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Susceptibility of Friend Erythroleukemia Cells to Natural Cytotoxicity after *In Vitro* Treatment with Dimethyl Sulfoxide (41354)

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Abstract. Growth of a continuous cell line of Friend erythroleukemia cells (FLC) in medium containing 2% dimethyl sulfoxide (DMSO) resulted in a marked increase in susceptibility to natural cell-mediated cytotoxicity by spleen cells from unimmunized mice. The transition of FLC to sensitive targets was not observed until 2 days after culture initiation in DMSO. Cytolytic reactivity was not restricted by products of the major histocompatability complex since both syngeneic and allogeneic effector cells responded similarly. Preincubation of effector cells in mouse fibroblast interferon augmented the cytotoxic reactivity in a dose-dependent manner against DMSO-grown FLC but had no influence on the ability of spleen cells to lyse untreated FLC. The adherence properties of effector cells on nylon wool, glass wool, and plastic surfaces were consistent with those of macrophages. Amphotericin B seemed also to be required for the transition of FLC to sensitive targets. The increased susceptibility of the DMSO-treated leukemia cells to natural cytotoxicity may be due to enhanced recognition by splenic macrophages.

Leukemias induced in experimental animals by retroviruses are widely studied as models for oncogenic processes and immunosuppression associated with neoplasia (1–3). Friend leukemia virus (FLV) induces a fatal erythroblastic leukemia in susceptible mouse strains (4, 5). Mice infected with this virus have been used as a source of readily transplantable erythroleukemia cell lines. Such Friend leukemia cell (FLC) lines have been widely studied in regard to physiological and oncogenic properties (6–8). Many FLC lines can be induced to differentiate along the erythroid pathway by cultivation for several days in medium containing 2% dimethyl sulfoxide (DMSO). Differentiation of FLC is characterized by *de novo* synthesis of hemoglobin (6, 9) and expression of erythrocyte membrane antigens (10, 11). Since DMSO is thought to affect cell surface characteristics and antigenicity, it was of interest to determine whether DMSO-induced changes in FLC would alter the sensitivity of these normally resistant cells to natural cytotoxicity by spleen cells from unimmunized syngeneic or allogeneic mice. As demonstrated in this report, cultivation of FLC (clone GM979) in medium containing 2% DMSO resulted in a marked susceptibility of the cells to natural cytotoxicity mediated by adherent spleen cells from normal mice.

Methods and Materials. *Mice.* Inbred DBA/2 mice (H-2^d) of either sex were obtained from Jackson Laboratories, Bar Harbor, Maine, and were maintained under specific pathogen-free conditions on mouse chow and water *ad libitum*. For some experiments, A/J (H-2^a), C57B1/6 (H-2^b), and BALB/c (H-2^d) mice (Jackson Lab.) were also used as a source of effector cells.

Tumor cell lines. The Friend erythroleukemia cell line, GM979, derived from DBA/2 mice, was the kind gift of G. H. Lyman (Department of Internal Medicine, Univ. of South Florida). These cells were maintained *in vitro* by cultivation in basal medium Eagle's (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 15% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin (Flow Laboratories, Rockville, Md.), and 2.5 μg/ml amphotericin B (Flow). YAC-1 lymphoma cells, described elsewhere (12), were maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum and 100 units/ml penicillin–100 μg/ml streptomycin.

