

CELLULAR REGULATION IN FRIEND VIRUS INDUCED ERYTHROLEUKEMIA

Studies with Anemic Mice of Genotype Sl/SI^d*

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Host genetic determinants that influence leukemia virus replication and expression play an important role in deciding the outcome of virus-host interactions (1). The *Steel* locus is one locus that has been shown to influence both normal differentiation processes, as well as sensitivity to the murine leukemia virus, Friend spleen focus-forming virus (SFFV).¹ Mice carrying two mutant alleles at the *Steel* locus (Sl/SI^d) are severely anemic, radiosensitive, sterile, and their hair is unpigmented (2). Although these mice are anemic, their hematopoietic tissues contain normal numbers of hematopoietic stem cells capable of repopulating lethally irradiated (+ / +) hosts (3). In contrast, neither normal (+ / +) nor mutant (Sl/SI^d) hematopoietic cells are capable of curing the Sl/SI^d anemia (3, 4). Mice of genotype Sl/SI^d have also been reported to be completely resistant to the induction of spleen foci and erythroleukemia by Friend SFFV (5). The experiments described in this study were undertaken to determine the mechanism of resistance of Sl/SI^d mice to Friend SFFV, and to determine whether Sl/SI^d mice are suitable hosts for enumeration of possible tumor colony-forming units (TCFU) present in Friend SFFV-infected + / + spleens. The development of such a TCFU assay is of particular interest because others have recently shown that spleen colony formation by Friend virus-infected spleen cells in secondary recipients results from virus infection of host cells, rather than proliferation of leukemic donor cells (6, 6a; A. A. Axelrad and H. C. Van der Gaag, University of Toronto, personal communication). This background of host foci arising by recruitment from infectious centers in the donor spleen can be eliminated by prior irradiation of the host; however, hematopoietic stem cells (CFU-S), present in the SFFV-infected donor spleens (7), form spleen colonies in irradiated mice (8). We attempted to develop a TCFU assay by using mutant Sl/SI^d mice as recipients because these mice have been reported to be totally resistant to spleen foci induction by SFFV (5), and CFU-S does not form macroscopic spleen colonies in lethally irradiated Sl/SI^d mice (3). Thus, either unirradiated or irradiated Sl/SI^d secondary recipients might eliminate both the infectious center and CFU-S background and hence be a suitable host for an unambiguous TCFU assay. The results presented in this study indicate, however, that the spleens of normal mice infected with SFFV do not contain cells capable of colony

* Supported by the Medical Research Council and the National Cancer Institute of Canada.

¹ *Abbreviations used in this paper:* CFU-E, colony-forming unit that responds to Epo; CFU-S, spleen colony-forming units; Epo, erythropoietin; FFU, focus-forming unit; PBS, phosphate-buffered saline; FCS, fetal calf serum; SFFV, spleen focus-forming virus; Sl, Steel; TCFU, tumor colony-forming units.

formation in Sl/SI^d hosts, although the disease typical of the polycythemic strain of Friend SFFV can be induced in these mice.

Materials and Methods

Mice. Female mice of strain DBA/2J, aged 2-3 mo, and female F_1 hybrids produced by crossing mice of strains WC/Re- $Sl/+$ and C57BL/6J- $+/SI^d$ (WCB6F1) were obtained from The Jackson Laboratory, Bar Harbor, Maine. This cross yields mice of four different genotypes: $+/+$, $Sl/+$, $+/SI^d$, and Sl/SI^d . Mice of genotype Sl/SI^d have a severe macrocytic anemia; the other genotypes have a normal ($+/+$) or near-normal ($Sl/+$, $+/SI^d$) phenotype.

Irradiation. Mice were irradiated in a ^{137}Cs biological irradiator at a dose of 79.9 rads/min (9).

Virus. The virus used for infection was a NB-tropic SFFV preparation harvested from the medium of chronically infected NIH/3T3 cells (10). Friend SFFV titers were determined by the spleen focus assay method (11) by using appropriate dilutions of the virus preparation in phosphate-buffered saline (PBS). The virus stock contained between 1 and 2×10^4 focus-forming units (FFU)/ml as titrated in DBA/2J mice.

