

MECHANISMS OF GENETIC RESISTANCE TO FRIEND VIRUS LEUKEMIA IN MICE

I. ROLE OF ^{89}Sr -SENSITIVE EFFECTOR CELLS RESPONSIBLE FOR REJECTION OF BONE MARROW ALLOGRAFTS*

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Friend murine leukemia virus, which induces malignant erythropoiesis in mice, is a complex containing spleen focus-forming virus (SFFV)¹ and lymphatic leukemia virus (LLV-F) (1). SFFV is defective and is dependent upon its associated helper, LLV-F, which supplies type-specific virus envelope antigen (2, 3). At least two genes determine the host response to Friend leukemia virus (FV) complex. The *Fv-1* gene controls host resistance to LLV-F and appears to be identical to the N-B locus conferring resistance to XC plaque formation in embryo cells by murine leukemia virus (4). The *Fv-2* gene controls susceptibility to SFFV. Mice homozygous for resistance (*Fv-2^{rr}*), including those of strains C57BL and C58, are completely resistant to splenomegaly and erythroblastosis induced by most strains of the FV complex (SFFV [LLV-F]) (5). Such "resistant" mice are also refractory to the extremely suppressive effect of the FV complex on antibody formation (6), but remain sensitive to the moderate and transient suppression induced by LLV alone (1). Furthermore, since resistance to leukemia in *Fv-2^{rr}* mice was not abrogated by treatment with cortisol (7) or antilymphocytic serum (8), it appeared that the *Fv-2* resistance was not immunologically mediated by thymus-dependent (T) cells or bursa equivalent-dependent (B) cells.

Resistance to FV complex could be transferred to susceptible mice by bone marrow cell grafts (9, 10). Thus, it appeared that *Fv-2*, the gene for resistance to leukemia, mediated its action through a hemopoietic cell which was either present in or could be generated from the bone marrow. It was assumed from the above experiments and other data (7, 8) that the hemopoietic cells through which resistance to leukemia was

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¹ *Abbreviations used in this paper:* FFU, focus-forming units; FV, Friend virus; *Fv-1*, Friend virus 1; *Fv-2*, Friend virus 2; *Hh*, hybrid or hemopoietic histocompatibility; LLV-F, Friend lymphatic leukemia virus; M cells, marrow-dependent effector cells; PFC, plaque-forming cells; SFFV, spleen focus-forming virus; SRBC, sheep red blood cells; TCFU, tumor colony-forming units.

manifested were the erythropoietic stem cells which are the target cells for FV-induced transformation.

When analyzing studies on the immunobiology of bone marrow transplantation, we were impressed by the similarity that exists between the ability of various strains of mice to reject allogeneic bone marrow grafts, and their ability to resist erythroleukemia induced by FV (11). Thus, adult mice of the strains C57BL, B10.D2, and C58, which are good responders to most bone marrow allografts are also extremely resistant to Friend SFFV-induced erythroleukemia. Conversely, *Fv-2^{ss}* mice, such as DBA/2, BALB/c, and 129 strain mice, are both poor responders to most bone marrow allografts (reference 12 and unpublished observations) and susceptible to SFFV. This and other evidence presented here suggested to us that resistance to Friend leukemia and to hemopoietic allografts may be mediated by a common effector system under *Fv-2* genetic control.

Immune cells responsible for the rejection of bone marrow allografts are thymus-independent effector cells which recognize transplantation antigens that are apparently restricted to primitive hemopoietic cells, called hybrid or hemopoietic histocompatibility (*Hh*) antigens (12). Agents which suppress B cells (cortisol) or T cells (rabbit antimouse thymocyte serum) do not affect these effector cells. However, ⁸⁹Sr, a radioactive bone-seeking isotope with a half-life of 62 days, selectively suppresses the ability of mice to reject hemopoietic cell grafts without affecting B cells, T "helper" cells, or T cells involved in graft-vs.-host reactions (13). These cells have been termed marrow-dependent effector (M) cells, since marrow appears to be their central lymphoid organ. In mice treated with ⁸⁹Sr, the spleen takes over stem cell function (14), and the erythropoietic progenitor cells, which are the target cells for FV, are maintained at near normal numbers. Antibody production appears to be normal (15).

The experiments reported here were performed to test the hypothesis that the *Fv-2* mediated genetic resistance to FV complex (SFFV[LLV-F]) is mediated through M cells and not at the level of the erythropoietic stem cells, i.e., "target cells." The results indicate that target cells from *Fv-2^{rr}* mice can be transformed by FV under appropriate conditions and the selective abrogation of M-cell function seems to confer susceptibility to mice otherwise genetically resistant to FV leukemia. Preliminary experiments leading to this work have been reported (11).

Materials and Methods

Mice.—Adult male mice of the strains C57BL/6J (B6), DBA/2J, B10.D2/nSn (B10.D2), 129/J, and C58/J were purchased from Jackson Laboratories, Bar Harbor, Maine. Mice were 6- to 8-wk old when used in most cases.

Virus and Virus Titration.—SFFV, as contained in the Mirand strain (16) of the FV complex, was originally obtained from Charlotte Friend (Mount Sinai School of Medicine, New York) and has now undergone over 200 cell-free passages in Ha/ICR Swiss mice. More recently, it has been serially passaged through adult BALB/c mice and initiates infection with equal efficiency in DBA/2 and BALB/c mice, and is therefore designated as being NB-tropic. All SFFV preparations were titrated in vivo with the spleen focus assay method (17). Briefly, 1-ml samples of diluted SFFV were injected intravenously into susceptible mice. 9 days later,

their spleens were removed and fixed in Bouin's solution. Discrete foci on the splenic surface were counted by macroscopy, and virus titers (mean numbers of foci per spleen \times dilution factor) were expressed in focus-forming units (FFU)/ml, where 1 FFU is the amount of virus required to induce an average of 1 focus/spleen.

Assay for Tumor Colony-Forming Units (TCFU).—Transformation of *Fv-2^{rr}* bone marrow cells after transplantation into the infected *Fv-2^{ss}* primary recipient was assayed as follows. Washed spleen cells of the *Fv-2^{ss}* primary recipient were infused into unirradiated adult *Fv-2^{rr}* secondary recipients which were syngeneic with the marrow donor. 9 days after cell transfer, the spleens were removed from the secondary recipients and fixed in Bouin's solution. The surface nodules were enumerated macroscopically and each nodule was taken to represent one TCFU. The histologic examination of such nodules revealed foci of malignant erythropoiesis, which was unaffected even if the secondary recipients were previously hypertransfused to suppress physiological erythropoiesis (18). Such foci of malignant erythropoiesis could not have been generated by any free extracellular virus transferred along with the spleen cells because the *Fv-2^{rr}* recipients are solidly resistant to focus formation by FV complex. A TCFU assay described earlier utilized *Fv-2^{rs}* (F₁ hybrids) as recipients (19). In such recipients which are only partially resistant to focus formation by SFFV, the splenic nodules represented not only TCFU but also some virus-induced foci. Therefore the assay described here is more discriminatory. Experiments described in this paper also rule out any contribution of the spleen cells of the primary recipient in generating the tumor colonies seen in the secondary recipient.

