

MECHANISMS OF GENETIC RESISTANCE TO FRIEND VIRUS LEUKEMIA IN MICE

II. Resistance of Mitogen-Responsive Lymphocytes Mediated by Marrow-Dependent Cells*

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Friend leukemia virus, which produces a rapidly developing erythroleukemia in adult mice of susceptible strains, is a complex of defective Friend spleen focus-forming virus (SFFV)¹ and its helper lymphatic leukemia virus (1). Resistance to leukemogenesis by Friend virus complex (FV) is controlled by a number of genes (2). The Friend virus-2 (*Fv-2*) locus controls host resistance to SFFV. Adult homozygous resistant mice (*Fv-2^{rr}*) such as C57BL/6 (B6) and C58, are completely resistant to the erythroleukemic effects of FV (2) and relatively resistant to immunosuppression by FV (3).

In the first paper of this series (4), we reported that genetically resistant mice possess erythropoietic target cells susceptible to transformation by FV, but seem to be protected from developing leukemia by a special class of effector cells which are responsible for bone marrow allograft rejection. The effector cell system responsible for marrow allograft rejection differs in several characteristics from thymus (T)- and bursa equivalent (B)-dependent cells of the mouse (5). Two cell types, which may or may not be related, seem to be involved in the rejection of bone marrow allografts. These are marrow-dependent (M) cells and macrophages. The M cells appear to require a bone marrow microenvironment for a critical stage in maturation since their function is suppressed by the long-lived bone-seeking isotope ⁸⁹Sr (6). T-, B-, and macrophage-cell functions, e.g. humoral immunity, graft-vs.-host reactivity, response to T- and B-cell mitogens, and antibody-dependent cellular cytotoxicity, appear intact in mice treated with ⁸⁹Sr (6, 7). The macrophages involved in marrow allograft rejection are suppressible by injection of silica particles (8). Treatment of mice with ⁸⁹Sr not only abrogates their ability to reject allogeneic bone

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¹ *Abbreviations used in this paper:* B, bursa equivalent-dependent; Con A, concanavalin A; DS, dextran sulfate; FFU, spleen focus-forming units; FV, Friend virus complex; *Fv-1*, Friend virus-1; *Fv-2*, Friend virus-2; Hh, Hemopoietic histocompatibility; LPS, lipopolysaccharide; M, marrow-dependent; MC, methylcholanthrene-induced; MuLV, murine leukemia virus; NMS, normal mouse serum; PHA, phytohemagglutinin; SFFV, spleen focus-forming virus; T, thymus-dependent.

marrow cells, but also the genetic resistance of B6 mice to Friend virus-induced erythro-leukemia, suggesting a surveillance role for M cells (4).

The mechanism of M-cell-mediated surveillance is not clear. M cells recognize Hemopoietic histocompatibility (Hh) antigens which are expressed normally on primitive hemopoietic cells. Expression of certain Hh antigens is increased several fold on erythro-poietic progenitor cells transformed by FV (9). Therefore, M cells of resistant mice may act by "rejecting" transformed cells. Alternatively or additionally, M cells may act indirectly by antagonizing the suppressive effects of FV on T and B cells, thus permitting the latter to reject leukemic cells. Suppression of T- and B-cell function *in vivo* seems to be a consistent effect of FV in strains of mice susceptible to leukemia, but not in genetically resistant strains of mice (3, 10).

We have been able to investigate the regulatory role of M cells on suppression of T and B cells by FV using an *in vitro* technique. We have observed that FV, *in vitro*, can suppress the proliferative responses to mitogens of lymphocytes taken from mice susceptible to the immunosuppressive and erythro-leukemic effects of FV *in vivo*, and vice versa. In this paper we describe experiments which indicate that functional M cells are responsible for the resistance of lymphocytes to FV *in vitro*. In the accompanying paper (11) experiments are described which indicate that a subpopulation of T cells mediate the FV-induced suppression of lymphocyte mitogenesis. In both these papers the terms "in vitro susceptibility/resistance" are intended to imply the suppression or lack of suppression of lymphocyte mitogenesis by FV.

Materials and Methods

Mice. Adult male mice of the strains B6, DBA/2J, B10.D2/nSn (B10.D2), 129/J, and C58/J were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c and C3H/He mice were bred in our own animal facility. Mice were 6- to 8-wk old in most instances.

Virus. NB-tropic FV, as contained in the Mirand strain (12) was used. The virus is maintained by serial *in vivo* passages in BALB/c mice and virus stocks are prepared as 20% wt/vol cell-free extracts of leukemic spleens. The virus is titrated by the spleen focus assay (13).

