Roles of Helper and Defective Retroviral Genomes in Murine Erythroleukemia: Studies of Spleen Focus-Forming Virus in the Absence of Helper

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Retroviruses that cause acute oncogenesis are generally complexes of a replication-competent helper virus and a replication-defective component. However, the pure defective components have not been previously available. We prepared the defective spleen focus-forming virus component of Rauscher erythroleukemia virus (R-SFFV) by transfecting a colinear R-SFFV DNA clone into a retroviral packaging cell line (ψ2 cells). The transfected cells released virus (ψ2/SFFV) that was free of helper virus and that induced erythropoietin-dependent erythroid burst formation in bone marrow cultures. When injected into normal adult NIH/Swiss mice in moderate doses, ψ2/SFFV caused a rapid splenic erythroblastosis that regressed. Extensive erythroblastosis could be maintained by repeated injections of ψ2/SFFV into anemic mice or by the addition of a helper virus. We conclude that R-SFFV alone causes proliferation but not immortalization of a population of erythroblasts that is normally replenished from a precursor stem cell pool. Because these precursor cells are inefficiently infected, a single moderate inoculum of ψ2/SFFV causes a wave of erythroblastosis. The properties of the proliferating erythroblasts are substantially determined by the R-SFFV viral component.

Retroviruses that cause acute neoplasia generally consist of a replication-competent helper virus and a replication-defective component that contains an oncogene. Although the defective components are essential for the disease, they have only been studied in the presence of the helper, and their individual roles therefore remain uncertain. The phenomena of defectiveness and interference contribute to a complex series of interactions between the two viral components (20). In addition, recent evidence suggests that retroviral oncogenesis may be a multistep process (3, 7, 22). Because normal cells in vivo have only a limited self-renewal capability, a single cycle of infection may cause a small and transient neoplasm. Growth of the neoplasm may require infection of a rare premalignant cell, continued viral replication with infection of neighboring cells, or a secondary immortalization event that enables a subclone in the primary neoplasm to grow indefinitely (3, 7). Analyses of pure defective retroviruses would contribute to our understanding of these issues.

The Rauscher and Friend erythroleukemia viruses provide excellent models for analyzing the roles of viral and cellular genes in the multistep process of oncogenesis (13). The viruses contain two components, a replication-competent helper murine leukemia virus (MuLV) and a replication-defective spleen focus-forming virus (SFFV). The viruses cause an initial proliferation of erythroblasts in the bone marrow and spleens of infected mice and an associated anemia or polycythemia (6, 17, 19). Weeks later, immortalized leukemia cells form within the primary neoplasm, and these cells ultimately grow to kill the host (21). The SFFV components clearly perform an essential pathogenic role in adult mice. For example, SFFV *env* gene mutants are nonleukemogenic (8–10, 14), and pathogenic revertants contain second-site suppressor mutations in this same gene (8a;

J.-P. Li, R. K. Bestwick, C. Machida, and D. Kabat, J. Virol., in press). The SFFVs also cause formation of erythroid bursts in bone marrow cultures (4, 6). These results suggest that the SFFVs may be responsible for causing erythroblast proliferation during the initial phase of disease, but they do not indicate whether SFFV gene expression also contributes to subsequent leukemic progression or to the phenotype of advanced leukemia.

Recent development of retroviral packaging cell lines has made it feasible to produce pure preparations of replication-defective murine retroviruses (11). We report the use of such cells to prepare Rauscher SFFV (R-SFFV), and we describe the pathogenic properties of this virus. An independent study of a pure Friend SFFV was recently described (23).

MATERIALS AND METHODS

Cells and viruses. All cell lines were grown in Dulbecco modified Eagle medium (GIBCO Diagnostics, Madison, Wis.) supplemented with 10% fetal calf serum and antibiotics. Virus stocks of Rauscher MuLV (R-MuLV) and the Rauscher erythroleukemia viral complex were prepared from infected NIH/3T3 cells. All viral preparations were isolated from actively growing, subconfluent cell cultures 24 h after provision of fresh growth medium. ψ2 cells (11) and the plasmid pMSVneo (2) were obtained from R. Mulligan (Massachusetts Institute of Technology, Cambridge, Mass.). The ψ2/pMSVneo cell line was generated by transfecting ψ2 cells with pMSVneo DNA. This cell line produces >10^5 G418-resistant CFU/ml of culture media.