Administration of ⁸⁹Sr.—Doses of 25–100 μ Ci of ⁸⁹Sr (Oak Ridge National Laboratory, Oak Ridge, Tenn.) were infused intravenously on two separate occasions 4 wk apart. Mice were used for experiments 3–5 wk after the last injection. This protocol successfully prevents otherwise genetically resistant mice from rejecting incompatible parental strain or allogeneic marrow cell grafts (13).

Cell Transfer Procedures.—The methods of harvesting, counting, and intravenous infusion of marrow and spleen cell suspensions have been described (12). When more than 10⁷ nucleated cells/inoculum were to be infused, 50 U of heparin were added to each 5 ml of cell suspension to prevent embolic death. In some experiments the spleen cells were washed once in a refrigerated centrifuge in 15-ml vol at 800 g for 10 min.

Cytology.—Differential cell counts of imprints of the cut surfaces of spleens stained with Wright's stain were performed in three experiments. In another experiment, differential counts of paraffin sections stained with hematoxylin and eosin were performed. The purpose of these cytological studies was to determine the percentage of morphologically identifiable erythropoietic cells containing a nucleus, i.e., proerythroblast to normoblast. At least 1,000 nucleated cells/spleen were counted to obtain the values presented.

Suppression of Physiological Erythropoiesis by Hypertransfusion.—In one experiment, B6 mice treated with ⁸⁹Sr as well as control B6 mice were infused intravenously with 1 ml of whole mouse blood on the 5th and 6th day after infection (or not) with FV. The spleens of these mice were removed for assays 3 days after the second infusion of blood and 9 days after infection.

Immunizations and Assays for Antibody-Forming Cells.—Mice previously treated with ⁸⁹Sr and infected (or not) with FV were injected intravenously with 5 \times 10⁸ sheep red blood cells (SRBC). 4 days later, the spleens were removed and spleen cell suspensions (0.001 spleen equivalent) were assayed for anti-SRBC hemolytic plaque-forming cells (PFC), using a modification of the Jerne technique (7).

Protocol Involving Transplantation of Resistant Marrow Cells into Infected Susceptible Hosts.—Genetically susceptible *Fv-2^{ss}* mice were infected with 10⁸ FFU of NB-tropic FV complex 4 days before exposure to lethal doses of γ -rays (860 R) and intravenous infusion of genetically resistant *Fv-2^{rr}* marrow cells (Fig. 1). 5–7 days later, the spleens of the primary hosts were assayed for the presence of SFFV and SFFV-induced tumor cells (TCFU). Spleen

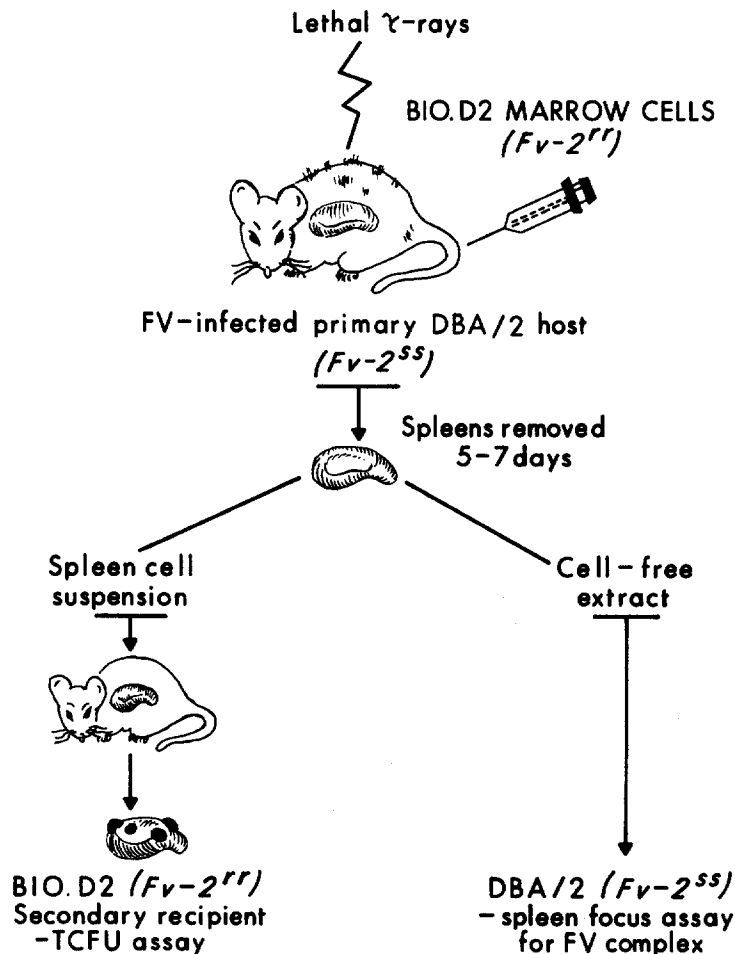


FIG. 1. The experimental design to study transformation of B10.D2 bone marrow cells ($Fv-2^{rr}$). Susceptible DBA/2 ($Fv-2^{ss}$) mice were infected with 10^8 FFU of FV. 4 days later they were lethally irradiated and grafted with 10^6 or 10^7 B10.D2 bone marrow cells. 5-7 days later, the spleens of the DBA/2 primary recipient mice were removed. Spleen cell free extracts were assayed for virus content by the spleen focus assay in DBA/2 mice. Spleen cells were assayed for B10.D2 TCFU by transplanting the cells into unirradiated adult B10.D2 secondary recipients.

cell suspensions were washed once; various dilutions of the supernate were assayed for content of infectious SFFV by injecting the material into susceptible DBA/2 mice. The spleen cells ($\frac{1}{4}$ or $\frac{1}{8}$ spleen equivalent) were infused into unirradiated secondary recipient mice ($Fv-2^{rr}$) syngeneic with marrow donor to assay for TCFU.