Cytotoxicity assay. Specific cytotoxicity

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was determined by a modification of a previously described procedure (13). Briefly, the spleens of at least three mice per group were teased into single cell suspensions in HEPES-buffered RPMI supplemented with 10% fetal calf serum, 5×10^{-5} *M* 2-mercaptoethanol, and antibiotics. Erythrocytes were removed by lysis in 0.86% NH_4Cl. After washing, the effector cells were assessed for viability using a hemacytometer and trypan blue dye exclusion and were then adjusted to cell densities that yielded the appropriate effector to target ratios. In most studies, 4-day-old cultures of DMSO-treated or untreated GM979 cells or cultures of YAC-1 were labeled for 1 hr in $Na_2{}^{51}CrO_4$ (New England Nuclear, Cambridge, Mass.) and adjusted to 10^5 viable cells/ml. One hundred microliters of effector cells were added to the individual wells of microtiter plates (Nunclon, Roskilde, Denmark) containing 100 μl of ^{51}Cr-labeled target tumor cells. The plates were centrifuged at 250*g* for 2 min and then incubated for 18 hr at 37° in a humidified atmosphere of 95% air/5% CO_2. To terminate the assay, the plates were centrifuged at 4° for 10 min at 500*g* and the amount of released radioactivity in 100 μl of supernatant was determined by liquid scintillation spectrometry. The percentage cytotoxicity was calculated from the formula: $(EXP_{cpm} - SR_{cpm}/MAX_{cpm} - SR_{cpm}) \times 100$, where spontaneous release (SR) was the amount of radioactivity released from target cells incubated in the absence of effector cells over the assay period and the maximum counts (MAX) were determined after the addition of 100 μl of 10% sodium dodecyl sulfate to 10^4 ^{51}Cr-labeled target cells. Representative experiments are presented since daily variation in basal levels of cytotoxicity were encountered; however, replicate experiments always yielded similar results. Within any single experiment, standard deviations were less than 2% and are therefore, not included in the data shown. Spontaneous release of ^{51}Cr from target cells was always less than 20% of the maximum ^{51}Cr release.

Interferon activation of effector cells. Spleen cells, 10^7/ml, were pulsed for 1 hr with various doses of mouse fibroblast interferon, Mu IFN-β (Calbiochem–Behring, La Jolla, Calif.), and incubated at 37° for 1 hr. The effector cells were then washed free of unbound interferon and assayed for cytotoxic activity.

Nylon and glass wool fractionation of spleen cells. Spleen cells, $1.0-1.5 \times 10^8$ in 2.0 ml RPMI 1640 were layered over 1.5–2.0 g of prewarmed nylon or glass wool and washed into the columns with 2.0 ml of warm medium (14). After 30 min of incubation at 37°, 5% CO_2 the nonadherent cells were eluted from the columns with 25 ml of warm RPMI 1640.

Fractionation by plastic adherence. Spleen cells, 5×10^7 in 10 ml RPMI 1640, were incubated in 60 × 100-mm plastic petri dishes for 30 min at 37°, 5% CO_2. Nonadherent cells were decanted and saved. The monolayers were then washed three times with warm medium. The wash fractions were discarded. Adherent cells were scraped from the plastic surface using a rubber policeperson.

Experimental Results. Growth of FLC for 4 days in medium containing 2% DMSO resulted in a marked increase in the susceptibility of these cells to the natural cytotoxic reactivity of normal, unimmunized syngeneic spleen cells (Fig. 1). Whereas untreated FLC were completely resistant to natural cytotoxicity, DMSO-treated FLC demonstrated a similar sensitivity to natural cytotoxicity as YAC-1 in 18-hr assays. The combined results of five such experiments indicate that the degree of killing of DMSO-treated FLC at 50:1 was significantly different ($P < 0.001$) from untreated control FLC (Student's *t* test). However, the results of 4-hr ^{51}Cr release assays failed to demonstrate specific cytotoxicity of DMSO-treated FLC although YAC-1 was found to be exquisitely sensitive (data not shown).

The transition of resistant FLC to targets which were sensitive to natural cytotoxicity was observed on the second day of cultivation in 2% DMSO (Fig. 2). FLC remained sensitive to killing when cultured in 2% DMSO up to 4 days. In contrast, FLC cultured up to 24 hr in medium containing DMSO, or cultivated up to 4 days in

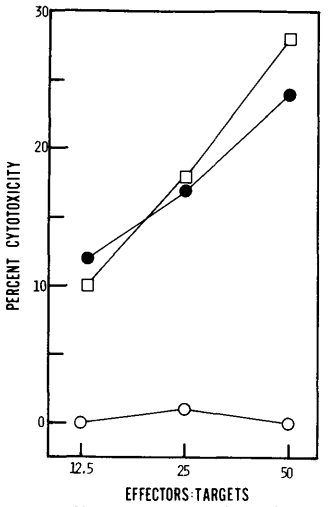

FIG. 1. The effect of growth in 2% DMSO on the susceptibility of GM979 to the natural cytotoxicity of DBA/2 spleen cells. GM979 cells were cultured 4 days in medium alone (○) or in medium containing 2% DMSO (●) before assessing susceptibility to natural cytotoxicity in 18-hr ^{51}Cr release assays. YAC-1 (□), highly sensitive to both natural killer cell- and macrophage-mediated cytotoxicity was also included.

