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Hemoglobin Synthesis in Murine Virus-Induced Leukemic Cells *In Vitro*: Stimulation of Erythroid Differentiation by Dimethyl Sulfoxide

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ABSTRACT **Cells of a cloned line of murine virus-induced erythroleukemia were stimulated to differentiate along the erythroid pathway by dimethyl sulfoxide at concentrations that did not inhibit growth. A rise in the number of benzidine-positive normoblasts was accompanied by increased synthesis of heme and hemoglobin and a decrease in the malignancy of the cells. This action of dimethyl sulfoxide, which was reversible, may represent the derepression of leukemic cells to permit their maturation.**

The opportunity to explore the possibility that leukemia is a disease resulting from a block in the process of maturation of hematopoietic cells has been provided by established tissue culture lines of murine virus-induced erythroleukemic cells (1–3). These cells, which grow in suspension, have continued to exhibit a limited degree of differentiation along the erythroid line throughout their 4-year serial passage history. Although they synthesize hemoglobin (4, 5), they are malignant as tested by bioassay in syngeneic hosts. They produce virus which, although low in leukemogenic activity, is a highly effective immunizing agent (6).

During the course of studies to determine the effect of superinfecting these cells with Friend leukemia virus, dimethyl sulfoxide (DMSO) was added to the medium. DMSO had been demonstrated to enhance infectivity of both poliovirus RNA (7) and mengovirus RNA (8), as well as transformation by polyoma virus (9). It is also known to stabilize enveloped viruses (10). The wide range of biological activities of DMSO has been described (11). The present report describes an effect of DMSO on the differentiation of established lines of murine virus-induced leukemic cells and illustrates still another property of this compound.

In the dose–response experiments initially set up to determine the toxicity of DMSO on the leukemic cell lines, a striking effect on the differentiation of these cells was noted. Of the cells allowed to grow in medium containing 2% DMSO for 4 days, a majority of the erythroblasts had matured to normoblasts which stained benzidine-positive (B+). The increase in the number of cells maturing along the erythroid series in DMSO-containing medium was accompanied by an increase in the amount of hemoglobin synthesized.

Abbreviation: DMSO, dimethyl sulfoxide.

This study is dedicated to the memory of Dr. Austin S. Weisberger.

MATERIALS AND METHODS

The origin of the cell lines of murine Friend leukemia virus-induced leukemic cells and their clonal derivatives has been described previously (1, 2). Methods for maintaining the cell culture, cloning on semisoft agar, and the medium were also detailed in these earlier reports.

The present experiments were done on a clone designated 707, in which 1–2% of the cells are B+ (5). Cells were generally seeded at a concentration of 10^5 per ml (except where otherwise indicated) either in 32-oz. (900 ml) prescription bottles containing 60 ml of medium or in Falcon plastic Petri dishes (60 × 15 mm) containing 10 ml of medium. Dehydrated Eagle's basal medium diluted with Earle's balanced salt solution and supplemented with 15% fetal calf serum was used. The cultures were grown in a humidified incubator containing 5% CO_2 in air.

Certified reagent grade DMSO (Fisher) was stored in brown bottles and just prior to use was added, unsterilized, to the medium in the concentrations cited in the text on a volume-per-volume (v/v) basis.

For the dose–response experiments, replicate Petri dish cultures were incubated with 10^6 cells in 10 ml of medium with or without DMSO. At designated intervals the number of cells in each of two replicate cultures was determined by counting in a hemocytometer. Cell counts were done with trypan blue added to estimate the ratio of living to dead cells. Smears were prepared with Ralph's hemoglobin stain (12), then counterstained with Leischman, and the percent of benzidine-positive (B+) cells was determined.

For malignancy tests, the cells were inoculated subcutaneously into 8-week-old DBA/2J SPF mice of either sex (Jackson Laboratory).

The techniques for studying iron metabolism in these cells are described elsewhere (5). In brief, cells grown in medium containing ferrous (^{59}Fe) citrate (0.15 μCi of ^{59}Fe per ml) at an iron concentration of 6.8×10^{-9} M were washed in saline, lysed by freezing and thawing, and analyzed for iron as: intracellular iron (radioactivity of the total lysate), heme iron (cyclohexanone-extractable radioactivity after acid dissociation of heme and globin), and hemoglobin iron (radioactivity of B+ bands, separated by discontinuous acrylamide gel electrophoresis, of hemoglobin precipitated with $(NH_4)_2SO_4$ between 60 and 85% saturation).

RESULTS

Effect of DMSO on differentiation

After we had observed that the number of differentiating normoblasts increased among the leukemic cells grown in DMSO-containing medium, we studied the effect of several concentrations of the compound on cell growth. The dose–response curve obtained from a representative experiment of 4 days duration is shown in Fig. 1.