Culture of Lymphoid Cells. Cell suspensions were made from spleens, lymph nodes, bone marrow, and thymuses of normal mice in chilled RPMI 1640 medium with 10% fetal calf serum. Lymph node and thymus cells were routinely washed once in cold medium. Spleen cells were washed or not, dependent upon the experimental protocol as described in the Results. Cells were cultured in Falcon Microtest II plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) (10^6 nucleated cells per well). Each cell suspension was cultured in 0.2 ml vol in triplicate with (a) mitogen only, (b) mitogen plus FV, (c) FV only, or (d) neither FV nor mitogen. In each experiment at least three doses of FV ranging from 100-500 spleen focus-forming units (FFU)/well were used. The virus, after appropriate dilution in the culture medium, was added in 25- μ l vol at the onset of culture. [3 H]thymidine (0.5 μ Ci; New England Nuclear, Boston, Mass.) was added to each well 48 h after plating and the cultures were harvested 18 h later by a MASH II cell harvester (Microbiological Associates, Bethesda, Md.) Incorporation of [3 H]thymidine into DNA, as detected by a standard liquid scintillation counting procedure, was the measure of cell proliferation. Results are expressed as Δ blastogenesis which represents mean counts per minute in cultures stimulated with mitogen minus mean counts per minute in cultures without the mitogen. To calculate Δ blastogenesis in the presence of FV, the counts per minute in wells with FV alone were subtracted from counts per minute in wells with FV and the mitogen. FV without mitogen did not have any significant effect on incorporation values of [3 H]thymidine. Therefore, FV-induced changes in Δ blastogenesis could not be attributed to changes in "background" counts. The percentage (%) suppression of Δ blastogenesis in cultures with FV was calculated as follows: % suppression = Δ blastogenesis (control-FV)/(control) \times 100.

Mitogens. Concanavalin A (Con A) (Calbiochem, San Diego, Calif.) was used as a T-cell

mitogen and after preliminary experiments, 2, 1, and 0.25 $\mu\text{g}/\text{well}$ were selected as optimal doses for thymus, spleen, and lymph node cells, respectively. *Salmonella typhi* lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) was used as one of the B-cell mitogens in a dose of 2.5 $\mu\text{g}/\text{well}$ both for spleen and lymph node cells. Bone marrow and splenic B cells were also stimulated with dextran sulfate (DS) (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) at a dose of 300 $\mu\text{g}/\text{well}$.

Transplantation of Methylcholanthrene-Induced (MC) Sarcoma in B6 Mice. Details of this have been published (15). Briefly, B6 mice inoculated subcutaneously in the thigh with 10^5 syngeneic MC sarcoma cells. Mice were used when the tumor reached a diameter of 2.0–2.5 cm 15 days after inoculation of tumor cells. Under these conditions, B6 mice lose the ability to reject bone marrow allografts (15).

Neutralization of FV Preparation. Stock FV as contained in the leukemic spleen cell extract was incubated at 37°C either with an equal volume of normal Swiss mouse serum (NMS) or with an anti-FV serum prepared in Swiss mice. The details of preparing this antiserum have been published (14). At 40 and 80 min after the onset of incubation, 0.1-ml aliquots of FV incubated with NMS or anti-FV serum were diluted to achieve a concentration of 200 FFU/25 μl and then tested in vitro for ability to suppress the response of splenic T cells to Con A. Two other control groups were included to determine if NMS or anti-FV serum had any effect on the Con A response at the dilutions equal to those used in the incubation mixtures in culture wells.

UV Light and Heat Treatment of FV. A 1.5 dilution of stock FV was exposed to UV irradiation (6×10^3 ergs) and then tested for its suppressive activity after dilution to an amount that would have been 200 FFU/25 μl before UV irradiation. Similarly, a 1:5 dilution of stock virus was incubated at 56°C for 45 min and then tested for its ability to suppress the Con A response.

Administration of ^{90}Sr . 100 μCi of ^{90}Sr (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 abrogates genetic resistance to incompatible marrow allografts (6) and to FV-induced leukemia in B6 mice (4).

Administration of Silica. Silica particles (5 μm average size, gift from Dr. K. Robock, Steinkohlenberg Vauverein, 43 Essen-Krey, Germany) were suspended in medium and injected intravenously (5 mg/animal) 24 h before sacrifice of mice. This treatment in mice overcomes hybrid resistance and allogeneic resistance to incompatible hemopoietic cell grafts (8).

Results

Suppression of Lymphoid Cell Mitogenesis by FV In Vitro. It has been reported that spleen cells from mice infected with Friend or Rauscher leukemia viruses respond poorly to phytohemagglutinin (PHA), a T-cell mitogen (16, 17). The leukemic spleens, used 20 days after infection as a source of cells in such experiments are markedly hypercellular due to proliferation of neoplastic erythroid cells and contain large amounts of virus released from the transformed cells. Thus, a reduced PHA response might have been due to marked dilution of T cells by the erythroid cells and/or a direct suppressive effect of extracellular virus on the PHA-responsive T cells. To find out whether FV directly suppressed T- and B-cell mitogenesis, we cultured normal spleen, thymus, lymph node, and bone marrow cells from several strains of susceptible mice with appropriate mitogens and added FV to the cultures at the onset. Results collected from several experiments are shown in Table I.

Addition of FV suppressed the mitogenic effects of Con A on splenic, thymic, and lymph node T cells in a dose-related fashion. Responses of lymph node and splenic B cells to DS or LPS and of primitive B cells in the bone marrow which respond only to DS, were also suppressed by FV. Although the virus effect was always dose related, the absolute degree of suppression at a given dose of virus varied from one experiment to another by about 10–20%.