Preparation of helper-free SFFV. The pBR322-derived plasmid pBC-10, a nonpermutated molecular clone of R-SFFV (1), was cotransfected with pSV2neo (16) into the ψ2 cell line by previously described methods (1). G418 (GIBCO)-resistant colonies were isolated and screened for expression of R-SFFV-encoded gp54 by L-[35S]methionine
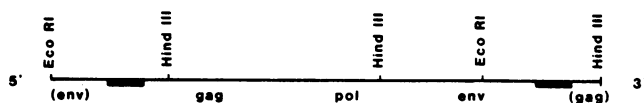
FIG. 1. Restriction endonuclease map of pBC-10 aligned with the approximate location of the R-SFFV-encoded genes and the long terminal repeats. Only the viral portion of the plasmid clone is shown. The viral genome was made colinear by duplicating the terminal EcoRI-to-HindIII fragment of a clone permutated at the EcoRI site as previously described (1). This construct results in portions of the env and gag genes (shown in parentheses) being duplicated at the 5′ and 3′ ends, respectively.

labeling, immune precipitation with anti-gp70 antisera, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (1, 15). The cell line that was selected for further analysis was named ψ2/pBC-10 and was shown to be releasing SFFV virions, called ψ2/SFFV (see below).

The ψ2/SFFV virus stocks were harvested 24 h after fresh medium (1 ml/5 cm^2 of culture) was added to cells grown to 80% confluency. We previously showed that the quantities of gp54 and gp70 synthesized in newly infected NIH/3T3 cells are proportional to the titers of R-SFFV and R-MuLV, respectively (9, 14). With this assay, we compared the ψ2/SFFV virus with Rauscher virus preparations for which the titer had previously been determined for SFFV and MuLV by limiting dilution (9) and determined that ψ2/SFFV stocks typically contained 10^5 infectious R-SFFV virions per ml.

Analysis of ψ2/SFFV pathogenic activity. Infection was by tail vein injection of either 4- to 6-week-old female NIH/Swiss mice or 4- to 6-week-old male NIH/Swiss mice that had been pretreated with 40 μg of gBW phenylhydrazine at −64, −48, and −40 h in a volume of 0.2 ml. The female mice were given a single injection of 0.5 ml of filtered ψ2/pBC-10 culture medium. Splenectomies were performed at various times after injection, and the spleens were weighed and prepared for gross pathologic analysis by previously described methods (1, 9, 15). The phenylhydrazine-treated male mice were injected with 0.5 ml of the same medium at 0, 1, 2, 3, and 4 days. Splenectomies were performed on day 6; the spleens were weighed, and a small fragment was used to make a single cell suspension for the determination of erythroid CFU (CFU-E) by the plasma clot assay of Stephenson et al. (18). Briefly, 5 × 10^4 cells in 0.1 ml of suspension were seeded in plasma clot cultures and incubated for 5 days in the presence or absence of 0.5 U of erythropoietin (EPO) per ml. The clots were then fixed, stained with benzidine, counterstained with hematoxylin, and scored for CFU-E.

In vitro analysis of ψ2/SFFV for erythroid burst induction was performed as described earlier (6). Briefly, 2.5 × 10^6 bone marrow cells from phenylhydrazine-pretreated NIH/Swiss mice (see above) were incubated with 0.375 ml of ψ2/SFFV at 4°C for 2 h. EPO, when added, was at 0.1 U/ml, and the cultures were incubated at 37°C for 5 days before being scored for bursts of erythroid colonies as described previously (12).

RESULTS

Preparation of R-SFFV from ψ2 cells. We used the ψ2 retroviral packaging cell line (11) for the purpose of isolating R-SFFV virions in the absence of helper MuLV. The ψ2 cells

are a derivative of NIH/3T3 fibroblasts that contain a Moloney MuLV mutant with a deletion in the ψ site that is required in cis for packaging the helper virus RNA into virions (11). However, the cells synthesize the Moloney MuLV proteins, and they therefore package RNAs that contain ψ sites to form helper-free virion particles (11). Our approach was to transfect ψ2 cells with the colinear R-SFFV molecular clone pBC-10 (1). A restriction map of pBC-10 is shown in Fig. 1. As described previously, pBC-10 was reconstructed from an R-SFFV molecular clone that was circularly permutated at its EcoRI site (1).