Preparation of Cell Suspensions. Normal and SFFV-infected mouse spleen cell suspensions were prepared in ice-cold α -minimal essential medium (12) and 2% fetal calf serum (FCS) by passage through a stainless steel mesh followed by gentle syringing with an 18 gauge needle to break up remaining cell clumps. Aliquots of the resulting suspension were taken for cell counting by using a hemocytometer. The nucleated cell concentration was then either adjusted to 1×10^7 /ml in cold α -minimal essential medium (MEM) and 2% FCS for in vitro culture or in cold PBS at 4×10^6 or 1×10^7 /ml for intravenous injection into assay mice (0.5 ml/mouse).

Preparation of Virus from Infected Spleens. The spleens from mice infected previously with SFFV were first weighed; then ice-cold α -MEM and 2% FCS (9 ml/g spleen) were used to make a cell suspension. The cells were disrupted by quickly freezing and thawing three times, followed by centrifugating at 8,000 g for 4 min to remove cell debris. The virus-containing supernate was then passed through a 0.45 μm Millipore filter and stored at $-70^\circ C$ until use. Cell-free filtrates were thawed quickly and diluted in cold PBS for virus titration.

Culture of Erythroid Colonies. The plasma clot method (13) was used with α -MEM as culture medium. Each plasma clot culture of 0.1 ml final vol contained 1×10^6 nucleated spleen cells. Epo (Connaught Med. Res. Labs., Willowdale, Ontario, Canada, Step III, lot 3018) was added at the onset of culture when required. After 2 d incubation, the clots were fixed and stained and colonies of eight or more cells showing a positive benzidine reaction were scored using a light microscope (magnification $\times 400$). In each assay at least six plasma clot cultures were prepared for each experimental point.

Results

Transplantation of SFFV-Transformed $+/+$ Spleen Cells into Sl/SI^d Recipients. Fig. 1 gives a diagrammatic outline of the experimental protocol. Normal WCB6F1 mice of $+/+$ genotype were injected intravenously with 10^3 FFU of SFFV (as assayed in DBA/2J mice). 9 d later, their greatly enlarged spleens were removed and single cell suspensions were injected into normal $+/+$ and anemic Sl/SI^d recipients. As shown in Fig. 2d, the spleens of the Sl/SI^d secondary recipients were greatly enlarged, suggesting that SFFV-transformed cells (either donor or host) could proliferate in Sl/SI^d mice.

To determine whether the cell proliferation in these recipient Sl/SI^d mice was due to growth of $+/+$ TCFUs, SFFV-transformed $+/+$ spleen cells were also transplanted into Sl/SI^d recipients that had been previously irradiated (700 rads) to prevent infection and transformation of Sl/SI^d host cells by virus released from the $+/+$ spleen cells. As shown in Fig. 2c, irradiation before transplantation totally prevented the appearance of splenomegaly or individual spleen colonies. During the

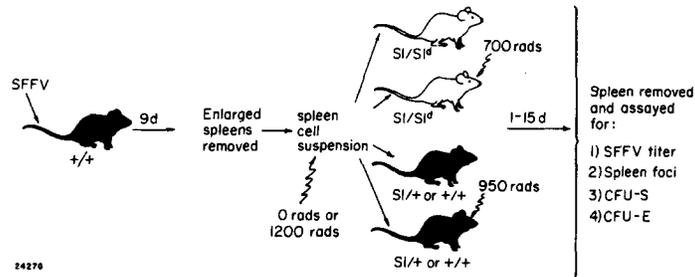


FIG. 1. Experimental protocol used to study the focus-forming or colony-forming ability of SFFV-infected +/+ spleen cells in secondary recipients.