Relation of Suppressive Activity of FV stocks to Presence of Infective Viri-

TABLE I
Suppression of Mitogenic Response of Various Lymphoid Cells from Susceptible Mice by FV

Mouse strain	Lymphoid cell* source	Mitogen†	Δ Blastogenesis§				Suppression with:		
			+ FV (FFU/per well)						
		No FV	100	200	400	100	200	400	FFU/ well
BALB/c	Thymus	Con A	47,110 ± 2,933	30,195 ± 1,203	12,907 ± 1,120	9,230 ± 506	36	73	91
129	Thymus	Con A	73,540 ± 4,367	59,212 ± 2,518	31,212 ± 1,467	9,166 ± 591	20	58	88
C3H	Spleen	Con A	60,342 ± 1,256	48,254 ± 1,772	27,937 ± 1,012	15,679 ± 472	21	54	75
BALB/c	Spleen	Con A	51,805 ± 2,852	36,756 ± 1,089	18,784 ± 636	13,026 ± 1,003	30	64	75
DBA/2	Spleen	LPS	24,653 ± 159	20,508 ± 1,346	8,845 ± 293	201 ± 69	17	65	99
DBA/2	Spleen	DS	52,037 ± 5,536	37,715 ± 4,291	31,411 ± 4,318	13,883 ± 97	28	40	74
DBA/2	Bone Marrow	DS	6,727 ± 62	3,903 ± 140	2,728 ± 192	0	42	60	100
BALB/c	Lymph node	Con A	150,487 ± 1,899	NT¶	70,610 ± 6,307	NT	—	54	—
BALB/c	Lymph node	LPS	6,420 ± 337	NT	37.5 ± 4.5	NT	—	99	—

* 1×10^6 cells from various lymphoid organs were cultured for 66 h in Microtest II plates with or without mitogens and with or without FV. [3 H]thymidine was added during the last 18 h of culture.

† Optimal concentrations of mitogens used were for Con A - 2, 1, and 0.25 μ g/well for thymocytes, spleen cells, and lymph node cells, respectively; for LPS - 2.5 μ g/well and for DS - 300 μ g/well.

§ Δ Blastogenesis, (cpm with mitogen - cpm without mitogen). In groups with FV, Δ blastogenesis, (cpm + mitogen + FV - cpm + FV).

|| Response less than in unstimulated cultures.

¶ NT, not tested.

TABLE II
Requirement of Infective FV* to Suppress Con A-Induced Mitogenesis of C3H Spleen Cells

Exp.	Prior treatment of FV		Δ Blastogenesis		Suppression
	Mode of treatment	Duration	No FV	FV (200 FFU/well)	
		<i>min</i>	<i>mean cpm ± SE</i>		<i>%</i>
1	None		69,731 ± 826	32,141 ± 309	54
	UV light ‡	40		63,168 ± 1,016	10
2	None		53,740 ± 282	23,076 ± 1,183	58
	Heat§	45		44,475 ± 558	18
3	None		64,556 ± 1,142	33,219 ± 754	49
	Anti-FV antiserum	40		49,916 ± 1,355	23
	Anti-FV antiserum	80		61,858 ± 2,382	5¶
	Normal mouse serum	80		34,624 ± 1,459	47

* FV extracted as a 20% cell-free suspension from spleens of leukemic BALB/c mice.

‡ A 1:5 dilution of FV was exposed to UV light (6×10^3 ergs/mm²).

§ A 1:5 dilution of FV was heated at 56°C in a water bath and diluted to 200 FFU/25 μl in RPMI 1640.

|| 0.2 ml FV preparation was incubated with equal volume of an anti-FV serum at 37°C. 40 and 80 min after incubation aliquots of 0.1 ml were removed and diluted to the desired concentration (200 FFU/25 μl) with ice-cold RPMI 1640; the virus was then tested for ability to suppress the Con A-induced mitogenesis of C3H spleen cells. Controls consisted of FV not treated with antiserum or treated with normal mouse serum.

¶ Not significantly different from control (No. FV) value ($P > 0.05$). Suppression induced by FV treated with heat, UV light, and anti-FV serum at 40 and 80 min was significantly ($P < 0.05$ as determined by Student's *t* test) less than untreated virus preparations.

ons. Leukemic spleen extracts, which were the sources of our FV preparations, might have contained nonviral soluble immunosuppressive factors. Therefore, it was important to determine if the effects of FV on the mitogen response was virus associated. If the suppressive effect of leukemic spleen extracts was a function of live and infective virus, it would be expected to be sensitive to heat and UV irradiation.

UV irradiation and heating at 56°C for 45 min largely abolished the suppression of Con A response of C3H spleen cells by FV (Table II, exps. 1 and 2). To confirm that it was FV and not other nonspecific immunosuppressive factors or contaminating viruses which are responsible for the in vitro suppression of mitogenesis, we tested if prior incubation of our fully suppressive FV stock with an anti-FV serum would abolish the effect on Con A-responsive T cells. 40 min after incubation with anti-FV serum, the suppressive activity of FV was significantly reduced from 49 to 23%, and 80 min after incubation with the antiserum, the suppressive activity of FV was almost completely abrogated (Table II, exp. 3). Incubation with NMS for 80 min did not affect the immunosuppressive function of FV. Since the anti-FV serum used neutralizes spleen focus-forming activity of FV, the results suggest that infectious virus is necessary for lymphocyte suppression in vitro.

