PERSISTENCE AND PATHOGENICITY OF DEFECTIVE FRIEND SPLEEN FOCUS-FORMING VIRUS

Decreased Transplantability of Hemopoietic Cells as a Marker for Preleukemic Change*

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Since the original description of the continuous replication of defective Friend spleen focus-forming virus (SFFV)[1] in mouse embryo fibroblasts (MEF) (1), nonproducer mouse and rat cell culture systems have been developed for the purpose of estimating the coding capacity of the SFFV genome and to determine the relatedness of the SFFV genome to its associated Friend lymphatic leukemia-inducing helper virus (LLV-F) and other murine leukemia viruses (MuLV) (2–4). Although it has been shown that the genome of fibroblastic cell grown SFFV contains two sets of distinguishable RNA sequences, one related to a portion of the LLV-F genome and one related to murine xenotropic MuLV (4), several issues obscure the meaning of these observations with respect to the role of xenotropic-related sequences in the induction of Friend erythroleukemia. First, fibroblastic cells in culture are not a target for the expression of SFFV transformation markers and it has been assumed that the replication defective component of the Friend virus (FV) complex carries the oncogenic potential for the induction of classic Friend disease. However, to date there has been no direct evidence put forth demonstrating that SFFV, when purified free from standard MuLV helper and inoculated into a susceptible host, is able to initiate a series of pathogenic events which ultimately lead to the induction and maintenance of an erythroleukemia. Also, the rapidity with which SFFV (as contained with LLV-F in the FV complex) is able to induce a fatal erythroleukemia in both newborn and adult mice has limited attempts to unravel those pathogenic events which precede the development of leukemia and which might be characterized as preleukemia changes. This is in contrast to the LLV-F (5) and other MuLV model systems in which preleukemic syndromes have been defined (6, 7), and in which xenotropic MuLV expression has been correlated with the onset of leukemia (8).

In a previous study we have demonstrated that replication defective Friend SFFV particles can be physically separated from standard MuLV helper and that SFFV is able to persist in vivo for a 3-d period (9). Here we present a series of experiments which document the oncogenic potential of purified SFFV particles and define for the first time an extensive preleukemic phase during which SFFV function(s) and host anti-leukemic reactivity may be investigated.

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[1] *Abbreviations used in this paper*: CFU-S, pluripotent hemopoietic stem cell; DI, defective-interfering; DMEM, Dulbecco's modified medium; FFU, focus-forming units; FIU, focus-inducing units; FMR, Friend-Moloney-Rauscher; FUdR, 5-fluoro-2′-deoxyuridine; FV, Friend virus; GLV, Gross leukemia virus; Hh, hemopoietic histocompatibility; IUdR, 5-iodo-2′-deoxyuridine-^{125}I; LLV-F, lymphatic leukemia-inducing virus; MEF, mouse embryo fibroblasts; MuLV, murine leukemia virus; PBS, phosphate-buffered saline; SFFV, spleen focus-forming virus; TCFU, SFFV-induced tumor cells.

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Materials and Methods

Mice. Male and female 4- to 6-wk-old SIM ($Fv\text{-}1^{nn}$) and SIM.R ($Fv\text{-}1^{bb}$) mice are maintained in our breeding colony. These highly inbred mice (10) were originally obtained through the courtesy of Arthur A. Axelrad, University of Toronto, Canada. C57BL/6 ($Fv\text{-}1^{bb}$, $Fv\text{-}2^{rr}$) and BDF_1 i.e., (C57BL/6 × DBA/2)F_1 ($Fv\text{-}1^{nb}$, $Fv\text{-}2^{rs}$) were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Viruses. All viruses used in this study were derived from in vitro (MEF) cultures. Exposure of NIH/3T3 cells to infectious FV complex has resulted in the productive infection of these cells by both defective Friend SFFV and NB-tropic LLV-F helper (1). Several preparations of FV complex have been subsequently derived from MEF-grown FV complex and provide homogenous stocks of FV complex for the studies presented here. Virus from one particular clone of NIH/3T3 cells was used throughout the study. This virus has an LLV-F to SFFV ratio of approximately 3:1.

Isolation and maintenance of LLV-F (11) and Gross leukemic virus (GLV) (12, 13) has been previously described. In all instances, the helper virus used to reveal the presence of SFFV in gradient samples and used for in vivo rescue of SFFV consisted of homogenous stocks of either LLV-F or GLV each having an NB-tropic host range and titers of greater than 5×10^5 FIU per ml. All in vitro rescues of SFFV were done using LLV-F derived from NIH/3T3 cells chronically infected with cloned LLV-F.

All virus stocks used in these experiments were free of detectable xenotropic virus since sedimentable RNA-dependent DNA polymerase activity could not be detected in 200-fold concentrated culture fluids of MvlLu (CCL64) mink cells 30 d after exposure of cultures to each of the viruses in question. Finally, the method of purifying SFFV free from LLV-F helper has been described in detail (9). Briefly, cloned FV harvested from NIH/3T3 cells was disaggregated by using ultrasonic shock (1 pulse per s for 8 s using a Branson sonifier and microtip model W-200P) and layered onto a preformed 0–20% sucrose sedimentation gradient. After centrifugation (SW27 rotor for 30 min at 20,000 rpm at 4°C), the tube was bottom-punctured and 1.0-ml samples each were collected. Those samples seen to contain only SFFV (see spleen focus assay below) were layered onto preformed 20–40% sucrose equilibrium gradients and centrifuged for 24 h at 4°C (SW27 rotor at 26,500 rpm). Samples were again taken by bottom-puncture and those shown to contain pure SFFV (density ≈ 1.14 g/ml) were pooled and used in the studies presented below.

Cultured Cells and Maintenance Medium. Swiss mouse 3T3FL cell lines were originally obtained from Dr. R. H. Bassin (National Cancer Institute, Bethesda, Md.). The origin of these 3T3FL cells has been described as has the origin of the murine sarcoma virus-transformed cells termed sarcoma-positive leukemia-negative (S+L−) mouse cells (14). 3T3FL cells, and their S+L− counterparts (clone C-243-3) are maintained by routine trypsinization every 3–4 d. Culture medium consists of McCoy's 5A (Grand Island Biological Co., Grand Island, N. Y.) containing 10% heat-inactivated fetal calf serum.

NIH/3T3 cells were obtained from P. Besmer, Center for Cancer Research, MIT, Cambridge, Mass. This cell line is passaged by trypsinization before confluence in Dulbecco's modified medium (DMEM, Grand Island Biological Co.) supplemented with antibiotics and 10% heat-inactivated calf serum.

Clone III6A of feral mouse embryo fibroblasts (SC-1 cells) (15) was kindly provided by P. Besmer, as was the mink cell line MvlLu (CCL64) (16). These cell lines are maintained in DMEM with 10% heat-inactivated fetal calf serum and antibiotics and are passaged every 4–5 d before forming a confluent monolayer.