RESULTS

Abrogation of Genetic Resistance to Friend Leukemia Virus by ^{89}Sr Treatment.— Since it appeared that the genetic resistance to Friend erythroleukemia may

be mediated by an effector system common with that responsible for rejection of bone marrow allografts (11), the first set of experiments were designed to ask the question whether administration of ^{89}Sr which selectively abrogates the ability of mice to reject allogenic bone marrow would also abolish genetic resistance to FV leukemia. In a preliminary experiment, four B6 mice treated with ^{89}Sr were infected with 100 FFU of FV complex and were killed 9 days later. Their spleens were moderately to severely enlarged. On fixation in Bouin's solution, no discrete foci of tumor cells could be seen grossly, since the entire surface of the spleens was bright yellow, as are individual tumor foci. However, microscopic examination of the enlarged spleens showed great expansion of the red pulp and almost complete effacement of the white pulp by large, primitive erythroid cells (so-called "Friend cells"). There were abundant mitoses and differentiated erythroid cells (normoblasts) with pyknotic nuclei. Since spleen foci were not countable, only histological evidence indicated that ^{89}Sr treatment abrogated the resistance of *Fv-2^r* mice to malignant erythropoiesis.

In subsequent experiments, therefore, evidence for leukemia induction included recovery of infectious SFFV and induction of erythropoietin-independent, i.e., malignant, erythropoiesis. Polycythemia induced in the test mice would be expected to suppress physiological erythropoiesis but not malignant erythroblastosis induced by SFFV (18). The spleens of mice treated with ^{89}Sr and infected with FV were enlarged, resembling those seen in the earlier experiment, while the spleens of normal B6 mice infected with FV were unaffected. Microscopic examination of the spleens showed extensive malignant erythropoiesis in ^{89}Sr -treated mice after FV infection, despite the fact that these animals were polycythemic (Fig. 2 *a*). This was also reflected in the greater than 90% count of erythroid cells (Table I). Erythropoiesis in the spleens of ^{89}Sr -treated mice not infected with FV and in normal B6 mice infected with FV was almost completely suppressed by hypertransfusion, indicating a lack of malignant erythropoiesis. The splenic histology of B6 mice treated only with ^{89}Sr did reveal extensive myelopoiesis (Fig. 2 *b*); this was expected, since the spleens of such mice had taken over all stem cell functions of the mouse (14). A large amount of SFFV was recovered from the spleen and plasma of mice treated with ^{89}Sr and infected with FV, whereas no detectable virus could be recovered from the normal B6 mice infected with FV at the dilution tested (Table I).

These results were consistent with the concept that M cells suppressible by ^{89}Sr are responsible for the genetic resistance of B6 mice to FV as well as for the genetic resistance to most bone marrow allografts (13). To seek confirmation of this apparent association between resistance to marrow allografts and resistance to malignant erythropoiesis, B6 mice were injected with 0, 25, 50, or 100 μCi of ^{89}Sr twice at a 1-mo interval. 5 weeks after the second injection, the mice were infected with 75 FFU of NB-tropic FV complex. The spleens were removed 9 days later. Imprints were stained with Wright's stain to determine

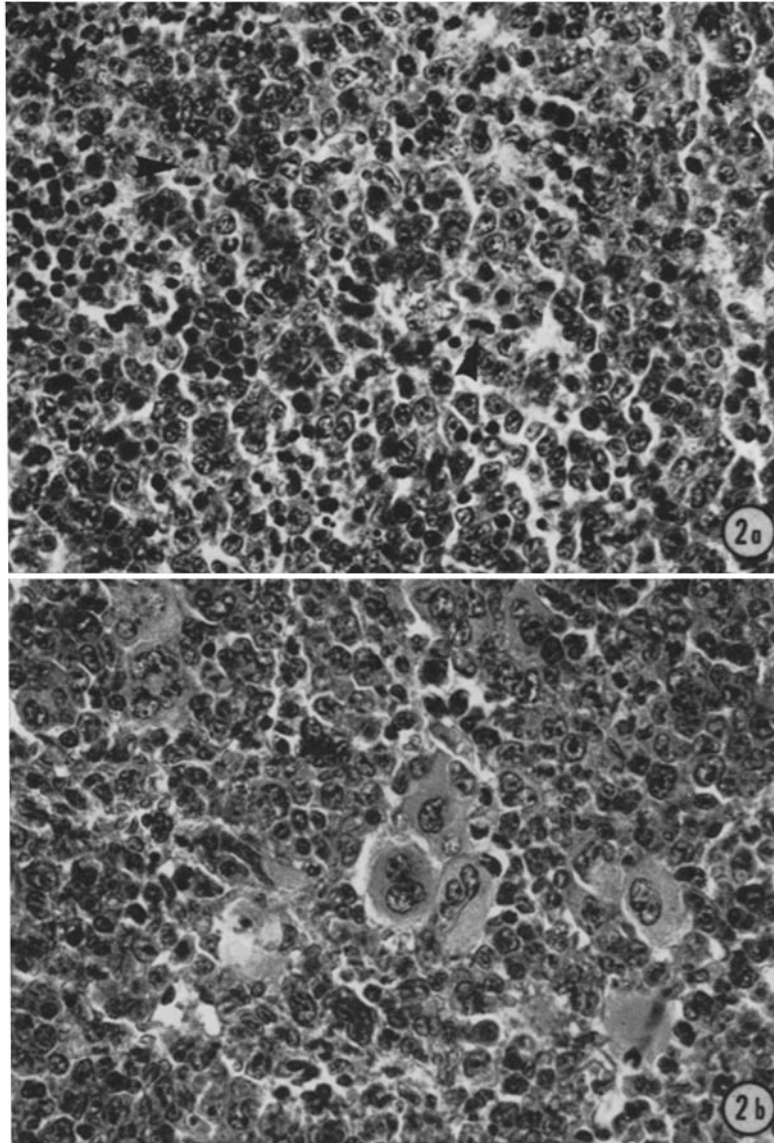


FIG. 2. (A) Section from spleen of polycythemic C57BL/6 mouse treated with ^{89}Sr and later infected with FV. The entire spleen is filled with erythroid cells, which efface the white pulp. The primitive erythroid cells (Friend cells) are large, have vesicular nuclei, and prominent nucleoli. The smaller, mature normoblastic erythroid cells have dark pyknotic nuclei. Numerous mitotic figures (arrows) could be seen (H and E \times 430). (B) Section from spleen of polycythemic C57BL/6 mouse treated with ^{89}Sr but not infected with FV. Megakaryocytes and granulocytic cells at various stages in differentiation were the predominant cells. Only rare erythroid cells were seen. These cells filled the subcapsular zone and the red pulp, but did not efface the white pulp (H and E \times 430).