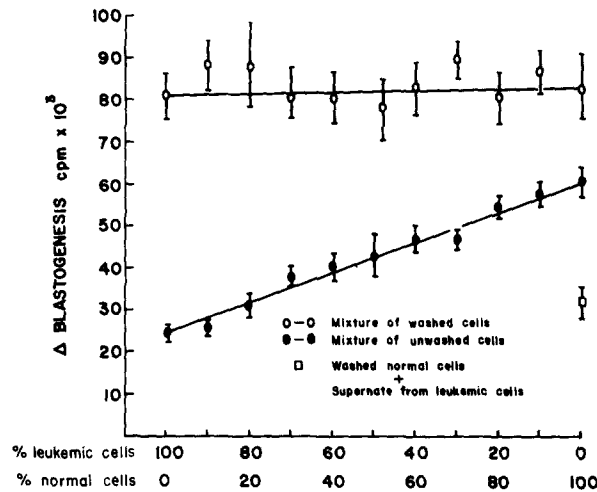


FIG. 1. The effect of adding washed or unwashed leukemic spleen cells on the proliferative response of normal splenic T cells. Spleen cells were harvested from normal BALB/c mice or those infected 4 days earlier with 10^3 FFU of FV complex. Half of the cells, from infected as well as control BALB/c mice, were washed three times in cold medium; the other half were used unwashed. Both washed and unwashed cells from normal and infected animals were cultured with Con A, either without mixing (100% normal and 100% leukemic) or in a series of mixtures containing nine different ratios of normal and transformed cells. The term "100% leukemic" indicates that all the cells were derived from mice infected with FV, and does not imply that every cell is malignant. Normal (100%) spleen cells were also cultured with Con A and a 100 μ l sample from the first washing of leukemic cells (which contained 100 FFU of FV by spleen focus assay). Result: Mitogenic response of T cells in unwashed cells from leukemic animals was suppressed; washing the cells brought it to control value. Neither washed nor unwashed leukemic cells showed any suppressive interaction with normal cells. Washing from leukemic cells suppressed response of normal cells.

Role of Leukemia Virus vs. Leukemic Cells in Suppressing the Con A Response of Spleen Cells. It has been suggested that the inability of splenic T cells from leukemic DBA/2 mice to respond to Con A may be due to a "suppressor" action of Friend leukemic cells (17). Since we found FV itself to be capable of suppressing Con A-induced mitogenesis, we decided to investigate whether the suppressive function ascribed to Friend leukemic cells may be due to the extracellular virus present in the leukemic spleen cell preparation. Spleen cells from BALB/c mice infected 4 days earlier with 1,000 FFU of FV and spleen cells from control BALB/c mice were harvested. Half of the cells, both from control and leukemic mice, was washed three times in cold RPMI while the other half was used unwashed. Samples were kept from each wash to test for virus activity. Both washed and unwashed normal and leukemic spleen cells were cultured either alone or in a series of mixtures containing nine different ratios of normal and leukemic spleen cells (Fig. 1). The cells were cultured with or without Con A, and no exogenous source of FV was added to the cell cultures. We reasoned that if addition of both washed and unwashed leukemic cells to normal cells lowered the Con A response, the suppressor function could be ascribed to leukemic cells. On the other hand, if after washing, the leukemic cells lost their suppressive effects on normal spleen cell response to Con A, the

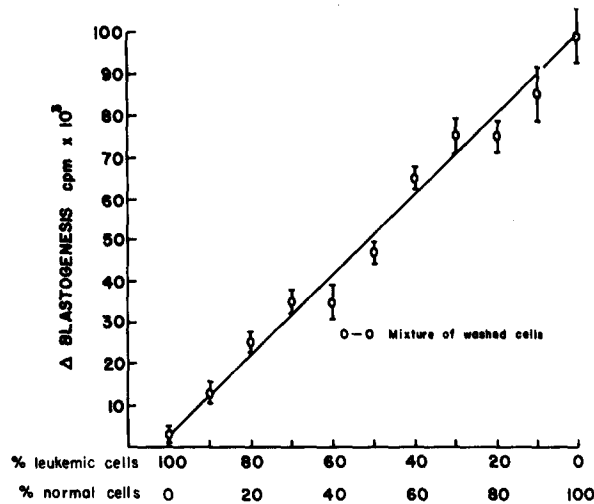


FIG. 2. Effect of adding washed leukemic spleen cells from 12-day infected BALB/c mice on the Con A-responsive cells of normal spleens. Experimental design same as in Fig. 1. Result: Con A response of washed 100% leukemic spleen cells markedly lower than control. Washed leukemic spleen cells did not interact with normal splenic T cells to suppress their response to Con A.

suppressive effect in such case would be due to a noncellular factor, probably FV.