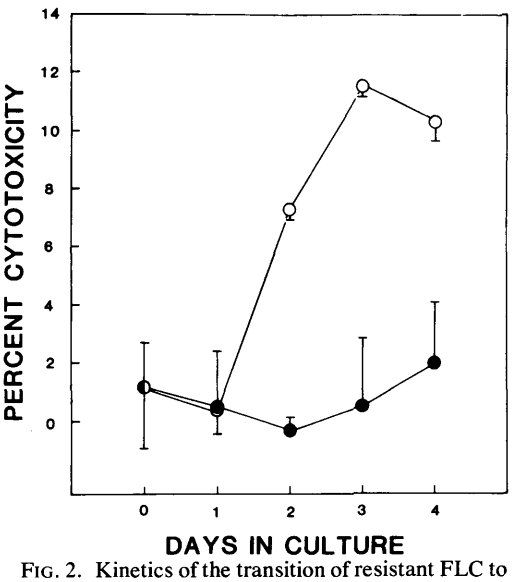

FIG. 2. Kinetics of the transition of resistant FLC to sensitive targets of natural cytotoxicity of unfractionated DBA/2 spleen cells. FLC were cultivated for the indicated times in the presence (●) or absence (○) of 2% DMSO. The effector to target cell ratio is 50:1 in an 18-hr assay.

medium lacking DMSO remained resistant. The results of this study suggested that the susceptibility of DMSO-treated FLC to natural cytotoxic activity of unimmunized spleen cells was not merely the result of chemical alterations in the target cell surface, but may have arisen as a consequence of cell-directed membrane alterations that accompanied cell differentiation.

Susceptibility of FLC to natural cytotoxicity after treatment with DMSO was not restricted to syngeneic effector cells. As is evident in Fig. 3, increased lysis occurred when DMSO-treated FLC were incubated with spleen cells from A/J or C57Bl/6 mice which differed from GM979 at the major histocompatability complex (MHC), or from BALB/c mice which are similar to DBA/2 in terms of histocompatibility. The results of this study also indicated that the strains of mice tested showed a similar level of cytotoxic reactivity against DMSO-treated FLC but not YAC-1.

Interferon has been shown to augment natural killer cell activity as well as macrophage-mediated cytotoxicity in a wide variety of model systems (15–17). Thus, it was of interest to determine if interferon pretreatment of normal mouse spleen cells would overcome their inability to lyse FLC which had not been cultivated in DMSO. Incubation of spleen cells in various doses of mouse fibroblast interferon for 1 hr resulted in a dose-dependent enhancement of natural cytotoxicity when susceptible targets were used in cytotoxicity assays (Fig. 4). However, reactivity against the resistant, untreated FLC remained unaltered.

To characterize the effector cell populations responsible for mediating natural cellular cytotoxicity against YAC-1 and DMSO-treated FLC, spleen cells were fractionated based on adherence properties and tested for their ability to kill ^{51}Cr-labeled target cells in 18-hr assays (Table I). Spleen cell suspensions depleted of cells that adhered to nylon wool, glass wool, or plastic were enriched in effector cells capable of lysing YAC-1 but not DMSO-cultivated FLC. In fact, two of these fractions were virtually devoid of effector cells

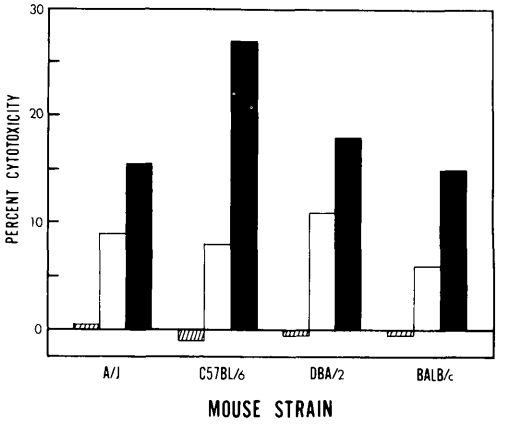

FIG. 3. The natural cytotoxicity of DMSO cultivated GM979 cells is not restricted by histocompatibility products. The spleen cells of inbred mice of different genetic backgrounds were tested in 18-hr assays for natural cytotoxic reactivity toward GM979 cells cultured in medium (▨), medium containing 2% DMSO (□), or against YAC-1 lymphoma cells (■). Although tested at three different effector to target ratios, only the results of the 50:1 cultures are shown.