To determine whether the virus released from ψ2/pBC-10 cells contained R-SFFV, helper MuLV, or both, we infected NIH/3T3 fibroblasts and analyzed them for the synthesis of the glycoproteins encoded by the viral envelope (env) genes. NIH/3T3 cells that had been infected with ψ2/SFFV synthesized gp54 but not the MuLV components gPr90, gp70, and p15E (Fig. 2, lane 2). The addition of MuLV in various proportions resulted in a synthesis of the MuLV-encoded proteins (lanes 3 through 6). Quantitative analysis of these results in comparison with other virus preparations that had known titers of each viral component (see Materials and
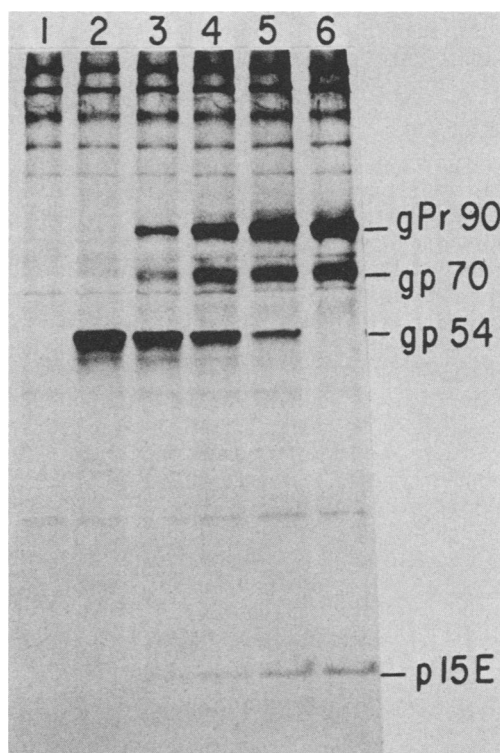

FIG. 2. Analysis of viral envelope glycoproteins from NIH/3T3 fibroblasts infected with ψ2/SFFV and with mixtures of ψ2/SFFV and R-MuLV. Cells were pulse-labeled with [^{35}S]methionine 2 days postinfection and subjected to immunoprecipitation and polyacrylamide gel electrophoresis as described previously (15). Lysates of cells infected with 1 ml of ψ2/SFFV only (lane 2), 0.8 ml of ψ2/SFFV and 4 × 10^4 PFU of R-MuLV (lane 3), 0.5 ml of ψ2/SFFV and 10^5 PFU of R-MuLV (lane 4), 0.2 ml of ψ2/SFFV and 1.6 × 10^5 PFU of R-MuLV (lane 5), or 10^6 PFU of R-MuLV (lane 6) were precipitated with goat antibody directed against Rauscher gp70. Lane 1 is a normal goat serum control from the lysate of cells infected with ψ2/SFFV only. The gel was autoradiographed for 12 days at −70°C.

TABLE 1. ψ2/SFFV-induced erythroid burst formation in vitro

Bone marrow treatment	Bursts/well[a]	No. of bursts/10⁶ cells[b]
Dulbecco modified Eagle medium	0, 0, 0	0
ψ2 medium	0, 0, 0	0
ψ2/SFFV	48, 58, 63	110
Friend MuLV[c]	0, 0, 0	0
Friend MuLV[c] + ψ2/SFFV	34, 44, 52	87
Heat-inactivated[d] ψ2/SFFV	0, 0, 0	0

[a] All wells contained 0.1 U of EPO per ml, and hemoglobinized bursts were counted.
[b] Based on average number of bursts per well.
[c] 5 × 10⁴ XC PFU were incubated with cells during the 2-h virus adsorption period.
[d] ψ2/SFFV was incubated at 56°C for 1 h before use in assay.

Methods) suggested that the ψ2/SFFV stocks contained approximately 10⁵ infectious R-SFFV virions per ml.

Although the titer of MuLV was very low in ψ2/SFFV stocks and in newly infected NIH/3T3 cells, prolonged passaging of the latter cultures resulted in the eventual spread of a replication-competent MuLV. From the rate of this spread, we inferred that the ψ2/SFFV stocks contained approximately 0.01% MuLV. NIH/3T3 cells clonally infected with SFFV do not release replication-competent MuLV (15). Such MuLV is also absent from the culture medium of ψ2/pMSVneo cells that contain high titers of the pMSVneo virus (data not shown). Since NIH/3T3 cells infected with the latter virus do not synthesize gp70, the failure of MuLV spread in this case cannot be ascribed to interference. We inferred that ψ-negative Moloney MuLV RNA may rarely copackage with R-SFFV (but not with pMSVneo) and that subsequent recombination may generate replication-competent MuLV.