Unwashed leukemic spleen cells responded poorly to Con A (Fig. 1). Addition of increasing numbers of unwashed leukemic cells lowered the response of unwashed normal spleen cells to Con A, proportionate to the percentage of leukemic cells in the mixture. However, washed leukemic cells responded as well as washed normal cells to Con A. Thus, 4 days after FV infection, the decreased response of unwashed leukemic cells to Con A was not due to a dilution or decrease in frequency of the Con A-responsive cells. The procedure of washing normal spleen cells enhanced the response to mitogens (Fig. 1). This is a consistent finding, but the supernatant fluids are not suppressive to the washed normal spleen cells. We also tested the supernatant fluid from the washing of leukemic cells, both for suppressive effect on the Con A response of normal spleen cells and for presence of FV complex. The first washing was both immunosuppressive (Fig. 1) and contained 1,000 FFU of FV/ml (total of 2,000 FFU/spleen).

We repeated similar experiments on days 8, 12, 13, and 21 after infection with FV. At these times, both virus titers and fraction of cells in leukemic spleen that were transformed are higher than at day 4. Results of an experiment using washed 12 day leukemic spleen cells is shown in Fig. 2. Two important points can be made from these results. Firstly, even after washing, the response of leukemic spleen cells is only 5% of control. By 12 days after infection with a large dose of FV, the spleen was markedly enlarged (800×10^6 cells per spleen in leukemic vs. 96×10^6 per spleen in control mice) with leukemic erythroid cells. Thus, the frequency of Con A-responsive T cells was greatly decreased. Secondly, the responses of the mixtures of washed leukemic spleen cells and normal

TABLE III
Strain Distribution of Susceptibility to FV-Induced Suppression of Mitogenic Response of T and B Cells

Exp.	Mouse strain*	H-2 type	Lymphoid cell source	Mitogen	Δ Blastogenesis		Suppression
					No Fv	+FV (200 FFU/well)	
					mean cpm ± SE		%
1	DBA/2	<i>d</i>	Spleen	Con A	66,525 ± 2,409	17,879 ± 77	84
	B10.D2/n	<i>d</i>	Spleen	Con A	34,720 ± 2,334	30,398 ± 1,196	13‡
2	129	<i>b</i>	Thymus	Con A	38,004 ± 457	8,432 ± 1,443	78
	B6	<i>b</i>	Thymus	Con A	22,370 ± 1,511	26,439 ± 1,177	-18‡
3	C3H	<i>k</i>	Spleen	Con A	23,291 ± 2,348	9,809 ± 192	58
	C58	<i>k</i>	Spleen	Con A	65,107 ± 7,786	62,741 ± 248	4‡
4	129	<i>b</i>	Spleen	LPS	66,152 ± 1,054	21,340 ± 487	68
	B6	<i>b</i>	Spleen	LPS	22,104 ± 98	17,295 ± 624	22
5	DBA/2	<i>d</i>	Spleen	LPS	30,127 ± 813	7,621 ± 195	75
	B10.D2	<i>d</i>	Spleen	LPS	12,992 ± 123	9,312 ± 906	29
6	129	<i>b</i>	Bone marrow	DS	11,295 ± 731	1,779 ± 319	85
	B6	<i>b</i>	Bone marrow	DS	23,119 ± 709	21,612 ± 1,171	7‡
7	DBA/2	<i>d</i>	Bone marrow	DS	6,423 ± 367	2,729 ± 192	58
	B10.D2	<i>d</i>	Bone marrow	DS	12,776 ± 458	11,651 ± 250	9‡

* DBA/2, 129, and C3H mice are susceptible to FV leukemia in vivo. DBA/2, 129, C3H, and C58 mice possess the *Fv-1^s* genotype. The rest, i.e., B10.D2 and B6 are *Fv-1^r*.

‡ Not significantly different from control (No FV) value ($P > 0.05$). Suppression of Δ blastogenesis was significantly greater ($P < 0.05$ as determined by Student's *t* test) in cells from susceptible donors in each experiment.

cells was directly proportional to the number of responding cells present in the mixtures, i.e., the washed leukemic cells did not suppress the normal spleen cells. Similar results (data not known) were obtained with spleen cells harvested on days 13 and 21 after infection.

Effect of FV on the Mitogenic Response of T and B Cells from Mice of Different Strains. In the experiments described so far, the effect of FV on mitogenesis of cells from mouse strains susceptible in vivo to leukemogenesis was tested. We next compared the effect of FV on B and T cells of several strains of mice to determine if the susceptibility to in vitro suppression of mitogenesis was under genetic control. In each pair, both strains were H-2 identical but were either susceptible (*Fv-2^{ss}*) or resistant (*Fv-2^{rr}*) to Friend virus leukemogenesis. The Friend virus-1 (*Fv-1*) type within each pair was not always identical. Since the FV stock used was NB-tropic, the *Fv-1* genotype was not taken into consideration in planning experiments.