TABLE I
Effect of ⁸⁹Sr on Genetic Resistance of C57BL/6 Mice to FV-Induced Malignant Erythropoiesis

Treatment with ⁸⁹ Sr*	Infection with FV*	Erythroid cells in spleen		Virus recovery	
		Polycythemic	Not polycythemic	Plasma‡	Spleen§
		%	%	FFU/ml	FFU/spleen
+	+	95 (3)¶	91 (4)	>10 ⁵ (7)	>10 ⁵ (7)
+	-	3 (2)	NT	0 (4)**	NT
-	+	1.5 (3)	12 (4)	0 (7)	0 (3)
-	-	0.3 (2)	13.2 (4)	NT	NT

* C57BL/6 adult mice with or without prior treatment with ⁸⁹Sr were infected with 100 FFU of NB-tropic FV complex. 9 days later all mice were sacrificed. Plasma and spleens were removed for various assays at this time.

‡ Plasma was tested for content of FV complex by injection of a 1 ml of 1:1,000 dilution into DBA/2 mice.

§ Splenic virus content was estimated by mincing spleens in Eagle's MEM and then assaying the first washing (1 ml of 1:1,000 dilution) for virus in DBA/2 mice.

¶ Numbers in parentheses indicate the number of animals used.

|| NT, not tested.

** To exclude activation of a latent virus in mice by treatment with ⁸⁹Sr, plasma samples from four such animals were assayed for presence of any FV complex before further treatment. Undiluted 0.1-ml plasma from each animal was injected into DBA/2 mice.

the percentage of erythroid cells and the washed spleen cell supernatant fluids were assayed for SFFV. The results are presented in Table II.

The amount of virus recovered from B6 mice was a function of the dosage of ⁸⁹Sr. Similarly, the percentage of erythroid cells in the spleen increased as the dosage of ⁸⁹Sr increased.

Susceptibility to Suppression of Antibody Synthesis Induced by FV.—Four ⁸⁹Sr-treated and four of eight normal B6 mice were infected with 50 FFU of NB-tropic FV complex. 7 days later all 12 mice were immunized with 5×10^8 SRBC intravenously to determine if FV would immunosuppress mice treated with ⁸⁹Sr. On the 11th day after infection with FV (4 days after immunization with SRBC), all the animals were killed and a direct PFC assay was performed with the spleen cells of all the mice to measure the primary IgM cellular antibody response to SRBC.

B6 mice treated with ⁸⁹Sr and infected with FV complex generated much fewer IgM PFC against SRBC than uninfected B6 mice ($P < 0.001$) and fewer PFC than infected B6 mice not treated with ⁸⁹Sr ($0.001 < P < 0.01$) (Fig. 3). The latter mice were moderately, but significantly ($0.001 < P < 0.01$), immunosuppressed, presumably because they were successfully infected with the NB-tropic LLV in the virus preparation (1).

To confirm and extend previous data (13, 15) indicating that ⁸⁹Sr does not, in itself, suppress antibody production under the conditions used here, four additional mice were treated with ⁸⁹Sr, as described above. These mice and 8 age-control mice were immunized with 5×10^8 SRBC, and the spleen cells

TABLE II
*Effect of Varying Doses of ^{89}Sr on Genetic Resistance of C57BL/6 Mice to FV**

Dose of ^{89}Sr	Erythroid cells in spleen	SFFV recovered/spleen ($\times 15$) \ddagger
μCi	%	Mean \pm SE
0	13.5	0.6 \pm 0.39
25	32	15.2 \pm 2.43
50	90	69.8 \pm 5.64
100	93.5	76.4 \pm 4.15

* C57BL/6 adult mice were injected with different doses of ^{89}Sr on two occasions 4 wk apart; 5 wk after the last injection of ^{89}Sr they were infected with 75 FFU of FV. 9 days later all mice were sacrificed and spleens were removed for various assays. Only when the virus yield was 8-10 times greater than the input dose, it was considered that active virus replication occurred in the spleen.

\ddagger Results are expressed as virus/spleen $\times 15$, since supernate from $\frac{1}{15}$ spleen fractions was used to infect susceptible mice for virus titration.

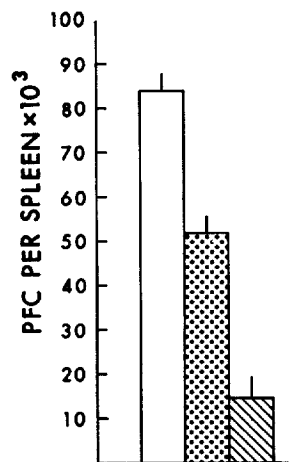


FIG. 3. Anti-SRBC antibody synthesis in B6 mice infected with FV, with or without prior treatment with ^{89}Sr . Four each of ^{89}Sr -treated and normal B6 mice were infected with FV (50 FFU). 7 days later these and four uninfected control B6 mice were immunized with SRBC. 4 days after immunization a direct PFC assay was performed. Result: FV markedly suppressed antibody synthesis in B6 mice treated with ^{89}Sr . Blank column, uninfected; stippled column, FV infected, but not treated with ^{89}Sr ; and diagonal column, ^{89}Sr -treated and infected with FV.

were assayed for PFC 4 days later. The mean (\pm SE) numbers ($\times 10^3$) of PFC/spleen were 69.7 ± 21.5 and 39.0 ± 3.6 in mice treated with ^{89}Sr and in control mice, respectively ($0.1 < P < .2$). Thus, ^{89}Sr alone does not suppress antibody production.

In some of the above experiments, spleen cells from B6 mice treated with ^{89}Sr and infected with FV were transplanted into adult unirradiated B6 mice to

see if tumor colonies would develop in the recipient mice by proliferation of the transformed cells from the ^{89}Sr -treated donor. No discrete tumor colonies were seen in the spleens of recipient B6 mice.