Biological activity of ψ2/SFFV. Rauscher erythroleukemia virus and certain strains of Friend virus cause splenic erythroblastosis associated with anemia, whereas other strains of Friend virus cause an associated polycythemia (4, 6, 20). Moreover, all of these viruses can infect bone marrow cultures and 3 to 7 days later cause the formation of clusters of erythroid colonies called bursts (4, 6). However, the bursts caused by the anemia-inducing viruses become hemoglobinized only in the presence of added EPO, whereas the polycythemia-inducing strains generate bursts that become hemoglobinized even in the absence of EPO (4, 6, 20). ψ2/SFFV was active in this assay, and the addition of MuLV did not increase the number of bursts (Table 1). Furthermore, the burst-forming activity of ψ2/SFFV was heat-labile,

TABLE 2. Effect of EPO on ψ2/SFFV-induced burst formation in vitro[a]

EPO (U/ml)	Bursts/well	Avg no. of bursts/10⁶ cells[b]
0	4, 2, 4	6
0.01	10, 5, 14	20
0.03	23, 28, 21	48
0.10	54, 38, 42	90
0.30	52, 50, 61	110
1.00	48, 32, 58	92

[a] Infections of 5 × 10⁵ bone marrow cells with ψ2/SFFV were as indicated in Materials and Methods, and hemoglobinized bursts were counted.
[b] The average number of hemoglobinized bursts per 10⁶ cells was 0 at all the EPO concentrations listed when culture medium lacking virus was used.

TABLE 3. Dose response of ψ2/SFFV on in vitro burst formation

Dilution[a]	Bursts/well[b]	Avg. no. of bursts/10⁶ cells
1:32	1, 0, 2	2
1:16	6, 5, 4	10
1:8	12, 11, 9	22
1:4	20, 25, 27	48
1:2	42, 30, 34	70
Undiluted	59, 61, 62	122

[a] The volume of ψ2/pBC-10 culture medium used in the undiluted assay was 150 μl.
[b] All cultures contained 0.1 U of EPO per ml and 5 × 10⁵ cells per well as described in Materials and Methods. The hemoglobinized bursts are indicated.

which suggests that it was not caused by the heat-stable hormone EPO (6). The formation of hemoglobinized bursts was dependent upon added EPO (see Table 2). Moreover, the number of bursts was proportional to the concentration of ψ2/SFFV (Table 3), suggesting that each burst was caused by a single virus particle. The bursts induced by ψ2/SFFV formed transiently in the cultures 3 to 7 days after infection (Fig. 3), which was consistent with earlier studies of the viral complex (5). These results provide clear evidence that the burst-promoting characteristics of Rauscher virus are entirely due to its R-SFFV component.

In addition to its activity in the above assay, injection of ψ2/SFFV into adult mice caused erythroblastosis. A single injection of 0.5 ml of ψ2/SFFV into normal mice induced an erythroblast hyperplasia of small magnitude (ca. 50% increase in spleen mass 3 to 6 days after injection) that subsequently regressed. Substantial splenomegaly was induced by repeated injections of ψ2/SFFV into phenylhydrazine-pretreated mice (Table 4). This splenomegaly also regressed after the cessation of injections (data not shown). Moreover, these enlarged spleens contained substantial numbers of CFU-E that hemoglobinized only in the presence of EPO (Table 4). Conversely, the inoculation of a polycythemic strain of Friend virus caused splenomegaly and CFU-E colonies that hemoglobinized in the absence of EPO (Table 4). EPO-dependent CFU-E formation is characteristic of anemia-inducing strains of SFFV, whereas the polycythemia-inducing strains cause the growth and devel-
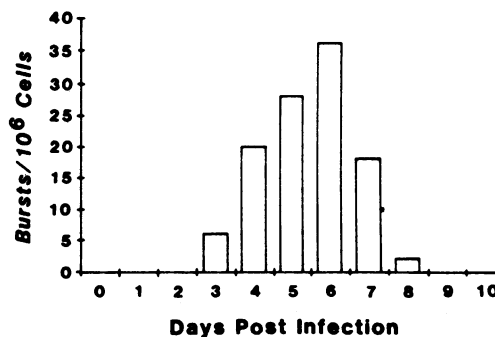

FIG. 3. Transient appearance of ψ2/SFFV-induced erythroid burst formation. The assay was performed as described in Materials and Methods, except that clusters of erythroid colonies were scored in situ on the days indicated. The values shown are averages of three wells.

TABLE 4. Erythroid colony-forming assay of enlarged spleens from mice injected with ψ2/SFFV

Virus	Spleen wt (mg)[a]	EPO[b]	Hemoglobinized colonies per clot[c]
ψ2/SFFV[d]	2,070	−	0
		+	225
	310	−	2
		+	211
	671	−	3
		+	423
FvP[e]	1,760	−	71
		+	96
	310	−	25
		+	74
	1,840	−	74
		+	112
No virus	110	−	0
		+	30
	90	−	0
		+	27
	130	−	0
		+	19

[a] Splenectomies were performed 6 days after the first viral injection.
[b] The concentration of EPO, when used, was 0.5 U/ml.
[c] The values shown are averages of three separate clots from the same spleen section.
[d] ψ2/SFFV was injected into the tail veins of phenylhydrazine-pretreated male Swiss mice as described in Materials and Methods.
[e] Phenylhydrazine-pretreated mice were given a single injection of 10[4] spleen focus-forming units of a polycythemic Friend erythroleukemia viral preparation (FvP).