The results (Table III) indicate that regardless of the H-2 type, the type of lymphoid cell (thymus, bone marrow, or spleen), or the mitogen used (Con A, LPS, or DS), there was a good correlation between the susceptibility to suppression of mitogenesis in vitro and susceptibility to leukemia induction in vivo by

FV. Thymocytes, splenic T and B cells, and bone marrow B cells from mice of resistant strains such as B6, B10.D2, and C58 were at least three times as resistant as similar cells from DBA/2, 129, and C3H mice. The data in Table III suggests that the *Fv-2* gene controls this property of lymphocytes, but a genetic analysis has indicated that this is not so.²

Effect of ⁸⁹Sr on Genetic Resistance of B6 Mice to the Suppression of Con A Response by FV. We next sought to determine whether the genetic resistance of mitogen-responsive lymphocytes to the suppressive effect of FV was an intrinsic property of T- and B-cell populations or was mediated by some other cell type. Since suppression of M cells in adult B6 mice with ⁸⁹Sr abrogated resistance to both the leukemogenic and immunosuppressive effects of FV in vivo (4), it seemed conceivable that M cells sensitive to suppression by ⁸⁹Sr treatment may also be responsible for the in vitro resistance of T cells to the suppressive effects of FV. Therefore, adult B6 (>12-wk old) mice were treated with 100 μ Ci of ⁸⁹Sr twice at 4-wk intervals, a regimen which abrogates resistance of such mice to bone marrow cell allografts and to the leukemogenic effects of FV (4). The effect of FV on the mitogenic response of spleen cells from control and ⁸⁹Sr-treated B6 mice was compared.

The mitogenic response to Con A of spleen cells from B6 mice treated with ⁸⁹Sr in the absence of FV was vigorous (Table IV, expts. 1 and 2). The Δ blastogenesis in cultures treated with Con A without the virus was 30-35% lower in spleen cells from ⁸⁹Sr-treated mice, apparently due to the dilution effect on T cells by the increased myelopoietic activity of spleens from ⁸⁹Sr-treated mice (18). Spleens from ⁸⁹Sr-treated animals were enlarged and contained 50% more cells than controls in these experiments indicating that the absolute number of responsive T cells were very similar in spleens of the two groups of mice. However, unlike control adult B6 spleen cells, proliferation of spleen cells from B6 mice treated with ⁸⁹Sr was as susceptible to suppression by FV as was mitogenesis of spleen cells from mice whose spleen cells are genetically susceptible. Thus, abrogation of M-cell function by ⁸⁹Sr was associated with a loss of the genetic resistance of T-cell populations to the suppressive effects of FV in vitro.

We next assessed the relevance of M cells to this phenomenon by an ontogenic approach. M-cell function, as measured by the ability to reject bone marrow allografts, does not develop until 3 wk of age (5). Moreover, spleen cells of infant genetically resistant B10.D2 mice are less resistant to malignant transformation by FV as compared with adult B10.D2 mice (19). Since M-cell function is deficient in untreated infants as in adults treated with ⁸⁹Sr, we tested the susceptibility of thymus cells from 2-wk-old B6 mice to suppression by FV in vitro. In two such experiments (Table IV, expts. 3 and 4), the blastogenic response of thymocytes from infant B6 mice was suppressed by FV. Thymocytes from 8- or 10-wk-old B6 mice were resistant. The mitogenic response of infant B6 thymocytes in the absence of FV was vigorous, indicating that Con A-responsive cells had developed by this age.

Effect of Silica and MC Sarcomas on Resistance of B6 Mice to FV-Induced

² Kumar, V., L. Goldschmidt, and M. Bennett. Mechanism of genetic resistance to Friend virus leukemia in mice. IV. Lack of correlation between *Fv-2* genotype and susceptibility of mitogen-responsive lymphocytes to Friend virus *in vitro*. Manuscript in preparation.

TABLE IV
Effect of Various Treatments or Age on the Susceptibility of T Cells from B6 Mice to FV

Exp.	Treatment	Lymphoid cell source	Δ Blastogenesis		Suppression
			No FV	+ FV (200 FFU/well)	
			<i>cpm</i> \pm <i>SE</i>		<i>%</i>
1	—	Spleen	26,988 \pm 6,723	31,300 \pm 2,910	-15*
	⁸⁹ Sr‡	Spleen	15,040 \pm 2,221	7,486 \pm 869	50
2	—	Spleen	31,113 \pm 2,566	28,121 \pm 856	10*
	⁸⁹ Sr	Spleen	22,219 \pm 1,216	10,565 \pm 674	53
3	Adults	Thymus	27,173 \pm 2,716	22,084 \pm 5,930	19*
	Infants§	Thymus	27,943 \pm 1,219	10,868 \pm 1,284	62
4	Adults	Thymus	39,677 \pm 1,138	37,520 \pm 2,887	6*
	Infants§	Thymus	18,962 \pm 1,518	7,714 \pm 2,177	60
5	—	Spleen	40,211 \pm 588	37,015 \pm 1,032	8
	Silica	Spleen	42,806 \pm 203	39,203 \pm 152	9
6	—	Spleen	67,891 \pm 892	62,200 \pm 940	9
	MC sarcoma¶	Spleen	27,470 \pm 543	27,116 \pm 536	2*

* Not significantly different from controls (No FV) ($P > 0.05$). The suppression of Δ blastogenesis was significantly greater ($P < 0.05$) in cells from infant mice and adult mice treated with ⁸⁹Sr but not significantly greater in cells from adult mice treated with silica or MC sarcomas.