All virus-producing cell clones were derived from cells infected with an initial multiplicity of infection (moi) of not greater than 0.02. SC-1/LLV-F cells are maintained in a manner identical to the parental SC-1 cell line and chronically produce LLV-F with a titer of approximately 5×10^5 infectious units per ml of unconcentrated culture medium. In addition, NIH/3T3 cells chronically infected with cloned LLV-F (clone NIH/LLVB6) are maintained in DMEM supplemented with 10% heat-inactivated calf serum and are subcultured every 4–5 d as above. This cell line was used preferentially for all in vitro rescues of SFFV from SIM spleen cells since, in that way, both spleen cell and fibroblast were of Swiss origin.

Preparation of Hemopoietic Cell Suspensions. Spleen cell suspensions were prepared by flushing

the spleens with DMEM until the majority of the spleen cells had been removed from the spleen stroma. The cells were then passed through a wire mesh (110/in), nucleated cells were counted using a Coulter counter (ZBI), the percent viability was determined via trypan blue dye exclusion (usually ≈90%), and cells were adjusted to the desired concentration in DMEM with fetal calf serum. The cells were kept in tubes in ice water until used.

Virus Detection Assays

SPLEEN FOCUS ASSAY. All SFFV preparations as well as culture media and sucrose gradient samples suspected to contain SFFV were titrated in vivo with the spleen focus assay (17). Samples of SFFV diluted into phosphate-buffered saline (PBS) alone, or diluted into PBS with exogenous LLV-F added (10^3 FIU) were injected i.v. into susceptible mice, seven per group. 9 d later, their spleens were removed and fixed in Bouin's solution. Discrete foci on the splenic surface were counted macroscopically, and virus titers (mean number of foci per spleen × dilution factor) are expressed in focus-forming units (FFU) per ml where one FFU represents that amount of virus required to induce an average of one focus per spleen. All LLV stocks were free of detectable SFFV.

S+L− INFECTIOUS CENTER ASSAY FOR DETECTION OF MULV HELPER ACTIVITY. The number of S+L− cells producing rescued MSV after exposure to NB-tropic MuLV helper only or FV complex (SFFV plus LLV-F helper) are measured by using an infectious center assay which has been described in detail previously (9). Briefly, 0.5 ml of each serial dilution of LLV-F only or SFFV and LLV-F is inoculated into 10^5 S+L− cells pretreated with DEAE-dextran. After an adsorption period of 1 h, 5 ml fresh medium containing 10% heat-inactivated fetal calf serum is added to each dish. 8–16 h postinfection, 10^4 cells are transferred into recipient plates already containing 10^5 normal 3T3FL cells. Transferred cells releasing infectious MSV give rise to large foci of recruited transformed 3T3FL cells which are easily enumerated 3–4 d after cell transfer. Virus titers (mean number of foci per dish multiplied by the dilution factor) are expressed in FIU per ml where one FIU represents that amount of virus required to induce an average of one focus per dish.

The S+L− focus assay is used for detecting and quantifying MuLV in the presence and absence of SFFV because SFFV neither provides a helper function to rescue MSV from S+L− cells nor interferes with LLV-F rescue function(s) in this assay (9). In addition, 3T3FL cells and S+L− cells are highly sensitive to MuLV helper infection and do not express *Fv-1* restriction (18). For all these reasons, this infectious center assay is the ideal method for the detection and quantification of ecotropic MuLV helpers.

ASSAY FOR VIRION-ASSOCIATED REVERSE TRANSCRIPTASE. Any fluids to be tested for RNA-dependent DNA polymerase activity were harvested and clarified by centrifugation at 10,000 rpm for 15 min. These fluids were then concentrated 200-fold via centrifugation at 39,000 rpm for 90 min and pellets were resuspended in 0.01 M Tris-HCl, pH 7.8. Reverse transcriptase reaction mixtures were incubated and quantified according to the technique of Fan and Baltimore (19).

In Vivo Rescue Technique. Animals were inoculated with 10^3 FFU of equilibrium gradient-purified SFFV. At the intervals indicated in Fig. 1, groups of these animals were inoculated with excess LLV-F in an attempt to reveal SFFV persistence. These animals were sacrificed 9 d after LLV-F inoculation, their spleens were removed, fixed in Bouin's solution, and observed for the presence of rescued SFFV-induced spleen foci. Since only a spleen cell infected with both SFFV and an MuLV helper produces SFFV progeny resulting in the rapid enlargement of a focus by spread of SFFV infection, this in vivo rescue technique is a valid approach for detecting latent SFFV infection (20).

In Vitro SFFV Rescue Technique. Animals suspected to be harboring latent SFFV were hemisplenectomized, and the spleen fragments were prepared as a single cell suspension as described above. 10^7 nucleated cells were pipetted into a 60-mm Falcon tissue culture dish already containing a mixture of normal and LLV-F-producing NIH/3T3 cells. The spleen cells were allowed to settle onto the monolayer and remained there undisturbed for 4–5 d. At that time, the culture fluids containing the spleen cells were harvested, clarified of all particulate matter by centrifugation at 10,000 rpm for 30 min, and assayed for the presence of SFFV and LLV-F by using the methods already described. The NIH/LLV cells onto which the spleen cells had been overlaid were maintained for 3 wk after this initial exposure and unconcentrated

culture fluids from the subcultures were also harvested and assayed for the presence of infectious SFFV and LLV-F.

Animal Irradiation and Spleen Cell Transplantation. Recipient mice were exposed to 800 rads of total-body irradiation in a small animal irradiator with two ^{137}Cs sources (Gamma Cell 400, Atomic Energy of Canada, Ltd., Toronto). Each mouse was infused i.v. with usually 2×10^6 spleen cells from normal Swiss, $SFFV^+$ preleukemic, or FV leukemic mice in a 0.5-ml vol within 2 h of irradiation. 7 d postcell transplantation, proliferation of the donor-derived cells in the spleens of recipient mice was assessed by measuring the incorporation of 5-iodo-2′-deoxyuridine-^{125}I (IUdR), a specific DNA precursor and thymidine analogue. The mice were each injected with 10^{-7} M 5-fluoro-2′-deoxyuridine (FUdR) i.p. in a 0.1-ml vol 1 h before the i.p. injection of 0.5 μCi IUdR. FUdR inhibits endogenous thymidylate synthesis and enhances IUdR uptake under these conditions. Spleens were removed 24 h after isotope injection and the ^{125}I radioactivity was measured in a crystal scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). In addition, the spleens were fixed in Bouin's solution to facilitate the enumeration of donor spleen cell growth in the form of spleen colonies.

Spleen Colony Assay for SFFV-Induced Tumor Cells (TCFU). Since the cell-free fluids derived from spleen cell preparations were free of detectable SFFV and ecotropic MuLV activities by using the assay techniques described above, and since the $SFFV^+$ erythroleukemic cells obtained in this study were transplantable in unirradiated mice, we were able to detect and quantify the numbers of SFFV-induced tumor cells in various spleen cell preparations on the basis of spleen colony formation in syngeneic SIM.R mice (20).