TABLE 5. Splenic erythroblastosis induced by ψ2/SFFV

Virus	Day postinfection	Spleen wt (mg)
None[a]	14	83, 76, 65
	21	144, 133, 98
	28	112, 91
ψ2/SFFV	14	185, 170, 133
	21	108, 96, 91
	28	157, 122, 98, 96
ψ2/SFFV/R-MuLV complex[b]	14	820, 607, 254
	21	298, 285, 224
	28	2,683, 290, 232, 166
ψ2/SFFV/R-MuLV mixture[c]	14	321, 317, 401
	21	181, 172, 156
	28	786, 161, 142

[a] Culture fluid from ψ2 cells.
[b] Culture fluid from NIH/3T3 fibroblasts that had been coinfected with ψ2/SFFV and R-MuLV.
[c] ψ2/SFFV (0.4 ml) mixed with 2 × 10^{4} PFU of R-MuLV. This is the same viral preparation used to produce the lysate shown in Fig. 2, lane 3.

opment of CFU-E colonies that hemoglobinize in the absence of EPO (6).

Thus, the injection of 4- to 6-week-old Swiss mice with ψ2/SFFV caused splenic erythroblastosis that regressed. Mixing ψ2/SFFV with R-MuLV before a single injection caused the more prolonged pathogenesis that is characteristic of Rauscher disease (Table 5).

DISCUSSION

Pathogenic effects of SFFV. Hematopoiesis involves a hierarchy of stem cells that exist primarily in the bone marrow of healthy adults (20). The most primitive precursors are pluripotential stem cells that apparently contribute to the maintenance not only of the blood cells but also of the lymphoid tissues. Within this hierarchy occurs a lineage of stem cells that derive from each other and are committed to erythroid differentiation (20). These committed erythroblasts have limited self-renewal capabilities, and their growth and differentiation are normally regulated by both hormonal and microenvironmental factors.

This study provides strong evidence that single SFFV virions can stimulate the proliferation and differentiation of infected erythroblasts. The most pathologically prominent erythroblast targets seem to be 4- to 7-day burst-forming units. These divide several times to produce CFU-E, each of which seeds a small colony of hemoglobinized descendant cells (Tables 1 and 4) (4). The CFU-E colonies that form in response to R-SFFV (and to other anemia-inducing strains of SFFV) become hemoglobinized only in the presence of substantial concentrations of EPO, whereas the CFU-E colonies induced by polycythemia-inducing strains of Friend SFFV hemoglobinize even in the absence of EPO supplements (Table 4) (6). The ability of SFFVs to cause

erythroblastosis is dependent upon their env genes that encode the gp54-55 membrane glycoproteins (8, 8a, 9, 10, 13, 14). Moreover, the differences between the polycythemia- and anemia-inducing strains are also encoded by these env genes (13, 23).

Although the major pathogenic effect of the Friend and Rauscher virus complexes is restricted to the erythroid lineage, there has been no clear explanation for this specificity. Conceivably, the viruses could infect all hematopoietic cells but could only cause substantial pathogenic alterations within the erythroblasts. This would occur if gp54-55 specifically affected erythroblasts or if the enhancer sequence in the SFFV long terminal repeat was only active in this lineage. Our observation that ψ2/SFFV induced a transient and regressing erythroblastosis is consistent with other evidence (20) that SFFVs cause proliferation of erythroblasts that have a limited self-renewal capability. If the multipotential stem cell precursors that replenish the erythroblast pools were efficiently infectable with SFFV, a single inoculum of ψ2/SFFV would be expected to cause a nonregressing disease. Our results therefore suggest that these precursor stem cells are less efficiently infected than the committed erythroblasts or that the precursors infected with ψ2/SFFV inefficiently enter the erythroid lineage. Conceivably, a larger inoculum of ψ2/SFFV would cause infection of more stem cells and a relatively prolonged pathogenesis. A recent study by Wolff and Ruscetti (23) indicated that a polycythemic strain of pure Friend SFFV can cause persistent erythroblastosis in a proportion of phenylhydrazine-pretreated mice. Their evidence implied that the persistent erythroblastosis was probably not caused by helper virus contamination. We believe that our results are compatible with theirs and that additional evidence will be required to explain the pathogenic differences between different strains of SFFV.

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