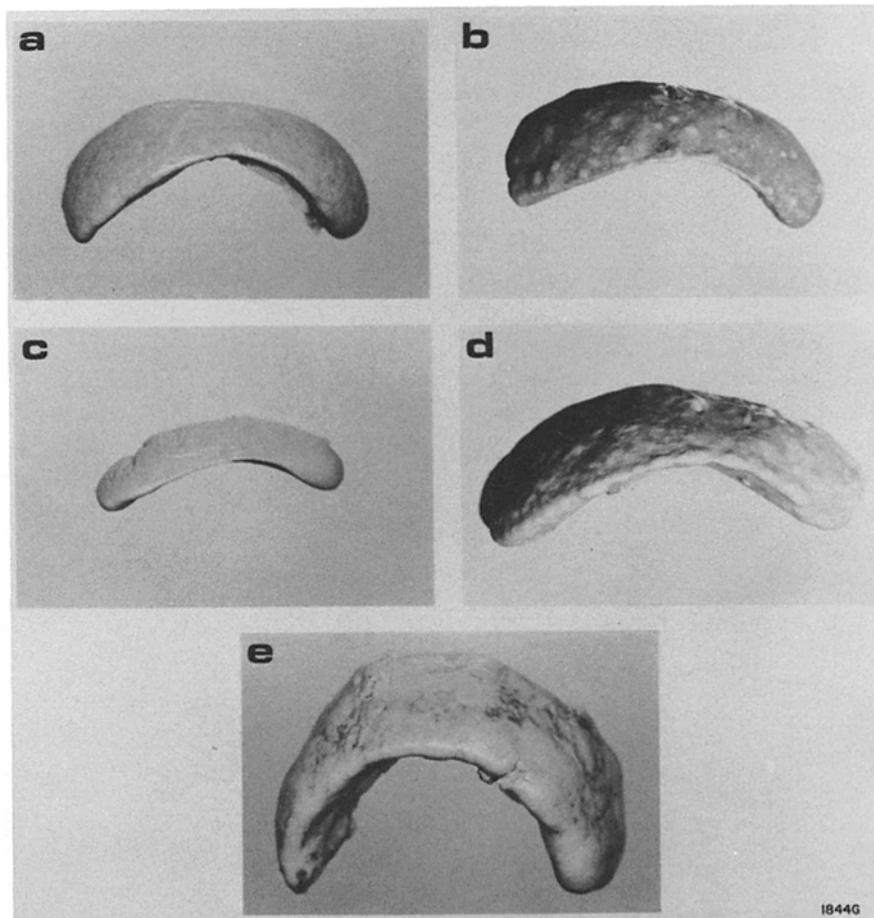


FIG. 2. Spleens of uninfected and SFFV-infected mice. a, normal SI/Si^d spleen; b, spleen from SI/Si^d mouse injected 9 d previously with 10^3 FFU of SFFV; c, SI/Si^d spleen, 9 d after whole-body irradiation (700 rads) and injection of 2×10^6 nucleated cells from the greatly enlarged spleens of +/+ mice injected with SFFV 9 d previously; d, same as c except that the SI/Si^d recipients were not irradiated; e, spleen from +/+ mouse injected 9 d previously with 10^3 FFU of SFFV.

TABLE I
*Effect of Irradiation on Focus-Forming and Spleen Colony-Forming Capacity of SFFV-Infected +/+ Cells**

Irradiation of		Recipient genotype	No. of spleen foci or spleen colonies
Cells‡	Recipients§		
—	—	SI/SI ^d	28, TMTC
+	—	SI/SI ^d	18, 27, 41
—	+	SI/+	29, TMTC¶
+	+	SI/+	0, 0

* See Fig. 1 for the experimental protocol. The number of spleen foci or spleen colonies were counted 9 d after injection of the SFFV-infected +/+ cells.

‡ Cells were irradiated with 1,200 rads.

§ Recipient mice were irradiated with 950 rads.

|| TMTC, too many to count.

¶ Colonies formed in the irradiated SI/+ mice had the characteristic large appearance of CFU-S colonies, as opposed to the smaller size of SFFV-induced foci.

course of three such experiments, no spleen colonies were observed after injection of a total of 5.0×10^7 SFFV-infected spleen cells into 12 irradiated SI/SI^d recipients. Thus, the SFFV-induced splenomegaly in the SI/SI^d secondary recipients appears to be due to infection and proliferation of SI/SI^d recipient cells, rather than clonal growth of +/+ TCFUs.

To confirm this conclusion, the donor SFFV-infected +/+ spleen cells were irradiated (1,200 rads) before transplantation into secondary recipients. If these donor cells were acting as infectious centers for the release of virus in the SI/SI^d recipients, then a dose of radiation sufficient to inhibit extensive cell division should have only a limited effect on the appearance of spleen foci in the SI/SI^d mice. As shown in Table I, this was indeed the case. Table I also demonstrates that the same irradiated cell suspension which was still capable of giving rise to spleen foci in SI/SI^d mice no longer contained viable CFU-S, as assayed in irradiated SI/+ mice. Thus, a dose of radiation which prevented the cell division of CFU-S had only a marginal effect on spleen focus induction, confirming that the secondary foci did not arise by clonal expansion of donor cells, but rather infection and recruitment of host SI/SI^d cells.