Results

Duration of Persistence of Rescuable Friend SFFV in Swiss Mice. We have recently demonstrated that defective Friend SFFV particles differ significantly from standard MuLV helpers with respect to their sedimentation properties and buoyant density in sucrose (9). Exploitation of these expressed differences in virion physical properties has made possible the purification of infectious SFFV particles free from standard helper virus. It has also been shown that SFFV can be separated from replicating MuLV helper by using cell culture techniques. Rescuable SFFV can persist in the absence of replicating helper for long periods of time in vitro in both NIH/3T3 and BALB/c nonproducer cells (2, 3).

In a series of preliminary experiments designed to investigate the infectivity properties of purified SFFV for hemopoietic target cells, we have observed that SFFV is able to persist in vivo for a 3-d period (9). This observation, together with the cell culture studies mentioned above, suggested to us that purified SFFV might be able to persist in vivo (i.e., in stem cells of a renewing hemopoietic cell population) for an extended period. To determine if a target cell for SFFV persistence is a hemopoietic stem cell or a differentiating cell population which would eventually come to the end of its life cycle and of necessity eliminate nonreplicating SFFV from the host, we inoculated 4-wk-old female SIM.R mice i.v. with gradient purified SFFV. Of the 40 animals inoculated, 4 were coinfected with 10^4 FIU of LLV-F helper to determine the focus-forming efficiency of our purified SFFV stock. 9 d after LLV-F inoculation, these four mice were sacrificed and their spleens were removed and fixed in Bouin's solution. The average number of SFFV-induced foci appearing on the surface of the fixed spleens was >100 (range of >100 to confluent) (Fig. 1, day 0). Four additional mice that had received only SFFV were also sacrificed. In all instances, their spleens, livers, and other lymphoid organs were free of macroscopic and microscopically detectable disease. We thus concluded that the entire group of SIM.R mice had initially received approximately $500–10^3$ FFU of infectious SFFV particles and that the remainder of the group could be used to study SFFV persistence and pathogenicity.

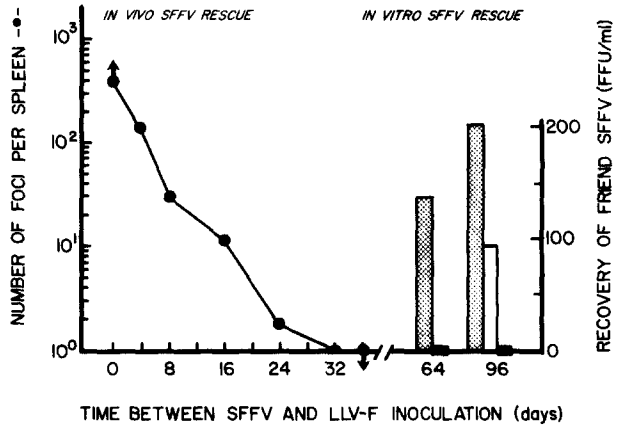

FIG. 1. Kinetics of rescue of defective Friend SFFV from the spleens of persistently infected SIM.R mice. On day 0, 40 mice were each inoculated with approximately 10^3 FFU of SFFV. At the time intervals indicated, groups of four mice each were inoculated with 10^4 FIU of NB tropic LLV-F helper. SFFV rescue and subsequent development of spleen foci of erythroleukemic cells was monitored on day 9 posthelper inoculation (●). Beyond day 36 post SFFV administration, only in vitro rescue techniques were employed. Hemisplenectomies were performed and histological spleen preparations were viewed for the presence of malignant change. The remaining tissue was prepared as a single cell suspension and exposed to helper virus in culture as follows: ■, spleen cells cultured alone; □, spleen cells cocultured with NIH/3T3 cells chronically infected with NB-tropic LLV-F; □, spleen cells cocultured with NIH/3T3 cells chronically infected with NB-tropic GLV.

We subsequently inoculated groups of four SFFV-pretreated mice with LLV-F helper on the days indicated. These animals were monitored for in vivo SFFV rescue by using the spleen focus assay since SFFV defectiveness for focus formation in vivo is a reflection of SFFV dependence upon its associated helper virus for rapid synthesis of infectious SFFV progeny and resultant spread of SFFV infection (20). As shown in Fig. 1, a consistent reduction in the numbers of rescued spleen foci was observed as a function of time between SFFV and LLV-F inoculations. Beyond 24 d, no spleen foci were observed. Further, we examined peripheral blood smears and histological spleen preparations obtained from SFFV-pretreated mice inoculated with LLV-F on day 32 and day 36, but could find no evidence of SFFV- or LLV-induced leukemic change. These data suggested to us that SFFV had been excluded from the hemopoietic system of these mice as the cells originally infected by SFFV differentiated. However, it was also possible that these mice had either become immune to LLV-F via the expression of cross-reacting SFFV-encoded antigen(s) (i.e., *gag* gene products [3]) or had restricted the expression of LLV-F via SFFV defective-interfering (DI) properties (9). Since individual serum samples prepared from these mice were not able to neutralize or otherwise interfere with SFFV or LLV-F infection (data not shown), we presumed SFFV-DI activity and continued to probe for evidence of SFFV persistence using in vitro rescue techniques (Materials and Methods). Four mice which had been inoculated 64 d previously with purified SFFV were anesthetized and one half of each spleen was removed. A section from each spleen was viewed histologically for evidence of leukemia and found to be free of detectable Friend disease (Fig. 2). The remainder of each spleen was pooled, prepared as a washed single cell suspension, and 10^7 nucleated cells were cultured in the presence of NIH/3T3 cells productively infected with NB-tropic LLV-F helper (NIH/LLV cells). The original spleen cell

