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Dimethyl Sulfoxide-Induced Inhibition of the Immunosuppressive Activity of Cultured Friend Erythroleukemia Cells (41874)

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Abstract. Two Friend leukemia virus-induced tumor cell lines lost their immunosuppressive properties *in vitro* when treated with dimethyl sulfoxide (DMSO), a known cellular differentiation agent. Incubation of the cell lines, GM 979 and GM 86, with DMSO for 4 days or longer, inhibited their ability to suppress the antibody forming capacity of normal murine spleen cells immunized *in vitro* with sheep red blood cells. Suppression of the inhibitory capability of the tumor cell lines by DMSO was time dependent. Three days incubation caused only slight, if any, inhibition, while a shorter period of treatment had no effect. Inhibition of the immunosuppressive properties of the tumor cell lines was not due to a decrease in tumor cell viability. The development of previously reported metabolic alterations in the treated cells, such as increased hemoglobin synthesis and other physicochemical alterations, paralleled cellular differentiation, and loss of immunosuppressive properties.

Oncogenic retroviruses such as the Friend leukemia virus (FLV) complex are known to have marked immunodepressive activities in susceptible mice (1–3). Acquired immunodeficiency induced in genetically susceptible animals by leukemia viruses is characterized by markedly depressed antibody responses following immunization with a wide variety of T-cell-dependent or -independent antigens. Cell-mediated immune responses also may be depressed in susceptible animals infected with such viruses. Leukemia virus-transformed cell lines have been established and are widely utilized in studies concerning the mechanisms of leukemogenesis. In this regard, a number of FLV-induced cell lines have been characterized in depth in terms of their biologic and physiologic activities (4, 5). Several of these cell lines, when incubated with relatively small concentrations of dimethyl sulfoxide (DMSO), a known differentiation agent, acquire some of the characteristics of normal erythroblastoid cells, including the ability to produce hemoglobin (6–9). DMSO treatment of such leukemic cells for 4 days results in differentiation of the cells and loss of many of their tumorigenic properties. Previous studies in this laboratory had shown that FLV-induced erythroleukemia cells from mouse spleens, when injected into normal susceptible animals or added to cultures of normal mouse spleen cells, markedly depressed antibody responses to antigens such as sheep red blood cells (2, 3). As shown in the present study, incubation of FLV induced cell lines with DMSO reversed their immunosuppressive properties for normal mouse spleen cells.

Methods and Procedures. *Animals.* BALB/c mice, 6–8 weeks old, were purchased from Jackson Laboratories, Bar Harbor, Maine. They were fed Purina mouse pellets and water *ad libitum* and kept in groups of 6–8 in plastic mouse cages.

Antibody assay. The *in vitro* induction of antibody-forming cells was performed as described previously (10). In brief, dispersed spleen cell suspensions were prepared in RPMI 1640 medium, fortified with 10% fetal bovine serum, plus 100 U penicillin and 100 μg streptomycin/ml medium (complete RPMI). The cells were washed once by centrifugation at 500*g* for 10 min at 4°C and suspensions of 5×10^6 nucleated spleen cells were placed in 1.0 ml of medium in individual wells of Linbro 24-well culture plates. To each culture was added 0.2 ml of a 0.1% suspension of washed sheep red blood cells (SRBC) so that each culture was immunized with 2×10^6 erythrocytes. The plates were incubated in an atmosphere of 5% CO_2, 95% air in a humidified chamber at 37°C for 5 days. At that time the number

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of antibody plaque-forming cells (PFC) in each well was determined by a standard Cunningham plaque assay (11). The average number of PFC per culture for three or more cultures was calculated.

Leukemic cells. FLV-induced leukemia cells, i.e., cell line GM 979 and cell line GM 86, were obtained initially through the courtesy of Dr. Gary Lyman, Division of Oncology, University of South Florida College of Medicine. The cell cultures were maintained *in vitro* by frequent passage in complete RPMI medium. Graded concentrations of each cell suspension were added to cultures of normal mouse spleen cells being immunized with SRBC. For differentiation, cell cultures were incubated at a concentration of 1×10^5 nucleated leukemic cells per milliliter in complete RPMI containing 2% DMSO for the various time periods defined in each experiment. Following treatment with DMSO, the cells were washed three times by centrifugation at 250*g* for 10 min at 4°C with medium and resuspended to the appropriate concentration in complete medium without DMSO.

Experimental design. The effect of DMSO on the ability of the leukemia cells to depress the antibody response of normal spleen cells was determined by coculturing the tumor cells in graded numbers with optimum numbers of normal spleen cells being immunized with SRBC. The effect of length of incubation of the tumor cells with DMSO on their immunosuppressive properties was determined. Following DMSO treatment, the tumor cell lines were examined for viability by microscopic examination, using trypan blue dye exclusion and a hemocytometer.