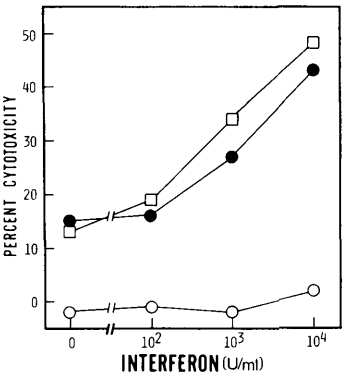

FIG. 4. Preincubation of DBA/2 splenic effector cells in various doses of mouse fibroblast interferon fails to elicit natural cytotoxicity against GM979 cells grown in medium alone (○). In contrast, the enhancement of natural cytotoxicity of DMSO grown GM979 (●) and YAC-1 (□) by interferon preincubation is dose dependent. The results shown are representative of 18-hr cytotoxicity assays with effector to target ratios of 50:1.

of FLC cytolysis. In contrast, enrichment of effector cells for FLC targets could only be demonstrated in the plastic-adherent fraction (nylon- or glass wool-adherent cells were not tested). Thus, the effector cells responsible for lysing YAC-1 or DMSO-treated FLC appeared to reside in different splenic populations. Furthermore, the adherence properties of the effector population mediating lysis of DMSO-treated FLC were consistent with those of macrophages.

It is also important to note the requirement for amphotericin B as well as DMSO in the culture medium in order to cause the alteration in FLC from resistance to sensitivity to natural cytotoxicity (Table II). Growth of FLC for 4 days in medium containing 2% DMSO or amphotericin B had no effect on target cell sensitivity. However, when FLC were grown in medium containing both 2% DMSO and amphotericin B, the FLC were lysed.

Discussion. The results of these studies indicate that a continuous cell line of mouse erythroleukemia cells transformed initially by FLV are quite resistant to natural cell-mediated cytotoxicity of normal syngeneic or allogeneic spleen cells but after treatment with 2% DMSO and 2.5 μg/ml amphotericin B for 4 days *in vitro*, acquire the property to be lysed by spleen cells from unimmunized mice. DMSO treatment of murine erythroleukemia cells is known to cause differentiation so that hemoglobin is synthesized and the cells acquire some of the characteristics of erythrocytes, including new cell surface markers. The nature and mechanism whereby DMSO causes these changes in leukemia cell populations is not known but is under active investigation in many laboratories. It is apparent, however, from the results of this study that DMSO-treated leukemia cells can become good targets for lysis by normal spleen cells. Although DMSO-treated FLC may be lysed to an extent similar to that of YAC-1 in 18-hr assays, the inability of splenic effector cells to kill FLC in 4-hr assays suggests that the effector cells are different for the two target cell lines used in these studies. Moreover, our results indicate that passage of spleen cells over nylon wool or glass wool columns effectively eliminates the effector cells responsible for the lysis of the DMSO-treated FLC while enriching for

TABLE I. ADHERENCE PROPERTIES OF SPLENIC EFFECTOR CELLS MEDIATING NATURAL CYTOTOXICITY OF YAC-1 AND DMSO-TREATED FRIEND ERYTHROLEUKEMIA CELLS

	Percentage cytotoxicity			
	YAC-1		FLC[a]	
Fractionation[b]	12.5:1	50:1[c]	12.5:1	50:1
None	10	23	7	14
Nylon wool column[c]	19	33	−2	0
Glass wool column[c]	12	25	−2	2
Plastic nonadherent[d]	13	31	4	12
Plastic adherent[d]	6	20	12	17

[a] FLC were cultivated 4 days in medium containing 2% DMSO.