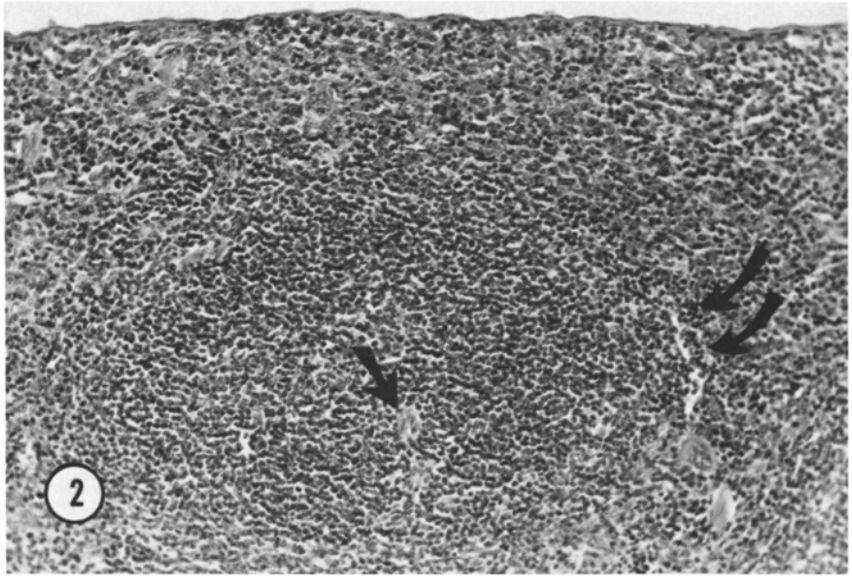

FIG. 2. Histological preparation of an adult SIM.R spleen. This animal received 10^3 FFU of purified defective Friend SFFV in the absence of detectable MuLV helper 64 d before splenectomy. A low power view of the spleen demonstrates the preservation of normal architecture. Note the prominent germinal center in the white pulp with the central arteriole (single arrow), the distinct marginal zone (double arrows), and red pulp (hematoxylin and eosin × 60).

wash as well as cell-free supernatant fluids from 4 and 5 d cocultures did not contain detectable SFFV. However, fluids harvested from 3-wk cultures contained $>10^2$ FFU of Friend SFFV (Fig. 1) and an excess of LLV-F helper. Since the fluids from the original spleen cell suspension contained no detectable virion-associated RNA-dependent DNA polymerase activity, and since fluids from 4 and 5 d cocultures contained only LLV-F activities, we presumed that an extremely limited amount of SFFV was initially rescued from the dying spleen cell preparation. Further, rescued SFFV together with LLV-F helper most likely coinfected the normal NIH/3T3 cells provided in the culture mixture (Materials and Methods) giving rise to chronic production of this FV complex. This same in vitro SFFV rescue technique was employed using four mice which had been inoculated 96 d previously with purified SFFV. Again, there was no histological evidence of leukemia detected in the donor spleens and SFFV was rescued and observed at 3 wk by using NIH/LLV cocultures (Fig. 1). To determine if SFFV could be rescued from these persistently infected spleen cells ($SFFV^+$ cells) more effectively by a helper virus not included in the Friend-Moloney-Rauscher (FMR) group, we cultured 10^7 $SFFV^+$ spleen cells in the presence of NIH/3T3 cells productively infected with NB-tropic Gross leukemia virus (NIH/GLV cells). The amount of infectious helper virus released from our NIH/LLV and NIH/GLV cultures were virtually identical based upon virus yield per cell. However, GLV was not as effective in rescuing SFFV in this culture system (Fig. 1). A similar result was obtained by Fieldsteel et al. (21) who attempted to rescue SFFV from FV-induced reticulum cell sarcomas using GLV helper. Although we do not know the reason for this reduction in expressed GLV helper function(s), it may be related to the differential expression of SFFV particle-associated defective-interfering

properties. Finally, both SFFV(LLV-F) and SFFV(GLV) pseudotypes were able to induce a pattern of Friend disease identical to that induced by the strain of in vitro (MEF)-maintained FV complex from which the SFFV was originally purified. This disease pattern included the induction of polycythemia (hematocrits $\geq$70%) in all infected mice.

Oncogenic Potential of Purified Friend SFFV Particles. Although we have not determined the type or the frequency of the persistently infected SFFV$^+$ spleen cell, it is clear from the data presented above that the target cell for SFFV persistence is either a very long-lived cell or a self-renewing cell, or both. Since SFFV particles are able to establish a latent form of persistent virus infection for a period of at least 90 d, we embarked upon a series of experiments to determine if SFFV particles alone carry the erythroleukemia-inducing potential traditionally expressed by the FV complex (i.e., SFFV in the presence of LLV-F). Although no histological evidence of Friend disease could be detected in SFFV$^+$ mice 96 d after virus inoculation, the possibility remained that mice harboring rescuable SFFV also harbored hemopoietic cells expressing preleukemic change or expressing SFFV transformation markers (i.e., persistently infected mice might contain small numbers of transplantable Friend tumor cells). It is reasonable to assume that at least in part host immune mechanisms are responsible for the disease-free status of these animals. The absence of disease could be explained by either active suppression of the SFFV$^+$ transformed phenotype on the part of the persistently infected mice, or by an effective killer cell mechanism directed against these cells. Therefore, we elected to conduct cell transplantation experiments using syngeneic mice $\leq$5 days of age in an attempt to circumvent possible immune functions which might be operative in mature adult animals and able to restrict the growth and resultant detection of SFFV$^+$ transformed cells. We inoculated a cell suspension freshly prepared from a spleen fragment obtained via hemisplenectomy from an SFFV$^+$ SIM.R mouse (96 d post SFFV). Each of 25 5-d-old recipient SIM.R mice received an i.p. injection of 10^7 cells. Within a 20-d period, the recipients of this attempted spleen cell transplant were observed to have enlarged and palpable spleens. Peripheral blood smear preparations were found to contain benzidine-positive, nucleated erythrocytes, large mononuclear cells, and a preponderance of extremely fragile smudged cells. Overall, the pattern of disease was identical to that originally described by Friend (22), and the histopathology of the disease induced is presented in Fig. 3 and 4. The clusters of leukemic cells seen within the hepatic sinusoids are at various stages of erythroid differentiation (Fig. 4). The average leukocyte count at day 20 postcell inoculation was 95,000/mm^3, and hematocrit values ranged from 27 to 32% (normal values for SIM.R are approximately 40%). We now have established an in vivo maintained tumor cell line from these leukemic animals and preliminary studies concerning the donor origin of this leukemia are presented below. The remaining SIM.R mice which had been hemisplenectomized and shown to harbor Friend SFFV (Fig. 1) began to die of typical Friend disease 134 d after SFFV inoculation. The disease observed in these SFFV-infected mice was identical to that described above. It is interesting to note that, although the SFFV used in this study was purified from a cloned stock of in vitro (MEF)-maintained FV complex which is a polycythemia-inducing strain (23), and although rescued SFFV-LLV complex induced a polycythemia, all leukemic mice inoculated only with SFFV were severely anemic. Finally, we were not able to detect mature virus particles in 100-fold