Experimental Results. The two leukemia cell lines used for the study were markedly immunosuppressive for normal mouse spleen cells *in vitro*. As is evident in Table I, coculture of graded numbers of GM 979 or GM 86 cells with 5×10^6 normal mouse spleen cells resulted in a dose-related suppression of the expected antibody response. Whereas normal spleen cell cultures, without added leukemia cells, developed large numbers of PFC when immunized *in vitro* with SRBC, cultures containing as few as 10^5 tumor cells (a ratio of 1 tumor cell to 50 normal cells) showed a consistent but noticeable depression in the expected number of PFC. A 5- to 10-fold larger number of tumor cells (ratios of 1:10 or 1:5) decreased the antibody response consistently to levels that were significantly lower than

TABLE I. ALTERATION OF *IN VITRO* ANTI-SRBC RESPONSE OF NORMAL MOUSE SPLEEN CELLS COCULTURED WITH FRIEND ERYTHROLEUKEMIA CELLS

Addition to cultures[a]	Antibody response[b]			
	Experiment No. 1		Experiment No. 2	
	PFC/culture	Percentage of control	PFC/culture	Percentage of control
None (control)	50 ± 17	—	88 ± 38	—
SRBC only	492 ± 82	—	1575 ± 139	—
Plus GM 979 10^4	613 ± 338	125	1100 ± 118	70
5×10^4	—	—	950 ± 201	62
10^5	513 ± 38	104	900 ± 113	57 (<0.002)
5×10^5	50 ± 5	10 (<0.05)[c]	267 ± 96	17 (<0.005)
10^6	0	0 (<0.02)	0	0 (<0.001)
Plus GM 86 10^4	325 ± 50	68	1542 ± 137	98
5×10^4	113 ± 38	23 (<0.05)	1540 ± 186	98
10^5	100 ± 50	20 (<0.005)	1467 ± 131	93
5×10^5	13 ± 13	3 (<0.02)	533 ± 145	34 (<0.001)
10^6	13 ± 10	3 (<0.02)	33 ± 8	2 (<0.001)

[a] Cultures of 5×10^6 spleen cells from normal mice immunized *in vitro* with 2×10^6 SRBC and incubated with indicated number of leukemia cells.

[b] Average PFC response, ±SD, for three cultures per group 5 days after *in vitro* culture initiation.

[c] Significance determined by Student's *t* test (two tailed).

controls. In contrast, when up to 10^6 tumor cells which were cultured for 4 days with 2% DMSO were added to normal spleen cell cultures, many more PFC developed (Table II). For example, addition of 10^5 DMSO-treated leukemia cells of either cell line to normal spleen cell cultures resulted in essentially no decrease in the number of antibody producing normal splenocytes. Although 10^6 untreated GM 979 cells severely suppressed the antibody response of normal spleen cell cultures, after treatment for 4 days *in vitro* with DMSO the same number of GM 979 cells had no depressive effect and actually resulted in some enhancement of the response (Table II). Although the GM 86 cells before treatment with DMSO were also markedly immunosuppressive, after treatment with DMSO they only partially suppressed the antibody response.

No difference between the number of viable leukemic cells was noted in either tumor cell preparation, regardless of whether or not they were first treated with DMSO. Although the GM 979 cell line appeared to be somewhat more suppressive in some experiments, both tumor cell lines had similar suppressive effects for normal spleen cells, since larger numbers completely suppressed antibody forming capacity of normal spleen cells while smaller numbers had less effect.

The ability of DMSO to reverse the suppressive effects of the leukemia cells depended upon the length of time the cells were exposed to this agent. Previous studies had shown that 2% DMSO was an optimum concentration for inducing cellular differentiation and hemoglobin formation (4). In the present study, hemoglobin was induced to a sufficient concentration so that cell pellets were light red after 4 days in 2% DMSO. Lesser amounts had little or no effect, while larger concentrations did not increase the effect. Similarly, previous studies had shown that DMSO treatment required a minimum of 3–4 days to result in normal amounts of hemoglobin synthesis and loss of tumorigenic properties. As is evident in Fig. 1, cultures treated for 4 days or longer *in vitro* with DMSO had the least immunosuppressive activity when added to normal spleen cells *in vitro*. Incubation of cultures with DMSO for 3 days resulted in much less or no effect on the immunosuppressive activity of the cells. Incubation of the tumor cells for 4–6 days resulted in the greatest loss of suppressive activity when treated leukemic cells were cocultured with normal spleen cells.

Discussion. FLV induced cell lines GM 979 and GM 86 were markedly immunosuppressive for normal spleen cells immunized *in vitro* with SRBC. DMSO resulted in a loss of their

TABLE II. EFFECT OF DMSO-INDUCED DIFFERENTIATION OF FRIEND ERYTHROLEUKEMIA CELLS ON *IN VITRO* SUPPRESSION OF ANTI-SRBC PFC RESPONSES OF NORMAL MOUSE SPLEEN CELLS

Addition to cultures[a]		Antibody response[b]			
		Untreated		DMSO-treated	
		PFC/culture	Percentage of control	PFC/culture	Percentage of control
None (control)		467 ± 82	—	—	—
SRBC only		1910 ± 21	100	—	—
Plus GM 979	10^4[c]	2692 ± 448	144	2259 ± 412	118
	10^5	1596 ± 176	84	1971 ± 264	103 (<0.005)[d]
	10^6	63 ± 31	3	2521 ± 364	131 (<0.025)
Plus GM 86	10^4	2321 ± 216	122	2117 ± 178	111
	10^5	879 ± 214	46	1829 ± 170	96 (<0.05)
	10^6	4 ± 4	1	584 ± 4	31 (<0.001)

[a] Indicated numbers of Friend leukemia cells added to cultures of 5×10^6 normal mouse spleen cells immunized *in vitro* with 2×10^6 SRBC.