Transformation of $Fv-2^{rr}$ B10.D2 Bone Marrow Cells Grafted into Irradiated $Fv-2^{ss}$ DBA/2 Recipient Mice Previously Infected with FV Complex.—Either one of two different hypotheses could explain why ^{89}Sr had rendered genetically resistant C57BL/6 mice susceptible to the leukemogenic action of FV. ^{89}Sr may have affected the susceptibility of otherwise genetically resistant erythropoietic target cells to malignant transformation by FV. In contrast, ^{89}Sr may have affected a regulatory cell of the hemopoietic system that prevents transformation of target cells or subsequent proliferation of transformed cells. To test whether or not “resistance” genes, e.g., $Fv-2$, primarily affect target cells, we exposed marrow erythropoietic target cells of $Fv-2^{rr}$ mice to the hemopoietic environment in $Fv-2^{ss}$ mice which is compatible with malignant erythropoiesis (Fig. 1). If genetic resistance is primarily a property of target erythropoietic stem cells, malignant transformation should not occur. However, if such genes affect hemopoietic stem cell regulation, malignant transformation may indeed occur. Therefore, $Fv-2^{ss}$ DBA/2 adult mice were infected with 10^3 FFU of FV complex 4 days before lethal irradiation (860 R) and transplantation of inoculum of 10^6 or 10^7 marrow cells from adult $Fv-2^{rr}$ B10.D2 donor mice (Fig. 1). DBA/2 and B10.D2 mice share the $H-2^d$ alleles. 5–30 days later, the spleens of the primary recipient DBA/2 mice were assayed for content of SFFV and TCFU. The results are presented in Table III.

Bone marrow cells (10^7) of B10.D2 origin supported both SFFV replication and generated TCFU (Exp. 1, Table III). However, the TCFU generated were too numerous to count accurately. No TCFU or SFFV were recovered when the primary recipients were B10.D2 rather than DBA/2 mice. In the subsequent experiments, only 10^6 donor marrow cells were grafted and only $\frac{1}{8}$ spleen equivalent of cells (instead of $\frac{1}{4}$ in Exp. 1, Table III) was retransplanted. B10.D2 bone marrow-derived cells growing in spleens of irradiated and infected DBA/2 mice again supported both virus replication and yielded transformed TCFU (Exp. 2, Table III). In a control group of primary recipient mice which were not infected, but transplanted with B10.D2 bone marrow cells, no TCFU were recovered. Also the participation of transplanted B10.D2 bone marrow cells in the generation of TCFU was indicated by the failure to recover TCFU from spleens of DBA/2 primary recipients, previously infected, but not transplanted with B10.D2 bone marrow cells (Exp. 2, Table III). Tumor colonies in B10.D2 secondary hosts were seen as slightly raised bright yellow nodules at the surface of the spleen (Fig. 4). Histologic examination of the secondary recipient spleens, revealed the typical picture of Friend erythro-leukemia, with colonies of transformed erythroid cells under the capsule and invading the white pulp. It may be re-emphasized that FV by itself does not induce erythro-leukemia in B10.D2 mice. These results indicate that the $Fv-2$

TABLE III
Transformation of B10.D2 Bone Marrow Cells

Exp.	No. of cells transplanted from a B10.D2 marrow donor	DBA/2 primary recipients*		Interval in days between transplantation of donor marrow cells and retransplantation of primary recipient spleen cells	B10.D2 secondary recipients	
		Virus treatment	SFFV recovered		Tumor colonies/spleen (× 8)	SFFV recovered
1	10 ⁷	SFFV(LLV)	400 (5)§	7	Mean ± SE >100 (5)	>500 (5)
	10 ⁷	No virus	0 (5)	7	0 (5)	0 (5)
	10 ⁷	SFFV(LLV)‡	0 (4)	7	0 (4)	NT¶
2	10 ⁶	SFFV(LLV)	>1,000 (7)	5	23.4 ± 3.3 (7)	>500 (7)
	10 ⁶	No virus	0 (5)	5	0 (5)	NT
	No cells	SFFV(LLV)	0 (5)	5	0 (5)	NT
3	10 ⁶	SFFV(LLV)	NT	5	35.2 ± 6.4 (5)	NT
	10 ⁶	SFFV(LLV)	NT	15	27.0 ± 5.4 (5)	NT
	10 ⁶	SFFV(LLV)	NT	30	0 (5)	NT

* Primary recipient mice were infected with 10⁸ FFU of NB-tropic FV complex. They were irradiated 4 days later and transplanted with B10.D2 bone marrow cells. On day 5, 7, 15, or 30 after transplantation, the primary recipient spleen cells were harvested and washed once in Eagle's MEM. The supernatant fluid was assayed for FFU in DBA/2 mice and the cells were assayed for TCFU by retransplanting ¼ (Exp. 1) or ⅛ (Exp. 2 and 3) fraction of the primary spleen into adult unirradiated B10.D2 mice. 9 days later the B10.D2 secondary recipient mice were killed and the spleens were fixed in Bouin's solution and examined for tumor colonies.

‡ In this group the primary recipients were B10.D2 mice infected with SFFV(LLV) 4 days before irradiation and transplantation of B10.D2 bone marrow cells.

§ Numbers in parentheses indicate number of animals used.

¶ NT, not tested.

gene does not control susceptibility of erythropoietic "target" stem cells to malignant transformation by FV.

In Exp. 3, Table III, the intervals between grafting of the resistant donor marrow cells and the assay for TCFU generated in the DBA/2 recipient spleens was 5, 15, or 30 days. If *Fv-2* controls the functions of M cells or other hemopoietic cell types, we reasoned that resistance to leukemia may develop in the bone marrow chimeras as these cells were generated by stem cells in the resistant bone marrow inoculum. There was no significant difference in the number of tumor colonies generated in the secondary recipients, whether retransplantation of DBA/2 spleen cells was done on day 5 or 15 (Exp. 3, Table III). However, when B10.D2 marrow cells were allowed to develop in DBA/2 spleens for 30 days before the retransplantation was performed, no TCFU could be recovered

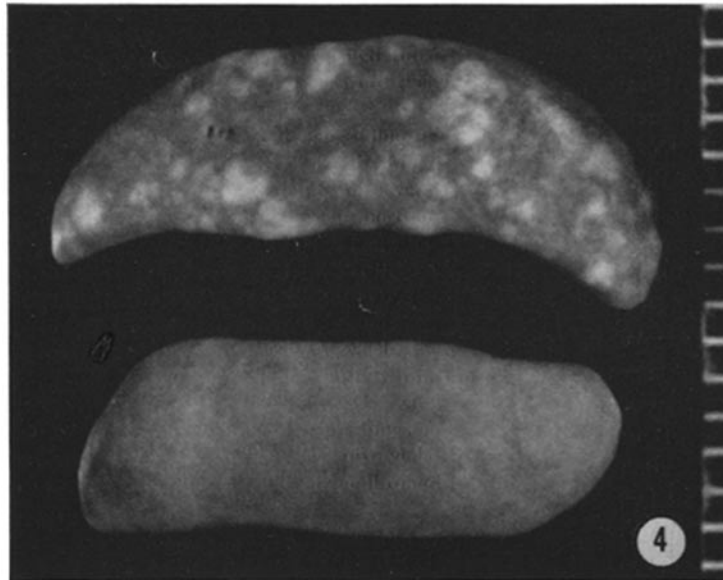


FIG. 4. Spleens fixed in Bouin's solution taken from B10.D2 secondary recipients. The upper spleen is from a B10.D2 mouse which received a spleen cell transplant from a DBA/2 primary recipient, previously infected and transplanted with B10.D2 bone marrow cells (outlined in Fig. 1). It shows numerous slightly elevated pale tumor colonies. The lower spleen, which is devoid of any tumor colonies, is from a control group in which the B10.D2 secondary recipient was transplanted with spleen cells from a DBA/2 primary recipient previously infected with FV, but not given the B10.D2 bone marrow cells ($\times 4.5$).

as indicated by total absence of tumor colonies in the secondary recipient spleens.