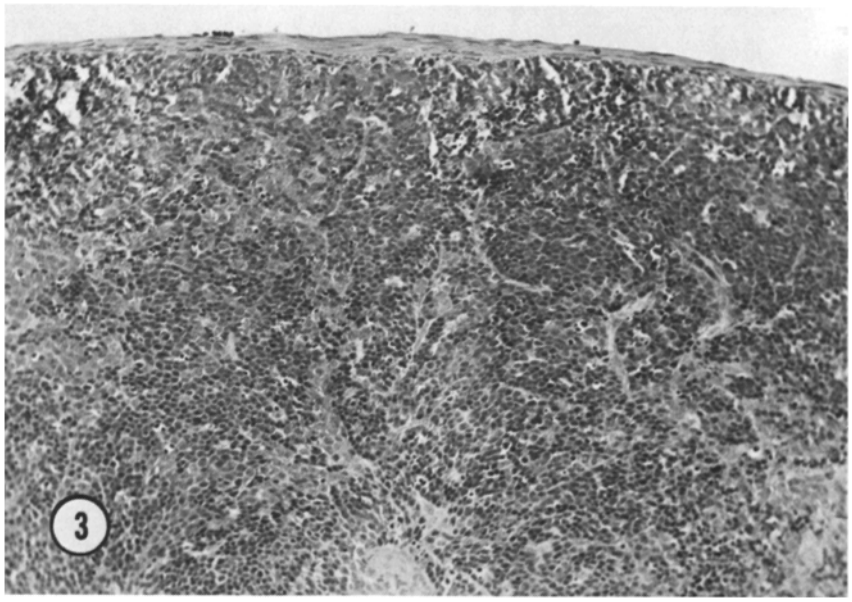

FIG. 3. Histopathology of leukemia induced within a 20-d period in an infant SIM.R who had received spleen cells prepared from an SFFV$^+$ yet histologically normal spleen cell donor. The large leukemia cells flood the splenic pulp and virtually replace the smaller lymphocytes (hematoxylin and eosin, × 60).

concentrated supernatent fluids from either liver or spleen cell suspensions prepared from leukemic mice using an assay for the detection of virion-associated reverse transcriptase activity and an S + L-helper assay for ecotropic MuLV.

Rejection of SFFV$^+$ Preleukemic Spleen Cells by Nonimmunized Lethally Irradiated Syngeneic Recipient Mice. A clear demonstration of the erythroleukemia-inducing potential of purified Friend SFFV particles allows us to define the SFFV latency period up to at least 90 d postinfection as a preleukemic phase. With the knowledge that preleukemia in other MuLV systems may be associated with changes in the expression of viral (e.g., xenotropic MuLV gp70) and host (e.g., *H-2* and Thy 1.2) cell surface antigens (6, 8, 24) and with the knowledge that FV-infected mice (25) and in vitro bone marrow cell cultures (26) either contain increased numbers or sustained proliferation of pluripotent hemopoietic stem cells (CFU-S), we were desirous to know if the spleen cells obtained from SFFV$^+$ mice expressed any detectable alteration in membrane surface markers. We elected to conduct cell transplantation studies because this would allow us to enumerate the frequency of CFU-S per SFFV$^+$ spleen, the transplantability of such cells, and their ability to protect lethally irradiated mice from hemopoietic death. Recipient SIM and SIM.R mice were exposed to 800 rads of total-body irradiation and infused intravenously with one of several different spleen cell preparations listed in Table I. The proliferation of donor cells in the spleens of recipient mice was estimated by measuring the incorporation of ^{125}IUdR and by enumerating the numbers of macroscopically detectable surface spleen colonies 8 d after cell inoculation. As shown in Table I, exp. 1, normal SIM.R donor spleen cells proliferated effectively in both SIM.R (*Fv-1^{bb}*) and SIM (*Fv-1^{nn}*) adults. We were thus able to

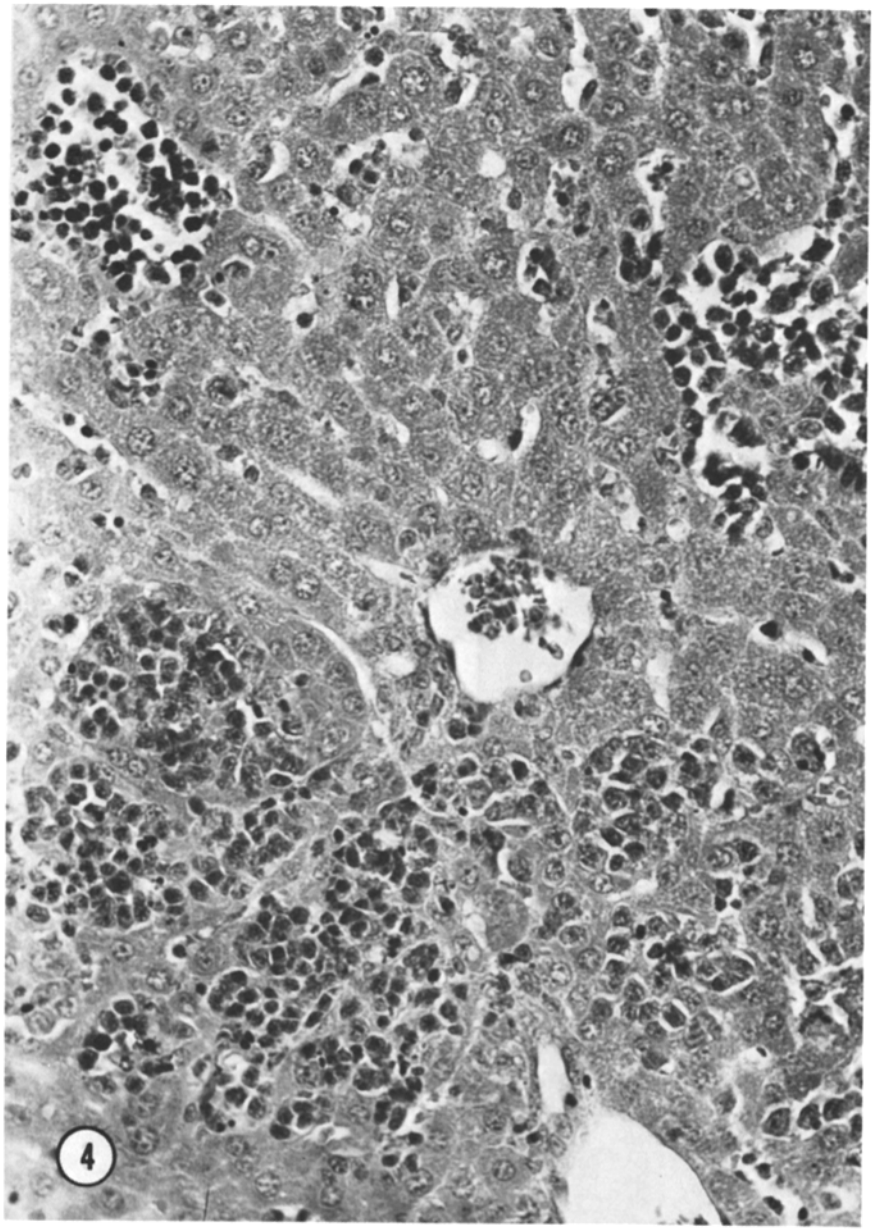

FIG. 4. A high power view of the liver of the same leukemic animal presented in Fig. 3 to demonstrate the infiltrate of large leukemic cells with large vesicular nuclei and prominent nucleoli. The clusters of leukemic cells within the hepatic sinusoids are seen as foci of normoblasts and more primitive erythroid cells at various stages of differentiation.