‡ B6 mice were injected with two doses of 100 μ Ci of ⁸⁹Sr 4 wk apart. Mice were sacrificed 3 wk after the last dose of ⁸⁹Sr.

§ 2 wk of age; all other mice were more than 8 wk of age.

|| Injection of 5 mg silica intravenously was given 24 h before sacrifice.

¶ Mice inoculated with 10⁵ tumor cells from a transplantable MC sarcoma 15 days before sacrifice (15).

Suppression. Treatment of mice with silica (a macrophage poison) and growth of MC sarcomas in B6 mice are both associated with loss of ability to reject bone marrow allografts (8, 15). Since both the above treatments mimic the effect of ⁸⁹Sr on bone marrow allograft rejection, we tested whether the Con A response of spleen cells from silica-treated mice and MC tumor-bearing B6 mice were suppressible by FV. However, neither of these two treatments rendered B6 lymphocytes susceptible to suppression by FV in vitro (Table IV, expts. 5 and 6). The low Con A responses of spleen cells from B6 mice bearing MC sarcomas have been described previously and shown to be due to splenomegaly with T-cell dilution (20).

Discussion

Friend leukemia virus, which can cause a rapidly developing erythroleukemia in genetically susceptible adult mice, has now been shown to be capable of suppressing the mitogenic response of T and B cells from lymphoid organs of adult susceptible mice. The observation that the FV-induced suppression was sensitive to heat and UV irradiation, two well-known methods of inactivating viruses, indicates that suppression of mitogenic response was probably not an effect of soluble factors also present in FV containing spleen cell extracts. Since UV irradiation prevents replication of the virus without destroying its ability to

adsorb and penetrate the cells, it follows that fully infectious virus is necessary for suppression *in vitro*. Abrogation of suppression by type-specific Friend antiserum makes it very unlikely that the suppression observed could be due to contaminating viruses in our FV stocks.

There are conflicting reports in the literature regarding the ability of murine leukemia viruses (MuLV) to suppress immune responses *in vitro*. In some other studies, FV failed to suppress humoral antibody synthesis *in vitro* (21) and certain FV preparations failed to suppress the Con A response of lymphocytes (17). However, clarified FV preparations directly suppressed humoral antibody synthesis against sheep red cells *in vitro* (22). Other MuLV stocks have been shown to suppress PHA responses of lymphocytes *in vitro* (23). Our FV preparations are consistently active in this assay. The inability of some investigators to detect suppression of lymphocyte functions by FV *in vitro* cannot be clearly explained. It is interesting to note that other investigators could detect suppression of immune responses and Con A mitogenesis *in vitro* by using unwashed Friend erythroleukemic cells, leading them to conclude that leukemic cells were suppressors (17). Prior treatment of FV leukemic cells with X rays or mitomycin C did not abrogate their suppressive effects (17) but treatment with anti-FV serum (without complement) did abolish the suppression (22). This supports our view that the virus *per se* and not leukemic cells are responsible for suppression. Experiments reported here using mixtures of washed and unwashed leukemic cells indicate that virus present in the washings of the erythroleukemic cells and not the cells themselves are suppressors. We deliberately chose to test the possible suppressor effects of spleen cells from early (4 days) as well as late leukemic mice. The reasons for testing spleen cells from mice infected 4 days earlier with FV were twofold. Within 4 days after infection, B-cell differentiation is already suppressed (24). In addition, early after infection, transformation of erythropoietic cells would have occurred (9) without a significant reduction in the frequency of Con A-responsive T cells. This would provide a natural mixture of transformed cells (candidate suppressor cells) and T cells (targets of suppression). Since the response of spleen cells from 4-day infected mice returned to normal after the cells were washed (Fig. 1), the low Con A response of unwashed cells could be attributed to extracellular material. However, this experiment alone does not exclude a role for cell-mediated suppressive effects at later times when the frequency of leukemic cells is greater. It was therefore necessary to test spleen cells from mice in later stages of leukemia, when the great majority of cells are known to have been transformed. The fact that washed spleen cells even from 8-, 12-, and 21-day infected animals failed to suppress normal T-cell response to Con A excludes the possibility that suppression can be mediated by Friend erythroleukemic cells alone without the intervention of cell-free factors. Furthermore, since thymus and lymph node, unlike spleen and bone marrow cell populations, do not include erythropoietic progenitor cells (the target cells for transformation) it follows that FV can exert biologic effects directly on immunocompetent cells without an intervening step of leukemic transformation.

From investigations of the effect of FV on B and T cells from several strains of mice, it became clear that the mitogenic responses of lymphoid cells from mice

resistant to FV leukemogenesis in vivo ($Fv-2^{rr}$) were also relatively resistant to FV-induced suppression in vitro. Table III shows that the differences between susceptibility and resistance in vitro could not be related to the $H-2$ genotype. The data is consistent with the possibility that $Fv-2$ controls the in vitro resistance of lymphoid cells to FV. However, since C58, B10.D2, and B6 mice belong to the same ancestry group of mouse strains (25), genes other than $Fv-2$ may control this property of lymphoid cells in vitro. Further genetic analysis using (DBA \times B10.D2)F₁ intercross and backcross mice has indeed indicated that the $Fv-2$ gene does not control the resistance of mitogen-responsive lymphocytes to FV.

