit burst induction in the BFU-E assay by helper-free SFFV(ψ -2), further experiments are needed to rule out the possibility that some Akv product that is nonfunctional in erythroid cells can compete with the homologous functional product of FMuLV for interaction with a target cell protein.

It is striking that full-strength preparations of Akv-rescued SFFV caused no mortality in FV-susceptible hosts, whereas similar preparations of FMuLV-rescued SFFV caused 100% mortality even when highly diluted. The erythroid hyperplasia characteristic of early stages of FV disease is polyclonal; participants in this hyperplasia include not only erythroid cells infected by virus in the original inoculum but also those recruited later by infection with virus produced in other cells infected in the first round. If SFFV(Akv) can replicate in and transform only a small fraction of those erythroid cells susceptible to SFFV(FMuLV), then the inability to attain a hypothetical threshold of infected cells necessary for development of the full-blown disease syndrome might be caused by the failure of recruitment by serial virus spread. In the later stages of FV disease, frankly malignant erythroleukemia cells frequently appear among the hyperplastic erythroid cells. The mono- or oligoclonal origin of these malignant cells in each mouse indicates that malignant transformation is a rare event; thus, if infection with SFFV(Akv) generates only small numbers of hyperplastic cells, the probability of the occurrence of malignant transformation is vastly reduced.

It seems unlikely that different immune responses against FMuLV or Akv antigens determine the different capacities of SFFV rescued by the two helpers to induce the earliest stages of FV disease, e.g., SFFV-induced proliferation of BFU-E cells. However, interactions of these helpers with the immune system could play a role in the later stages of the disease. Akv+SFFV(Akv) possesses a very weak capacity to induce spleen foci in vivo but cannot, even at high doses, induce the hyperplasia and lethal disease that routinely ensue from low doses of FMuLV+SFFV(FMuLV). This observation could be due, in part, either to a greater immunogenicity of Akv-infected cells or to a greater capacity of FMuLV to induce a state of immunodeficiency in the hosts.

One approach that might uncover clues to the mechanism of the polymorphism between FMuLV and Akv would be to locate the region of the viral genome in which the polymorphism maps. Our study provides a small beginning to this approach, since AF226, a genetically engineered recombinant in which much of the U3 region of the LTR of Akv was replaced by the homologous region of FMuLV (35), behaved identically as Akv in our experiments. Thus the regulatory functions located in the U3 region, including enhancer sequences, are not demonstrably involved in the phenomenon.

Studies with helper-free SFFV have shown that it is capable of in vitro infection of BFU-E cells, the target cells for FV disease, and that it can lead to the appearance of transformed cells in the spleens of mice pretreated with phenylhydrazine (2, 47, 49). Our observations indicate that, although SFFV is necessary for the induction of FV disease, replication-competent helper MuLV is also essential in the progression of the disease under normal conditions in vivo,

but strains of replication-competent MuLV differ widely in the ability to provide the needed help.

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