utilize both strains of Swiss mice as recipients of spleen cell grafts obtained from SIM.R donor mice inoculated 64 d previously and shown to contain rescuable Friend SFFV. The results of this attempted cell transplant (Table I, exp. 2) indicate that these preleukemic cells were not able to proliferate significantly in SIM or SIM.R

TABLE I

Proliferation of Normal, SFFV$^+$ Preleukemic, and FV Complex (SFFV-LLV)-Induced Leukemic Spleen Cells in Irradiated Syngeneic Recipient Mice

Donor SIM.R (cells grafted)	Recipient strain (no. of mice)	Mean splenic incorporation of ^{125}IUdR*		Mean no. of spleen colonies (CFU-S)‡
		cpm	*% uptake*	
Exp. 1				
Normal (2×10^6)	SIM.R (10)	7,860	(1.0)	54
	SIM (10)	6,840	(0.86)	44
None	SIM.R (5)	390	(0.05)	0
None	SIM (5)	560	(0.07)	0
Exp. 2				
Preleukemic: inoculated with SFFV +64 d§ (10^6)	SIM.R (5)	720	(0.08)	0
	SIM (3)	270	(0.03)	0
	SIM (4)	(Isotope not injected)‖		
Exp. 3				
Normal (2×10^6)	SIM.R (6)	8,220	(0.98)	40
Leukemic: inoculated with 10^3 FFU of FV complex +7 days (10^6)	SIM.R (6)	4,860	(0.58)	38
None	SIM.R (5)	830	(0.03)	1.6

* IUdR-labeled with radioactive ^{125}I was used to assess DNA synthesis in the spleens of irradiated mice engrafted with spleen cells (Materials and Methods). All recipient mice were exposed to 800 rads of total-body irradiation and proliferation was estimated 8 d after irradiation and spleen cell transplantation. Standard errors for mean splenic isotope uptake were ≈10% and the overall survival of the animals assigned to this series of experiments was 90%.

‡ The recipients' spleens were removed 8 d after irradiation and cell transplantation, fixed in Bouin's solution, and viewed macroscopically for the presence of surface colonies.

§ This cell suspension was prepared from the spleens of histologically normal (Fig. 2) donor mice which had received an inoculum of $\approx 10^3$ FFU of purified defective SFFV 64 d previously. These cells contained rescuable SFFV as shown in Fig. 1.

‖ These animals did not receive isotope and were not sacrificed, but monitored for potential hemopoietic repopulation and/or tumor development after i.v. injection of SFFV$^+$ spleen cells. All four mice died of hematopoietic failure 22-28 d after irradiation.

recipients. Four additional SIM mice were not injected with isotope but instead were monitored for potential hemopoietic repopulation and CFU-S development. All four mice died of hemopoietic failure 22–28 d after irradiation and cell transplantation indicating that the SFFV$^+$ donor spleen cells did not contain transplantable CFU-S and could not protect these animals from hemopoietic death. In contrast, spleen cells prepared from FV-leukemic SIM.R donors grew effectively in irradiated SIM.R recipients, and gave rise to the expected number of spleen colonies. The failure of SFFV$^+$ spleen cells to proliferate in syngeneic recipient mice was not due to the diminished viability of these donor cells since the number of cells transplanted was standardized to 100% viability based upon trypan blue dye exclusion test. Further, this SFFV$^+$ spleen cell preparation proliferated normally (Δ blastogenesis 2×10^4 cpm) in vitro in response to B- and T-cell mitogens.

From the data presented, it appeared that a radio-resistent host-versus-graft reaction was operative in these nonimmunized mice and directed only against SFFV$^+$ preleukemic cells. Rossi et. al. (27, 28) have previously investigated the proliferative capacity

TABLE II

*Effect of Silica Particles upon the Proliferation of SFFV$^+$ Preleukemic Spleen Cells in Irradiated Syngeneic Recipient Mice**

Donor cells (2×10^6 engrafted per animal)	Recipient SIM.R ± silica treatment	Mean splenic incorporation of ^{125}IUdR‡		Mean no. of spleen colonies (CFU-S)§	
		cpm	*% uptake*		
Normal SIM.R	−(6 mice)	10,700	(1.25)	52	
	+(3 mice)	10,900	(1.28)	57	
Preleukemic: SIM.R inoculated with SFFV +96 d‖	−(6 mice)	230	(0.03)	6	
	Individual mice				($P < 0.02$)
	+	6,630	(0.78)	25	
	+	7,930	(0.93)	34	
	+	3,220	(0.38)	16	
	+	1,010	(0.12)	5	Susceptible
	+	1,190	(0.14)	14	
	+	3,220	(0.38)	16	
	+	270	(0.03)	0	
	+	740	(0.09)	6	
	+	630	(0.07)	10	
	+	720	(0.08)	7	Resistant
	+	820	(0.09)	3	
None	+(6 mice)	260	(0.03)	0.3	

* Mice receiving silica particles were injected i.v. with 2.5 mg of sonicated silica 24 h before irradiation and cell transplantation.

‡ IUdR-labeled with radioactive ^{125}I was used to assess DNA synthesis in the spleens of irradiated mice engrafted with spleen cells (Materials and Methods). All recipient mice were exposed to 800 rads of total-body irradiation and proliferation was estimated 8 d after irradiation and spleen cell transplantation. Standard errors for mean splenic isotope uptake for each group (excluding values given for individual mice) were 15–20% and the overall survival of the animals assigned to this series of experiments was 80%.

§ The recipients' spleens were removed 8 d after irradiation and cell transplantation, fixed in Bouin's solution, and viewed macroscopically for the presence of surface colonies.

‖ This cell suspension was prepared from the spleens of histologically normal donor mice which had received an inoculum of 10^3 FFU of purified defective SFFV 96 d previously. These cells contained rescuable SFFV as shown in Fig. 1.

of FV-infected marrow cells in syngeneic, allogeneic, and hybrid mice. They concluded that within 3 h after FV infection, the enhanced expression of hemopoietic histocompatibility gene(s) (*Hh*) resulted in deficient growth of FV+ cells in irradiated recipient mice. Since this early signal of FV-induced infection and perhaps transformation may be analogous to the SFFV$^+$ preleukemic phase described here, we set out to determine if measures known to abrogate or prevent the rejection of hemopoietic allografts by lethally irradiated mice might also facilitate the growth of SFFV$^+$ spleen cells in syngeneic mice. Groups of SIM.R mice were injected intravenously with 2.5 mg of sonicated silica particles 24 h before irradiation and spleen cell transplantation. Pretreatment of mice with this anti-macrophage agent suppresses both hybrid and allogeneic resistance to hemopoietic cell grafts (29). As shown in Table II, normal SIM.R donor spleen cells proliferated equally well in silica and nonsilica treated SIM.R recipients. We were also able to repeat the observation that preleukemic SFFV$^+$ spleen cells fail to grow in syngeneic recipient mice. In this experiment, all SFFV$^+$ cells were obtained from SIM.R mice inoculated 96 d previously with purified