Cellular Composition of SFFV-Infected SI/SI^d Spleens. The spleens of normal mice infected with SFFV contain large numbers of cells capable of forming erythroid colonies in culture in the absence of Epo (14, 15). To determine whether the splenomegaly observed in SI/SI^d mice was typical of the disease induced by the polycythemic strain of Friend SFFV, spleen cells from SI/SI^d mice previously injected with SFFV-infected +/+ cells were plated in plasma clot culture in the presence or absence of Epo. After 2 d incubation, the cultures were observed for the presence of hemoglobin-positive colonies. As shown in Table II, the spleens of both uninfected +/+ and SI/SI^d mice contained cells that respond to Epo to form erythroid colonies. As expected from previous observations (16), a background level of apparently Epo-independent colonies were observed with uninfected SI/SI^d cells, presumably due to carry-over of Epo from the SI/SI^d mice, which have been shown to contain a high level of endogenous Epo in their serum (17). Spleen cells from both SFFV-infected +/+ mice, and SI/SI^d mice previously injected with SFFV-infected +/+ spleen cells, contained very high levels of cells capable of forming Epo-independent erythroid colonies in culture. Thus, by the criteria of both Epo-independence and splenomegaly,

TABLE II
Erythroid Colony Formation in Plasma Cultures by Spleen Cells from Normal and SFFV-Infected Mice

Mouse genotype	Treatment	Number of erythroid colonies/10 ⁶ nucleated spleen cells		
		0	Epo U/ml* 0.125	0.25
+/+	—	0	42‡	79
Sl/Sl ^d	—	30	51	95
+/+	Infected 10 d previously with 500 FFU of SFFV	1.1 × 10 ³	1.1 × 10 ³	1.0 × 10 ³
Sl/Sl ^d	Injected with 2 × 10 ⁶ SFFV-infected +/+ spleen cells 8 d previously	2.8 × 10 ²	2.9 × 10 ²	3.2 × 10 ²
Sl/Sl ^d	Injected with 2 × 10 ⁶ SFFV-infected +/+ spleen cells 16 d previously	2.3 × 10 ³	2.1 × 10 ³	2.1 × 10 ³
Sl/Sl ^d	Irradiated (700 rads) and injected with 5 × 10 ⁶ SFFV-infected +/+ spleen cells 8 d previously	0	0	0

* Epo added at start of 2-d plasma clot culture.

‡ Numbers are mean of six determinations; standard errors of the mean of all values shown did not exceed 10%.

TABLE III
Susceptibility of Normal and Mutant Mice to Spleen Foci Induction by SFFV

Host genotype	Virus titer* <i>ffu/ml</i>	Spleen weight‡ <i>g</i>
+/+	3.6 × 10 ⁴	1.68 ± 0.8
Sl/+	3.0 × 10 ⁴	0.68 ± 0.1
Sl ^d /+	2.0 × 10 ⁴	0.40 ± 0.05
Sl/Sl ^d	4.9 × 10 ²	0.12 ± 0.01

* A minimum of four mice/group were injected with various dilutions of a NB-tropic preparation of Friend SFFV. Their spleen were removed 9 d later, placed in Bouin's fixative, and counted for spleen foci.

‡ Spleen weights are the average weights ± standard error of the mean of the four spleens given 10³ FFU of SFFV (as assayed in DBA/2J mice) 9 d previously.

the disease induced in Sl/Sl^d mice appears to be identical to that previously described in +/+ mice.

Prior irradiation of the Sl/Sl^d host resulted in the total absence of cells capable of forming erythroid colonies (Table II). This observation is consistent with the experiment in Fig. 2c showing that neither donor CFU-S nor TCFU are present in irradiated Sl/Sl^d recipients.