Certain control experiments were performed to further establish that *Fv-2^{rr}* B10.D2 and not *FV-2^{ss}* DBA/2 TCFU were being produced and detected under these experimental conditions in the secondary recipient. A group of DBA/2 mice were infected with 10^8 FFU of NB-tropic FV complex and their spleens were removed 9 days later when massively enlarged with erythroleukemic cells. The spleen cells were harvested from these DBA/2 mice, were washed to remove excessive extracellular virus, and were transplanted ($\frac{1}{8}$ spleen equivalent) into five adult unirradiated B10.D2 recipients. 9 days later the B10.D2 recipient spleens were removed, fixed in Bouin's solution, and examined for TCFU. No tumor colonies were seen in any of the spleens of the five B10.D2 mice. Thus, *Fv-2^{rr}* B10.D2 mice did not support the proliferation of TCFU of DBA/2 origin.

In another experiment we followed the experimental protocol outlined in Fig. 1 except that the bone marrow donors of 10^6 cells/inoculum were DBA/2 mice instead of B10.D2. The primary recipients were DBA/2 mice. 5 days later spleen cells (after three washes) of the primary recipients were retrans-

planted ($\frac{1}{8}$ spleen equivalent) into both DBA/2 and B10.D2 secondary recipients. While DBA/2 secondary recipients supported the growth of DBA/2 TCFU generated in DBA/2 hosts (there were 50–100 tumor colonies/secondary recipient spleen), B10.D2 secondary recipients did not. In this experiment the surface nodules seen on the DBA/2 secondary recipient spleens would certainly include some foci formed by the transfer of a small amount of extracellular virus carried along with the spleen cells in addition to tumor colonies generated by transformed DBA/2 cells. However, the total absence of tumor colonies in B10.D2 secondary recipients, clearly proves the point that TCFU of DBA/2 origin when carried over into B10.D2 secondary recipients cannot proliferate to produce tumor colonies.

If the TCFU generated under these experimental conditions are of donor (B10.D2) origin, a linear relationship should exist between the numbers of TCFU generated and number of marrow cells transplanted within a certain range of cell inoculum sizes. Therefore, infected and irradiated DBA/2 primary recipient mice were infused with 1.5 , 4.5 , or 13.5×10^5 B10.D2 marrow cells. Spleen cells ($\frac{1}{8}$ spleen equivalent) were assayed for TCFU in B10.D2 secondary recipients 5 days after marrow transplantation. Under these conditions, a linear relationship between inoculum size and TCFU generated was obtained (Fig. 5).

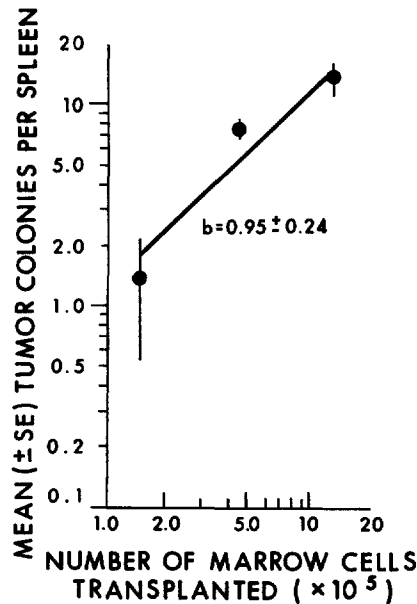


FIG. 5. Relationship between the number of B10.D2 bone marrow cells injected into previously infected DBA/2 primary recipient and the number of tumor colonies generated upon transplantation of spleen cells from the DBA/2 recipient into adult unirradiated B10.D2 secondary host (see Fig. 1 for details). Result: a linear relationship exists between the cell inoculum size of B10.D2 bone marrow and tumor colonies generated.

Preliminary experiments (not reported here) with other strain combinations confirmed the findings obtained with B10.D2 and DBA/2. B6 (*Fv-2^{rr}*) bone marrow cells could be successfully transformed by grafting into previously infected leukemic and irradiated histocompatible 129/J (*Fv-2^{ss}*) mice, and C58/J marrow cells could be transformed by transplantation into previously infected (C58 × C3H)F1 (*Fv-2^{ss}*) primary recipients. Whereas discrete tumor colonies could not be seen in the spleens of B6 secondary recipient mice, histologic evidence of transformation was obtained. The B6 and C58 marrow cells also supported extensive SFFV replication under these conditions.

DISCUSSION

The occurrence of splenomegaly with the histologic picture of malignant erythropoiesis independent of erythropoietin, the synthesis of infectious SFFV recovered from the plasma and spleen supernatant fluids, and the severe suppression of antibody formation indicate that ⁸⁹Sr treatment overcomes the genetic resistance of B6 mice to FV leukemia. Recovery of infectious SFFV and percentage of neoplastic erythroid cells in the spleens was a function of the dosage of ⁸⁹Sr administered. This finding is similar to the dose-related suppression of marrow allograft rejection by ⁸⁹Sr (13).

Since a similar treatment with ⁸⁹Sr also suppresses the ability of "good responder" mice to reject bone marrow allografts, one of the logical conclusions is that the cells responsible for expression of genetic resistance to this leukemia are the same as the M cells involved in rejection of hemopoietic allografts (13). Besides susceptibility to ⁸⁹Sr, some other properties of M cells also seem to go along with their suggested role in mediating resistance to FV complex. Thus, resistance to FV complex is not abrogated by thymectomy followed by irradiation and marrow reconstitution (unpublished results), treatment with anti-lymphocytic serum (8), or cortisol (7); such is also the case with rejection of bone marrow allografts (12). Before 21 days of life, when the ability to reject bone marrow allografts is still not developed (12), mice are also exquisitely sensitive to leukemia viruses (20). Even infant B6 mice infected with FV develop splenomegaly and high titers of circulating FV, both of which regress after the first 3 wk of life (unpublished observations).