TABLE III

Autonomous Growth and Transplantability of Hemopoietic Cells Harboring Rescuable SFFV

Source of donor cells (no. grafted)	Recipient strains (pretreatment)	Mean splenic incorporation of ^{125}IUdR*		Mean no. of spleen colonies (CFU-S)
		cpm	*% uptake*	
Exp. 1				
Enlarged spleens from SIM.R inoculated at birth with 10^7 SFFV$^+$ preleukemic spleen cells (2×10^6)‡	SIM.R (800 rads only)	11,900	(1.13)	30
	SIM.R (800 rads + silica)	9,950	(0.95)	41
	BDF_1 (800 rads only)	360	(0.03)	0

	Primary recipients (interval)§	Secondary adult recipients (no. of cells grafted)		Mean no. of spleen colonies (TCFU)‖
Exp. 2				
Same as above (5×10^7)	Infant SIM.R (10 d)	SIM.R	(10^5)	>100
		BDF_1	(10^5)	10 (blotchy)
	Infant BDF_1 (10 d)	SIM.R	(10^5)	>100
			(10^4)	39
		BDF_1	(10^5)	6.2
			(10^4)	0

* IUdR-labeled with radioactive ^{125}I was used to assess DNA synthesis in the spleens of irradiated mice engrafted with spleen cells (Materials and Methods). All recipient mice were exposed to 800 rads of total-body irradiation and proliferation was estimated 8 d after irradiation and spleen cell transplantation. Standard errors for mean splenic isotope uptake were 15% and the overall survival of the animals assigned to this series of experiments was 75%.

‡ This cell suspension was prepared from the spleens of leukemic SIM.R mice (confirmed histologically and presented in Figs. 3 and 4) which 20 d earlier as infants had received an i.p. inoculation of 10^7 histologically normal (Fig. 2) SFFV$^+$ preleukemic spleen cells. This transplant was conducted using cells harboring rescuable SFFV (Fig. 1, + 96 days).

§ 5-day-old SIM.R or BDF_1 mice were inoculated i.p. with SFFV$^+$ spleen cells since infant mice <21 d old are not able to reject foreign hemopoietic cell grafts. 10 d after inoculation, these mice were sacrificed and their spleens were used as a source of cells for the second transplant.

‖ 6- to 8-wk old adult secondary recipient mice were inoculated i.v. in an attempt to monitor for the presence of transplantable TCFU of SIM.R or BDF_1 origin.

SFFV particles. In contrast to this lack of cell growth (IUdR uptake not greater than that of radiation controls) approximately 50% of the silica-pretreated recipient mice permitted extensive growth of SFFV$^+$ cells. Although there was a variable silica effect, this is not unexpected (29) and there was a significant difference ($P < 0.05$) between the susceptible and resistant groupings of silica-treated mice. The cell proliferation and spleen colony formation observed in these mice did not result from the stimulation of endogenous spleen colonies (data not shown) since infusion of sonically disrupted cells into lethally irradiated and silica pretreated mice gave negative results. The observation that a single intravenous injection of silica particles greatly reduced the ability of irradiated mice to resist syngeneic SFFV$^+$ spleen cell grafts suggests that a radioresistant effector cell mechanism which has been characterized as marrow-dependent and free of T- and B-cell influences (30, 31) may be operative in the suppression or elimination of SFFV$^+$ preleukemic cells.

Transplantability and Donor Origin of an SFFV-Induced Leukemia. The 5-d-old SIM.R mice that received an intraperitoneal injection of 10^7 preleukemic SFFV$^+$ (plus 96 d) spleen cells developed palpable spleens within 20 d. Since SFFV and ecotropic MuLV

activities could not be detected in the cell-free fluids from either donor or recipient spleen cell suspensions, we presumed initially that the rapidly expanding population of leukemic cells was of donor origin. The availability of large amounts of leukemic spleen tissue enabled us to determine the efficiency with which these cells could be transplanted into irradiated and unirradiated recipient mice. As shown in Table III, exp. 1, spleen cells prepared from leukemic SIM.R mice, i.e., from mice who had received $SFFV^+$ preleukemic cells, proliferated effectively in irradiated syngeneic SIM.R but not in irradiated allogeneic BDF_1 recipients. This indicates that these leukemic spleen cells continue to express hemopoietic histocompatibility (*Hh*) antigens but, unlike their preleukemic predecessors, are no longer rejected by syngeneic recipients. This observed alteration of transplantability may be an expression of additional changes in cell surface antigen(s) which are relevant to the evolution of the SFFV-induced leukemia.

Donor cells (2×10^6) were also infused into lethally irradiated SIM.R mice who had been pretreated with silica. Under these conditions, any $SFFV^+$ leukemic or preleukemic cells which might have otherwise been rejected should now proliferate and contribute to the overall level of isotope incorporation and spleen colony formation. However, there was no such increase observed (Table III, exp. 1).

Finally, we inoculated $SFFV^+$ leukemic spleen cells into infant SIM.R and allogeneic BDF_1 mice to determine the genotype of the developing tumors in unirradiated mice. Mice <21 d of age are able to temporarily accept foreign hemopoietic cell grafts and a SIM.R tumor will grow transiently in infant BDF_1 recipients. Since the cellularities of the tumor-filled infant SIM.R and BDF_1 donor spleens were virtually identical, it is significant that intravenous injection of 10^5 nucleated cells from these enlarged leukemic spleens resulted in >100 colonies per SIM.R secondary recipient, but ≤10 colonies per BDF_1 secondary recipient (Table III, exp. 2). This indicates that the cells harvested from BDF_1 recipients did not proliferate extensively (i.e., grow as tumor colonies [TCFU]) in allogeneic adult BDF_1 secondary recipients and were therefore SIM.R in origin.

Discussion

The studies presented in this paper show conclusively that replication defective Friend SFFV particles purified free from standard MuLV helper are able to persistently infect hemopoietic target cells of susceptible adult Swiss mice and induce an erythroleukemia in the absence of overt virus replication. It has not been determined if the persistently infected cells or their differentiating progeny or both permit the expression of SFFV-induced malignant transformation. However, Friend disease was observed in $SFFV^+$ mice as a poorly differentiating erythroblastic leukemia. Tumor cells are intimately associated with a rescuable SFFV genome and are incapable of protecting lethally irradiated mice from radiation death. With the possible exception of the cytoplasmic expression of the *gag* gene product p15, other viral structural proteins have not been detected in $SFFV^+$ leukemic cells using indirect immunofluorescence techniques and antigen specific heterologous antisera. Further, we have not been able to reveal (using complement-mediated cytotoxicity) the presence of SFFV-induced antigen(s) on the membrane of transplantable tumor cells using a wide variety of murine antisera directed against syngeneic hemopoietic cells transformed by FV (13, 32). Future experiments must rely upon the availability of sera directed

against SFFV$^+$ hemopoietic and fibroblastic nonproducer cells.