Infection of Sl/Sl^d Target Cells Directly with SFFV. The experiments presented in Fig. 2 suggested that target cells in Sl/Sl^d mice can be transformed by SFFV. To confirm this directly, various dilutions of a high titered preparation of SFFV (10) were injected into Sl/Sl^d mice, and their Sl/+, Sl^d/+, and +/+ littermates. 9 d later, their spleens were removed, placed in Bouin's fixative, and counted for spleen foci. As shown in Table III and Fig. 2, normal +/+ mice and their heterozygous Sl/+ and Sl^d/+

TABLE IV
*Replication of SFFV in the Spleens of +/+ and Sl/SI^d Mice**

Experiment	Virus titer (FFU/spleen) recovered from mice of genotype	
	+/+	Sl/SI^d
1	7×10^4	8×10^4
2	1×10^5	4×10^5

* Spleen cells from three infected mice were pooled 9 d after infection with 5×10^2 FFU of SFFV. Virus was prepared from the spleen cell homogenates as described in Materials and Methods. SFFV titers were then determined by the spleen focus assay method (11) in DBA/2J mice, by using at least three mice per group.

littermates were highly susceptible to SFFV, as assayed by spleen focus induction and splenomegaly, whereas anemic Sl/SI^d were quite resistant to spleen focus induction by SFFV. The difference between the present observation and those reported earlier (5) can probably be explained by the much higher titer of virus used in the present study.

Replication of SFFV in Sl/SI^d Mice. The reduced efficiency of spleen focus formation in Sl/SI^d mice might be due to the inability of SFFV to replicate to sufficiently high titer in these mice. To test this possibility, the titer of SFFV recovered from the spleens of both +/+ and Sl/SI^d mice 9 d after injection of virus was determined. As shown in Table IV, SFFV grew equally well, if not somewhat better, in the spleens of Sl/SI^d mice when compared with virus recovered from +/+ controls. Thus, the reduced efficiency of spleen foci induction in anemic Sl/SI^d mice does not appear to be due to low in vivo titers of SFFV.

Discussion

The results presented in this study indicate that genetically anemic Sl/SI^d mice are 50–100-fold more resistant to spleen foci induction by SFFV, as compared to their normal or heterozygous littermates. This relative resistance to spleen foci and erythroleukemia induction does not appear to be due to the inability of SFFV to replicate in Sl/SI^d mice. Indeed, the titers of SFFV recovered from SFFV-infected Sl/SI^d spleens were consistently somewhat higher than the comparable titers from +/+ mice. This observation suggests that SFFV can replicate in vivo in cells other than transformed erythroid target cells, in agreement with previous observations demonstrating the infection and production of SFFV from nonerythroid cells in cell culture (10, 18, 19).

The apparent resistance of Sl/SI^d mice to SFFV can be overcome by the injection of SFFV-infected +/+ spleen cells. The extensive splenic enlargement and appearance of large numbers of Epo-independent CFU-E in the spleens of these Sl/SI^d mice appears to result from the infection and transformation of host Sl/SI^d cells rather than growth of donor +/+ cells. Thus, the donor +/+ cells appear to be acting as infectious centers resulting in high virus titers in the Sl/SI^d spleens. These results suggest that, at sufficiently high virus titers, the extrinsic factors in the *Steel* environment which limit the proliferation of virally-transformed cells can be overcome. The observation that SFFV can infect and transform cells in Sl/SI^d mice, albeit at a reduced frequency, makes it less likely that the proliferation of TCFU would be

completely blocked in these mice. In addition, previous work has shown that normal hematopoietic progenitor cells, including CFU-S and CFU-C, were capable of extensive proliferation in irradiated Sl/SI^d mice (20). The observation that normal hematopoietic cells can proliferate in irradiated Sl/SI^d mice makes it unlikely that TCFU, if they existed, would be totally inhibited from forming spleen colonies in these same mice. Together, these results suggest that, in its initial phase, the disease induced by the polycythemic strain of Friend SFFV is not the result of the clonal proliferation of transformed erythroid progenitor cells; rather, it would appear that the large increase in cell proliferation in the spleens of SFFV-infected mice results from continuous infection and recruitment of normal erythroid cells with only limited proliferative capacity. This conclusion is consistent with a previous observation from this laboratory, based on the multihit kinetics of titration of a Friend virus stock which contains SFFV in excess to its helper virus, that primary spleen foci also arise by a process of continuous infection and recruitment of newly transformed cells, rather than by clonal growth from a single transformed cell (10).