[b] Single cell suspensions of DBA/2 spleen cells were briefly exposed to 0.86% NH_4Cl to remove RBCs, assessed for viability by trypan blue dye exclusion, and diluted to the appropriate cell densities prior to fractionation. After fractionation, cells were counted, cell densities adjusted, and 100 μl added to 100 μl RPMI 1640 containing 10^4 ^{51}Cr-labeled target cells.

[c] 1.0–1.5 × 10^8 spleen cells in 2.0 ml RPMI were layered over 1.5–2.0 g of washed wool (nylon or glass) and washed into the columns with 2.0 ml warm RPMI. After 30 min incubation at 37°, 5% CO_2, the nonadherent cells were eluted with 25 ml warm RPMI.

[d] Spleen cells, 5 × 10^7 in 10 ml RPMI, were incubated in 60 × 100-mm plastic petri dishes for 30 min, 37°, 5% CO_2. Nonadherent cells were decanted and the monolayers were then washed three times with warm RPMI. The wash fraction was discarded. Adherent cells were scraped from the plastic surface using a rubber policeperson.

[e] Effector to target cell ratios.

TABLE II. REQUIREMENT FOR AMPHOTERICIN B IN THE DMSO-TRIGGERED TRANSITION FROM RESISTANCE TO SENSITIVITY OF FLC TO NATURAL CYTOTOXICITY

Target Cell Growth Conditions	Percentage cytotoxicity[a]		
	12.5:1	25:1	50:1[f]
Medium only[b]	1	0	1
Medium + DMSO[c]	1	−1	1
Medium + Amp B[d]	0	−1	−1
Medium + DMSO + Amp B[e]	6	7	9

[a] 18-hr assays.

[b] BME containing 15% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

[c] Medium as in *b* plus 2% DMSO.

[d] Medium plus 2.5 μg/ml amphotericin B.

[e] Medium plus 2% DMSO and 2.5 μg/ml amphotericin B.

[f] Effector to target cell ratios.

cells responsible for the lysis of YAC-1. Thus DMSO-treated FLC appear to be lysed by macrophages.

There are no dramatic differences in the sensitivity of DMSO-treated FLC to spleen cells from syngeneic mice (DBA/2), mice sharing the same major histocompatibility background (BALB/c), or to allogeneic mice (A/J or C57B1/6). This demonstrates that recognition and killing of DMSO-treated FLC were independent of MHC gene products as well as to levels of natural killer cell activity attributed to different mouse strains. In addition, since both male and female mice were used in these studies without demonstrable differences, the sex of the source of effector cells was ruled out as a major consideration for the differences observed in sensitivities of FLC to natural cytotoxic reactivity (data not shown).

Interferon enhanced the natural cell-mediated cytotoxicity of DBA/2 spleen cells in a dose-dependent fashion against both YAC-1- and DMSO-treated FLC targets but not untreated FLC. This demonstrates that the difference in sensitivity to natural cytotoxicity by treated and untreated FLC is not attributable to the selective ability of DMSO-treated FLC to affect interferon formation by spleen cells during the assay period.

It thus appears that recognition of target cells is a major factor influencing the susceptibility of FLC to natural cytotoxicity. In turn, the state of differentiation (DMSO treated or untreated) may play a vital role in regulating the phenotypic change in FLC leading to enhanced recognition by effector cells. The mechanism of FLC surface changes resulting in enhanced recognition is not known but may involve the expression of new plasma membrane structures (11) or the unmasking of preexisting structures normally not available for interaction with effector cells. It is also important to note that amphotericin B is used in the culture medium in which the FLC are grown. The evidence indicates that the presence of this antibiotic is necessary for alteration of

the leukemia cells to the state of sensitivity to natural cell-mediated cytotoxicity. The role of this antibiotic in combination with DMSO is not understood; although used alone, it has no effect on this system. Similarly, it has been recently reported (18) in preliminary studies that mouse L929 cells treated with subinhibitory concentrations of actinomycin D acquire sensitivity to cytotoxicity by endotoxin-stimulated macrophages of normal syngeneic mice. In the absence of this metabolic inhibitor, no lysis occurred.

The role of antibiotics (especially amphotericin B) and differentiation-inducing compounds leading to enhanced sensitivity of FLC to natural cytotoxicity are under current investigation in this laboratory.

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