The inability to see discrete tumor foci in adult B6 mice treated with ⁸⁹Sr is interesting. A similar situation exists in the case of infant mice less than 21 days old, in which erythropoiesis and virus replication occur but no discrete foci can be seen (unpublished observations). Perhaps extensive myelopoiesis, which occurs in the spleens of both infant mice and adult mice treated with ⁸⁹Sr, prevents the expression of malignant erythropoiesis in the form of discrete foci.

One of the natural corollaries of the postulated mechanism of action of ⁸⁹Sr would be that genetic resistance to FV leukemia is not due to resistant erythropoietic target cells as had been previously suggested (7, 9). The successful transformation of *Fv-2^{rr}* B10.D2 bone marrow cells in spleens of infected *Fv-2^{ss}*

mice strengthens and complements the conclusion that resistant mice do indeed possess susceptible target cells. These target cells are normally prevented from being rendered leukemic or proliferating by some influence of the ⁸⁹Sr-sensitive population of effector cells, i.e., M cells. These observations also imply that target cells of susceptible mice become transformed and continue to proliferate due to the genetically determined inability of their M cells to exert surveillance.

Since successful transformation of resistant B10.D2 target cells occurred during the first 15 days after marrow transplantation (Table III), it follows that the hemopoietic cells conferring resistance either were not immediately transplantable or were not present in bone marrow cell suspensions. We have tried unsuccessfully several times to adoptively transfer immediate resistance to bone marrow allografts with marrow, spleen, or lymph node cell suspensions, and tentatively conclude that these effector cells are not immediately transplantable (unpublished observations). However, precursors of M cells are transplantable as is indicated by the observation that the ability to reject bone marrow grafts can be detected in poor responder 129/J mice if 3 wk or more are allowed to pass after transplantation of bone marrow cells from good responder B6 mice (12). In the present experiments, therefore, when the retransplantation of primary recipient spleens was delayed until 30 days, no TCFU could be recovered. This finding could be explained by the generation of genetically resistant M cells from their precursors in the B10.D2 bone marrow inoculum between 15 and 30 days. Thus, observations that resistance to FV leukemia could be transferred to susceptible strains by means of bone marrow cell grafts (9, 10) can now be better explained by the development in the recipients of resistant effector M cells. This latter observation suggests that marrow grafts from histocompatible but genetically resistant donors may help cure leukemia in susceptible recipient mice. This possibility is now being tested.

In two separate experimental conditions (Table I and III), transformed B6 cells failed to produce grossly discrete tumor colonies in the spleen upon transplantation into unirradiated adult B6 mice, while transformed B10.D2 and C58 cells did produce discrete colonies. The B6 spleens fixed in Bouin's contained ill-defined bright yellow "areas" which histologically revealed malignant erythropoiesis. B6, B10.D2, and C58 mice are all *Fv-2^r* but differ at the *H-2* locus. It is conceivable that the gene for resistance to FV leukemia associated with the *H-2^b* allele (5) affects the development of tumor colonies in *Fv-2^r* mice.

The mechanism by which M cells confer resistance to FV leukemia is not entirely clear. As mentioned earlier, M cells recognize *Hh* antigens expressed normally on primitive hemopoietic cells. The expression of a certain type of *Hh* antigen was observed to be increased 10-fold on Friend erythroleukemic progenitor cells; that event appeared to occur almost simultaneously with the transforming events (21). It may be speculated that in resistant mouse strains the transformed cells with increased *Hh* expression on the surface are rejected by M cells. Experiments to verify this suggestion are in progress.

Another testable hypothesis developed during this study relates to the nature of the *Fv-2* gene. Since the *Fv-2* gene seems to mediate its effect by controlling properties of the M cells, it may be identical to one of the immune response genes which affect M-cell reactivity in rejection of marrow allografts (12, 22, 23). Although the strain distribution of "good responders" to marrow grafts and *Fv-2^{rr}* mice is similar (11), the real test for this hypothesis would be to compare the relative abilities of C57BL/6 (good responders and *Fv-2^{rr}*) and C57BL/6. *Fv-2^{ss}* mice to reject marrow allografts. The latter congenic C57BL mice would have the *Fv-2* susceptible gene serially backcrossed onto them. Breeding of such congenic mice is now being undertaken.

From the experiments reported here, the ⁸⁹Sr-sensitive population of effector cells does seem to be very important in mediating genetic resistance to Friend leukemia. However this does not exclude that other immunocompetent cells belonging to the T- and B-cell systems may also contribute to protection from leukemia in the resistant strains. In this connection, preliminary experiments² indicate that thymus cells may also be involved in genetic resistance. Thus mitogenic response of T cells from a number of *Fv-2^{rr}* donors is not suppressed by FV in vitro, while that of T cells from susceptible strains is significantly depressed. Also simultaneous transplantation of thymus and bone marrow cells from resistant donors into infected susceptible recipients, seems to offer partial protection to the resistant bone marrow cells from transformation. The exact relationship between thymus cells and M cells in mediating resistance to this leukemia is under investigation.

SUMMARY

Resistance to malignant erythropoiesis induced by Friend spleen focus-forming virus and resistance to marrow stem cell allografts are under genetic control. Strains of mice, e.g., C57BL/6 and B10.D2, which are homozygous for resistance at the *Fv-2* locus, are also good rejectors of most bone marrow allografts. ⁸⁹Sr, a bone-seeking isotope, irradiates marrow but not other lymphoid organs and abrogates resistance to marrow allografts without suppressing T- or B-cell functions. Thus, marrow-dependent effector cells (M cells) seem to resist allogeneic stem cells. To test if the genetic resistance to Friend virus (FV) is also mediated by M cells, B6 mice were treated with ⁸⁹Sr using a dosage schedule known to abrogate resistance to allogeneic marrow cells. 9 days after FV infection of such mice, the spleens showed malignant erythroblastosis which could not be suppressed by prior hypertransfusion, a procedure which suppresses physiologic erythropoiesis. Such ⁸⁹Sr-treated B6 mice also supported extensive virus replication, while control mice did not. FV markedly suppressed the ability of ⁸⁹Sr-treated B6 mice to produce antisheep red blood cell (SRBC)

² Vinay Kumar, Michael Bennett, Tin Han, and Robert J. Eckner. Mechanisms of genetic resistance to Friend murine leukemia virus. II. Role of thymus cells. Manuscript in preparation.

antibodies, a feature seen normally only in genetically susceptible mice. Thus, ⁸⁹Sr-treated B6 mice behaved in these respects as if they were susceptible to FV. When increasing doses of ⁸⁹Sr were administered to B6 mice, a dose-related loss of resistance to FV was seen. Therefore, it appears that ⁸⁹Sr-sensitive M cells mediate the genetic resistance to FV.