The investigation into the status of the persisting SFFV provirus was influenced by our belief that stable integration of SFFV into a renewing hemopoietic cell population (i.e., a stem cell) would result in the maintenance of rescuable SFFV for an indefinite period. In contrast, cells committed to a particular line of differentiation might permit the expression of a stable SFFV-host cell association but would by virtue of their limited life span necessitate the sequential elimination of SFFV from the hemopoietic system. Although we did observe a time-related reduction in SFFV rescued in vivo, rescuable SFFV continued to persist in spleen tissue and was detected in the fluids of mixed cultures containing spleen cells and NIH/LLV fibroblasts (Fig. 1). Spleen cell preparations were not fractionated before addition to NIH/LLV cells and we therefore cannot correlate the presence of rescuable SFFV with any one specific class of functionally or morphologically definable cell. It can be concluded, however, that the target cells for SFFV persistence are either very long-lived or have the potential for self-renewal because SFFV is replication defective and unable to persist via chronic or slow infections. Any amplification of SFFV presence in this model system must be due to hemopoietic cell proliferation. We shall pursue this matter further by coupling cell separation techniques with SFFV antigen detection assay systems.

We do not know why SFFV could not be rescued in vivo beginning ≈30 d after inoculation into adult Swiss mice. SFFV$^+$ mice may have mobilized both humoral and cellular immune mechanisms which were protective against infection by exogenous MuLV helpers. We have not been able to detect virus neutralizing antibodies in SFFV$^+$ preleukemic mice, but this does not preclude the possibility of rapid development of humoral immunity to MuLV infection. It has been shown that the induction of FV cytotoxic antibodies may be a contributing mechanism in the development of dormant FV infection (33, 34) and in the regression of FV disease (35). An important alternative explanation is based upon our previous observation that SFFV may be classified as a DI particle (9). DI function(s) alone may be responsible for the difficulties encountered in rescuing SFFV from spleens in vivo and in vitro.

We feel confident that the interval between original SFFV inoculation and onset of Friend disease (≈100 d) can be considered a preleukemic period. Other investigators have identified virus-induced alterations in splenic T and B cells (36), host immune functions (37–39), and in the expression of mouse histocompatibility antigens (27, 40–42) shortly after FV infection and these events may certainly be involved in the development of Friend leukemia. The present study was not designed to investigate such events, however, since we did not know if Friend leukemia would ultimately evolve in mice inoculated with SFFV. The only documented abnormality in SFFV$^+$ preleukemic mice was an increased number of immature smudged cells present in peripheral blood smear preparations beginning ≈60 d after SFFV inoculation and slightly elevated peripheral leukocyte counts (range of 12–20,000/mm^3). At the time of hemisplenectomy, SFFV$^+$ mice were shown to contain spleen cells capable of responding normally to both T and B cell mitogens (data not shown).

We have speculated that the inability of SFFV$^+$ preleukemic spleen cells to proliferate in lethally irradiated syngeneic recipient mice is due to the expression of SFFV-associated antigen(s) or to the altered expression of host *Hh* antigens. Gillis et al. (43) have recently shown that SFFV-encoded antigen(s) may be present on the

surface of SFFV-infected fibroblastic and erythroleukemic cells. However, SFFV-induced leukemic cells were not a target of syngeneic rejection in our study (Tables I and III) and it is therefore likely that the antigen(s) revealed by Gillis et al. using lymphocyte-mediated cytolysis assays is not involved in the phenomenon described here. There is also evidence for an *H-2*/viral protein complex on the surface of FV-induced tumor cells which may be responsible for an "altered self" molecule and *H-2* restriction of cytotoxic T cell activity in the Friend system (44, 45). The ability to abrogate syngeneic rejection of $SFFV^+$ preleukemic cells via silica pretreatment of recipient mice strongly suggests that this natural resistance is mediated by immunogenetically specific host antigraft reaction whose effector cells are relatively insensitive to radiation, are marrow-dependent, and are highly sensitive to elimination by the bone-seeking isotope ^{89}Sr (31). The work of Rossi et al. (27, 28) and Kiessling et al. (46), as well as studies concerning the role of macrophages in regression of FV leukemia (47–49) and our previous studies concerning *Fv-2* associated resistance to FV (50) have all implicated this effector cell system in surveillance over leukemogenesis.

Finally, culture systems have been established by several groups (26, 51–53) in an attempt to study hemopoietic microenvironments in vitro. Addition of MuLV to these cultures may facilitate the study of preleukemia. However, since some of these cultures may harbor leukemic cells (54), and others are deficient in the maintenance of erythroblastic differentiation (26) we feel that $SFFV^+$ preleukemic mice may provide a model for studying the leukemia-inducing potential of hemopoietic microenvironments in vivo in the absence of virus replication.

Summary

A latent form of persistent infection can be established in susceptible adult mice inoculated with a preparation of defective Friend spleen focus-forming virus (SFFV) purified free from standard leukemia-inducing helper virus (LLV-F). SFFV persistence was initially observed using an in vivo rescue technique in which SFFV could be directly rescued to form splenic foci of malignant erythropoiesis in mice. At ≈30 d after virus inoculation however, SFFV could not be rescued after inoculation of LLV-F indicating that persistently infected (i.e., $SFFV^+$) mice were either immune to exogenous helper virus or able to express SFFV-associated defective-interfering (DI) function(s). Persistent infection by SFFV was further documented using an in vitro rescue technique and ultimately resulted in the induction by SFFV of erythroleukemia in the absence of polycythemia or overt virus production. However, SFFV rescued by LLV-F from persistently infected normal and transformed hemopoietic cells was able to induce polycythemia in adult mice suggesting that this is a helper controlled property of the Friend virus complex. Transplantable SFFV-induced erythroleukemic cells could be retrieved from persistently infected yet histologically normal mice. The duration of SFFV persistence in normal spleen tissue suggests that the SFFV provirus resides in either a long-lived or pluripotent hemopoietic cell. Further, certain changes occured, presumably in the membranes of persistently infected cells, which preceded the overt development of Friend leukemia and facilitated the definition of an SFFV preleukemic phase. Cell surface alterations were revealed using cell transfer techniques. Hemopoietic cells harboring a rescuable SFFV failed to proliferate when inoculated into lethally irradiated, syngeneic adult mice. In contrast, the transformed

progeny of preleukemic cell populations and spleen cells transformed by FV complex (i.e., cells replicating both SFFV and LLV-F) were not rejected. This result suggests that histologically normal $SFFV^+$ preleukemic cells express an antigen recognition site which is not present on overtly transformed cells and which may be a pertinent surveillance target for host anti-leukemogenic reactions.

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