The most significant findings in the present set of experiments relate to the abrogation of in vitro genetic resistance of B6 T cells to FV by prior treatment of donor mice with ^{89}Sr , which selectively abrogates their M-cell function without affecting any known T, B, or accessory cell function. The lack of resistance in both ^{89}Sr -treated adult and untreated infant B6 mice suggests that the genetic resistance of spleen and thymus cells in vitro to FV depends upon functional M cells. Alternatively, ^{89}Sr may have altered the sensitivity of mitogen-responsive lymphocytes to FV. This latter possibility has been tested and excluded in the accompanying paper (11).

Treatment of B6 mice with silica or MC sarcomas suppressed marrow allograft rejection but did not weaken their genetic resistance to FV-induced suppression in vitro (Table IV). Such B6 mice also remain resistant to FV leukemia in vivo (unpublished observations). In this respect, the effect of ^{89}Sr treatment is distinguishable from the effects of silica- and MC sarcomas. Such data suggest as one possibility that resistance to FV is mediated by M cells suppressible by ^{89}Sr and not by macrophages affected by silica. Alternatively, M cells may be a subpopulation of macrophages and the differences between the effects of ^{89}Sr and silica may be quantitative rather than qualitative. ^{89}Sr prevents the generation of all cells that need to differentiate in the marrow while silica kills differentiated macrophages capable of phagocytosis.

What is the possible relevance of these findings to Friend virus leukemogenesis in vivo? Mice of strains highly susceptible to erythroleukemia induction by FV are severely immunosuppressed in vivo (26) and their lymphocytes responsive to mitogens are suppressed in vitro (Table III). B6 mice, which are highly resistant to erythroleukemia and are resistant to the immunosuppressive effects of FV in vivo (3), have lymphocytes which are not suppressed in vitro by FV to any great extent (Table III). Furthermore, treatment of B6 mice with ^{89}Sr weakens the resistance of B6 mice to the malignant erythropoietic and to the immunosuppressive effects of FV in vivo (4) and to the suppressive effect of FV in vitro (Table IV). These correlations suggest that the in vitro findings are relevant to Friend disease. Since the $Fv-2$ gene does not control the property of resistance of mitogen-responsive lymphocytes to FV in vitro,² it would be desirable to determine the resistance of $Fv-2^{ss}$ mice congenic for this "resistance" gene of the B6 strain to FV leukemogenesis in vivo. We are breeding such mice at the present time. The data in Table III indicates that the gene(s) controlling this property of lymphocytes in vitro is not linked to the $H-2$ genetic region. Recovery of mice from FV-induced splenomegaly appears to be influenced

favorably by functions of multiple genes of the C57BL/10 mice not linked to *H-2* (27). The gene(s) controlling resistance of lymphocytes to FV in vitro could be partly responsible for that recovery. It appears reasonable to consider that a gene protecting against the immunosuppressive effects of FV would also contribute to recovery from its pathological effects.

The mechanism by which M cells confer resistance to in vitro suppression of mitogenesis by FV are not entirely clear. In the following paper, we present evidence to indicate that M cells could act by regulating suppressor T cells. These suppressor cells, upon activation by FV infection in vitro, suppress the Con A-responsive T cells which are not directly sensitive to FV. In resistant mice, the function or numbers of suppressor T cells may be under the regulatory influence of M cells.

Summary

Friend leukemia virus suppresses the proliferative responses of normal thymus-dependent (T) and bursa equivalent-dependent (B) lymphocytes from spleen, thymus, lymph node, and bone marrow to mitogens. The suppressive effect of Friend virus complex (FV) requires fully infectious virions. Friend erythroleukemic cells, washed to remove extracellular virus, fail to suppress concanavalin A (Con-A)-induced mitogenesis of normal spleen cells. This indicates that FV does not mediate its immunosuppressive effect via transformed erythropoietic cells.

The in vitro suppressive effect of FV on lymphocyte mitogenesis is under host genetic control. Spleen, bone marrow, and thymus cells from strains of mice susceptible to FV-induced leukemogenesis in vivo were quite susceptible to the suppressive effects of FV in vitro. On the other hand, similar cells from strains of mice such as C57BL/6 resistant to Friend erythroleukemia, were quite resistant to in vitro immunosuppression by FV. Mitogenesis of splenic T cells from resistant B6 mice, previously treated with ^{89}Sr , became susceptible to suppression by FV. This indicated that the in vitro resistance of lymphocytes to FV-induced suppression is not an intrinsic property of T cells, but is controlled by marrow-dependent (M) cells which are selectively eliminated by treatment with ^{89}Sr . M-cell function does not develop in mice less than 3-wk old. The Con A response of thymus cells from 2-wk-old B6 mice was susceptible to suppression by FV, further supporting the concept that M cells may regulate the genetic resistance to FV.

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