The results of experiments with ⁸⁹Sr indicated that genetically resistant mice would be expected to possess target cells which are susceptible to transformation by FV. To verify this corollary, bone marrow cells from B10.D2 (*Fv-2^{rr}*) mice were transplanted into previously infected and lethally irradiated DBA/2 (*Fv-2^{ss}*) recipients which share the same *H-2^d* alleles. 5–15 days later, the spleens of DBA/2 primary recipients yielded transformed cells which were capable of producing splenic tumor colonies upon transplantation into adult, unirradiated B10.D2 secondary recipients. Various control experiments clearly indicated that the tumor colonies so induced were of B10.D2 marrow origin. This indicated that B10.D2 stem cells could be transformed when allowed to interact with FV in the spleens of susceptible DBA/2 mice. However, 30 days after transplantation of B10.D2 bone marrow cells into DBA/2 recipients, no transformed cells were detected. Apparently, in the 30-day interval precursors in the B10.D2 marrow gave rise to mature M cells which resisted the leukemic process. Since M cells recognize hybrid or hemopoietic histocompatibility antigens expressed on primitive normal and transformed hematopoietic cells, we suggest that M cells may exert surveillance by rejecting leukemic cells. Thus, marrow transplantation from genetically resistant donors may provide a new mode of treatment for leukemia, by providing precursors of M cells and other immunocompetent cell types.

REFERENCES

1. Steeves, R. A., R. J. Eckner, M. Bennett, and E. A. Mirand. 1971. Isolation and characterization of a lymphatic leukemia virus in the Friend virus complex. *J. Natl. Cancer Inst.* **46**:1209.
2. Eckner, R. J., and R. A. Steeves. 1972. A classification of the murine leukemia viruses. Neutralization of pseudotypes of Friend spleen focus-forming virus by type-specific murine antisera. *J. Exp. Med.* **136**:832.
3. Eckner, R. J. 1973. Helper dependent properties of Friend spleen focus-forming virus: Effect of the *Fv-1* gene on the late stages in virus synthesis. *J. Virol.* **12**:523.
4. Pincus, T., W. P. Rowe, and F. Lilly. 1971. A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to Friend murine leukemia virus. *J. Exp. Med.* **133**:1234.
5. Lilly, F. 1970. *Fv-2*: identification and location of a second gene governing the spleen focus response to Friend leukemia virus in mice. *J. Natl. Cancer Inst.* **45**:163.
6. Ceglowski, W. S., and H. Friedman. 1969. Murine virus leukemogenesis: rela-

- tionship between susceptibility and immunodepression. *Nature (Lond.)*. **224**:1318.
7. Bennett, M., and R. A. Steeves. 1970. Immunocompetent cell functions in mice infected with Friend leukemia virus. *J. Natl. Cancer Inst.* **44**:1107.
 8. Stutman, O., and J. Dupuy. 1972. Resistance to Friend leukemia virus in mice: effect of immunosuppression. *J. Natl. Cancer Inst.* **49**:1283.
 9. Axelrad, A., and H. C. Van Der Gaag. 1968. Genetic and cellular basis of susceptibility or resistance to Friend leukemia virus infection in mice. *Proc. Can. Cancer Res. Conf.* **8**:313.
 10. Odaka, T., and M. Matsukura. 1969. Inheritance of susceptibility to Friend mouse leukemia virus. VI. Reciprocal alteration of innate resistance or susceptibility by bone marrow transplantation between congenic strains. *J. Virol.* **4**:837.
 11. Bennett, M., and R. J. Eckner. 1973. Immunobiology of Friend virus leukemia. *In Virus Tumorigenesis and Immunogenesis*. W. S. Ceglowski and H. Friedman, editors. Academic Press, Inc., New York. 387-414.
 12. Cudkowicz, G., and M. Bennett. 1971. Peculiar immunobiology of bone marrow allografts I. Graft rejection by irradiated responder mice. *J. Exp. Med.* **134**:83.
 13. Bennett, M. 1973. Prevention of marrow allograft rejection with radioactive strontium: evidence for marrow dependent effector cells. *J. Immunol.* **110**:510.
 14. Fried, W., C. W. Gurney, and M. Swatek. 1966. Effect of Strontium-89 on the stem cell compartment of the spleen. *Radiat. Res.* **29**:50.
 15. Gurney, C. W., L. Klassen, J. Birks, and E. Allen. 1972. Hematopoietic and immunologic alterations in mice produced by ⁸⁹Sr-induced marrow hypoplasia. *Exp. Hematol.* **22**:27.
 16. Mirand, E. A., R. A. Steeves, L. Avila, and J. T. Grace, Jr. 1968. Spleen focus formation by polycythemic strains of Friend leukemia virus. *Proc. Soc. Exp. Biol. Med.* **127**:900.
 17. Axelrad, A. A., and R. A. Steeves. 1964. Assay for Friend leukemia virus: rapid quantification method based on enumeration of macroscopic spleen foci in mice. *Virology*. **24**:513.
 18. Mirand, E. A. 1967. Virus induced erythropoiesis in hypertransfused-polycythemic mice. *Science (Wash. D. C.)*. **156**:832.
 19. Thomson, S., and A. A. Axelrad. 1968. A quantitative spleen colony assay method for tumor cells induced by Friend leukemia virus infection in mice. *Cancer Res.* **28**:2105.
 20. Gross, L. 1951. "Spontaneous" leukemia developing in C3H mice following inoculation in infancy, with AK-leukemic extracts or AK-embryos. *Proc. Soc. Exp. Biol. Med.* **76**:27.
 21. Rossi, G. B., G. Cudkowicz, and C. Friend. 1973. Transformation of spleen cells three hours after infection in vivo with Friend leukemia virus. *J. Natl. Cancer Inst.* **50**:249.
 22. Bennett, M. 1971. Graft vs. host reactions in mice I. Kinetic and immunogenetic studies of alloantigen sensitive units of lymphoid tissue. *Transplantation*. **11**:158.
 23. Cudkowicz, G. 1971. Genetic control of bone marrow graft rejection. I. Determinant specific difference of reactivity in two pairs of inbred mice strains. *J. Exp. Med.* **134**:281.