[b] Average number of PFC, ±SD, for three cultures per group 5 days after *in vitro* culture initiation.

[c] Friend erythroleukemia cells cultured for 4 days with 2% DMSO.

[d] Significance determined in comparison to responses of cultures incubated with untreated leukemia cells by Student's *t* test (two tailed).

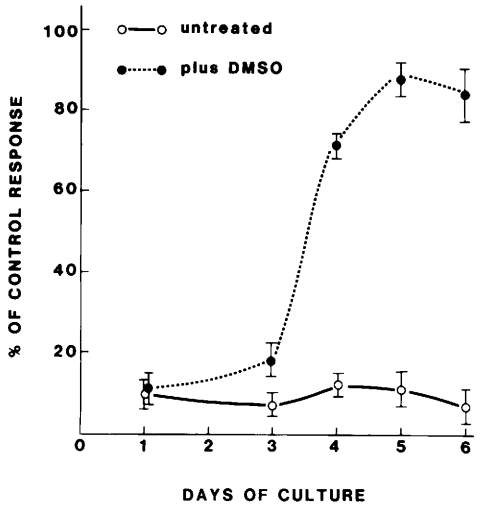

FIG. 1. Effect of DMSO treatment on GM 979 cell line *in vitro* for various lengths of time on immunosuppressive activities for normal mouse spleen cells. Each point represents the average anti-SRBC PFC response ± SD, 5 days after *in vitro* immunization of cultures of 5×10^6 spleen cells from normal mice incubated with 10^5 GM 979 cells, either untreated (solid line) or cultured for the indicated number of days with 2% DMSO (dashed line).

ability to suppress the antibody-forming capacity of normal spleen cell cultures. Previous studies had shown that DMSO treatment of various leukemia cell lines caused differentiation of the cells; hemoglobin synthesis became evident and histologically the cells appeared to be more like those of normal erythroblastoid cells (6, 7). Similarly, DMSO treatment of leukemic cells for 4 days or longer *in vitro* resulted in loss of leukemogenic properties as assessed by subsequent transfer of the cells into syngeneic animals, which failed to develop a tumor (unpublished observation). Normally a tumor results upon injection of these cell lines into susceptible syngeneic recipient mice.

Previous studies also showed that when mouse splenic FLV-induced leukemia cells were added to normal spleen cell cultures, even at ratios as low as 1:20 or 1:50, marked immunosuppression occurred (3). This was evident in the present experiments where a dose-related suppression of the normal immune response occurred. This suppression did not appear due merely to "overgrowth" of the normal lymphoid cells by the leukemia cells. Previous studies had shown that cell-free extracts or culture supernatants from the GM 979 cell line (unpublished observations) as well as from FLV-infected spleen cells per se, were markedly immunosuppressive (3). Thus, immunosuppression appeared to be associated with soluble factor(s) released from the tumor cells or possibly the virus itself. Furthermore, in coculture experiments with Millipore membranes it was evident that when the leukemia cells were grown in one chamber, separated by a cell-impermeable membrane from normal spleen cells being immunized with SRBC in a separate chamber, marked immunosuppression still occurred (12). Thus, Friend erythroleukemia tumor cells had the property of directly suppressing normal spleen cells *in vitro,* similar to the suppression induced by tumor cells or virus *in vivo.* It is interesting to note that GM 979 when examined by transmission electron microscopy, contain C-type virus particles, yet cell-free extracts do not cause leukemia in FLV-susceptible mice. This suggests that either the particles are not FLV or that they are not infectious virus. In addition, 2% DMSO treatment increased the number of particles visible by TEM (unpublished observations). Thus, it seems unlikely that the effect of DMSO was to inhibit production of virus.

In the present study it was evident that prior treatment of the leukemia cells with DMSO similar to the treatment which induces differentiation also diminished the immunosuppressive properties of the cells. It is not clear, however, whether this reduction in immunosuppressive activity was due to direct or indirect effects on virus infection of the cells, production or release of other intermediary factors, or alteration of a cell membrane component. Nevertheless, it is evident from the results of this study that differentiating leukemia cells after treatment with DMSO lost the ability to suppress antibody formation. It is possible that important metabolic activities of the leukemic cells could have been altered following treatment with DMSO or that the DMSO-treated leukemic cells became more susceptible to cell mediated lysis, even *in vitro* (13). Whether the effect of DMSO is due to cell differentiation or some other phenomenon is unclear. Thus, it will be of interest to determine whether other differentiating agents such as butyric acid have similar effects on

the immunosuppressive properties of viral induced leukemic cells.

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