The conclusion that the leukemic spleens of SFFV-infected mice do not contain TCFU is seemingly at odds with the observation that permanent cell lines can be established in cell culture from these spleens. These apparently contradictory findings can be reconciled as follows: (a) TCFU may only start to appear in SFFV-infected spleens at much later times than examined in this study. Because the disease regresses in the +/+ mice used in this study, we were unable to look for TCFU at later times. (b) Cells capable of forming permanent lines in cell culture (and also forming spleen colonies in irradiated recipients) may not pre-exist in these leukemic spleens, but rather may only arise in cell culture. While no direct evidence exists to support this hypothesis, it is relevant to note that these Friend cell lines do not emerge immediately after placing leukemic spleen cells in culture; rather, a dormant period of several weeks in culture, or subcutaneous passage in vivo (21, 22), appears to be first necessary before cells capable of rapid and extensive proliferation emerge in the culture dish.

The predominant class of cells which are transformed by SFFV appear to be the same as that which normally respond to Epo by proliferating and differentiating into mature erythroid cells (23, 24). These SFFV-transformed cells appear to become independent of the positive regulatory controls that Epo normally exerts on erythroid progenitor cells (25, 26). Genetically anemic Sl/SI^d mice are unresponsive in vivo to the stimulatory effects of Epo on erythropoiesis (17, 27), even though the in vivo levels of Epo are very high in Sl/SI^d mice (16, 17). It is of interest to note, therefore, that although the susceptible populations for both Epo and SFFV appear to be identical or very similar, and although the effects of Epo and SFFV resemble each other in many ways, cells in Sl/SI^d mice cannot effectively respond to high levels of Epo whereas high doses of SFFV are still effective in promoting erythroid differentiation in Sl/SI^d mice.

The regulation of erythroid progenitor cells by Epo, both in vivo and in cell culture, has been widely accepted as a normal feature of erythropoiesis. Conversely, the Epo-independent proliferation of erythroid cells in vivo and in cell culture, observed in both Friend disease and the human disease polycythemia vera (28, 29), has been associated with abnormal leukemic regulation. Recent observations by Johnson and Metcalf (30), however, suggest that normal cells from mouse fetal liver are also capable of extensive erythroid differentiation in the absence of Epo. These observations suggest that Friend SFFV, rather than initiating a new program of leukemic erythroid

differentiation, may be activating or amplifying normal regulatory events which are Epo-independent. This conclusion is consistent with observations to be published elsewhere from this laboratory that normal hematopoietic cells express cellular RNA sequences which are related to possible transforming sequences on the SFFV genome. It also would explain why, at least in the initial phase of Friend disease, SFFV-transformed cells, like their normal counterparts, are still subject to the regulatory influences of the *Steel* locus.

Summary

Genetically anemic SI/SI^d mice have been shown previously to have a defective hematopoietic environment which prevents extensive erythroid differentiation of normal hematopoietic stem cells and also confers resistance to the erythroleukemia-inducing virus, Friend spleen focus-forming virus (SFFV). In this study, we show that the relative resistance of SI/SI^d mice to transformation by SFFV is not due to the inability of SFFV to replicate, nor is it because SFFV cannot transform erythroid cells, in the spleens of these mice. Injection of syngeneic +/+ mouse spleen cells, previously infected in vivo with SFFV, into secondary SI/SI^d recipients resulted in marked splenic enlargement, and the appearance of large numbers of erythropoietin (Epo)-independent erythroid colonies in plasma clot culture. The cellular proliferation observed in these SI/SI^d secondary recipients appeared to be due to infection and transformation of host SI/SI^d cells rather than the growth of possible tumor colony-forming units (TCFU) present in the infected +/+ spleens, because preirradiation of the SI/SI^d recipients abolished the splenomegaly and appearance of Epo-independent erythroid colonies. Furthermore, prior irradiation (1,200 rads) of donor spleen cells from SFFV-infected +/+ mice only slightly reduced spleen focus formation in unirradiated SI/SI^d recipients. The conclusion that SI/SI^d target cells could be infected and transformed by SFFV was confirmed directly by injecting a high titered preparation of SFFV into SI/SI^d mice. SI/SI^d mice were not absolutely resistant to infection or transformation by SFFV. Nevertheless, cells from the spleens of SFFV-infected mice were unable to form tumor colonies (TCFU) in irradiated SI/SI^d recipients, suggesting that TCFU are either present at an undetectably low frequency in these spleens, or that they are still subject to the regulatory influences of the *Steel* locus.

The authors acknowledge helpful discussions with Dr. E. A. McCulloch during the preparation of this manuscript. Mr. George Cheong provided excellent technical assistance.

Received for publication 4 December 1978.

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