Cells grown in media containing 0.5 or 1% DMSO multiplied at approximately the same rate as those of the control (untreated) cultures. When the concentration was increased to 2%, there was a lag in the rate of growth during the first 48 hr, but by the 96th hour the number of cells approximated that of the controls. At a concentration of 3% DMSO, cell growth was inhibited, the cultures remaining almost stationary, and at 5% the compound was cytocidal, no living cells remaining after 72 hr.

When the cells were centrifuged for staining, it was noted that the cell pellets of the cultures exposed to DMSO became increasingly tinted with time. By the 4th day, the pellets were pink to red because of the presence of hemoglobin, whereas the control pellets were almost colorless.

In the cultures treated with 2% DMSO no clear-cut morphologic changes were detected for the first 48 hr. At 72 hr there was a slight but significant rise in the number of differentiating B+ cells and by the 96th hour a striking increase was apparent. Daily analysis of a representative experiment over a period of 5 days yielded consistently low frequencies of B+ cells in control cultures (0, 0.2, 0.2, 1.0, and 0.2%) and increasing frequencies in DMSO-treated cultures (0.4, 0.4, 2.8, 66.0, and 95.6%). The cells survived in 2% DMSO-containing medium as long as 7 days, at which time most of the living cells had reached the stage of orthochromatophilic normoblast. The appearance of the cells grown in the absence or presence of DMSO for 6 days is shown in Fig. 2. The blast cells characteristic of this leukemic cell line predominated in the untreated culture (Fig. 2*A*), whereas normoblasts were numerous in the treated cultures (Fig. 2*B*). These maturing cells were smaller, had condensed nuclei, and had a lower nuclear to cytoplasmic ratio than the cells from the control cultures.

Electron microscopic studies (manuscript in preparation) of the control and DMSO-treated cells revealed a marked difference in the number and association of ribosomes. Single ribosomes were numerous in the control cells, whereas fewer were seen in the treated cells, whose ribosomes often appeared

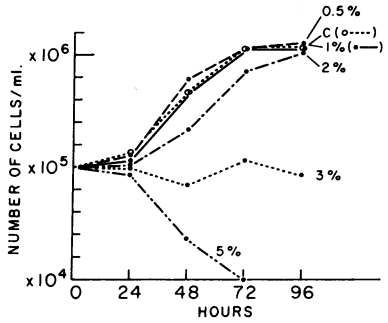

FIG. 1. Dose–response curves of leukemic cells of clone 707 in medium containing different concentrations of DMSO. Control: O- - -O; DMSO: 0.5% ●——●; 1% ●——●; 2% ●— — — —●; 3% ●- - -●; 5% ●- - — - - — -●.

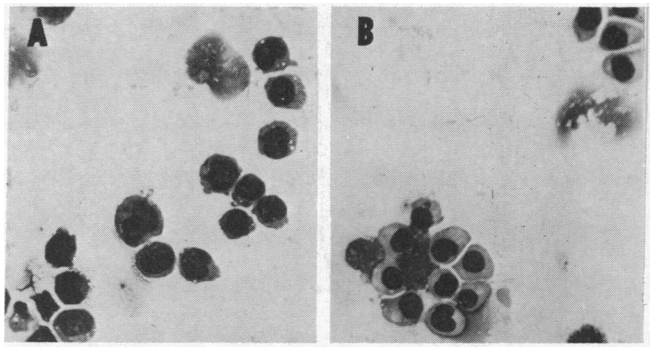

FIG. 2. Microphotographs of control and DMSO-treated cells of clone 707. (*A*) Control cells show pleomorphism and relatively large, basophilic erythroblasts. (*B*) Treated cells show many orthochromatophilic normoblasts with small, dense nuclei and more prominent cytoplasm. Both photographs were taken after 6 days of growth and are the same final magnification (×1250).

in clusters of 3 or 4. However, growth of the cells in DMSO does not eliminate the production of virus, since virus particles could be detected budding from the cell membranes and in the tissue-culture supernatant fluid.

The response of the cells to lower concentrations of DMSO, although less dramatic, also showed an increase in the number of B+ cells. Similarly, short exposures to the compound were sufficient to stimulate the mechanism involved in differentiation. The cells were seeded in replicate cultures in medium containing 2% DMSO and incubated for 1 and 24 hr, after which times aliquots of each were transferred to DMSO-free medium for an additional 4 days of incubation. As compared to the untreated cells, in which the frequency of B+ cells was 1.6% at the end of the observation period, the percentage in cultures exposed to DMSO for 1 hr had increased to 7.7 and in those exposed for 24 hr to 16.

In the experiments described, DMSO did not affect all of the cells since there were a few stem cells and early erythroblasts remaining in the cultures. Serially transferred in DMSO-free medium, these cultures gave rise to cell populations that were morphologically identical to those of the untreated cultures.

Thus far, the cells have not developed resistance to the treatment. When alternate passages were made in DMSO-containing and DMSO-free medium, there was no apparent change in the sensitivity of the cells to stimulation. The leukemic cells of the mass cultures, as well as of all clones tested, have demonstrated the ability to differentiate in the presence of DMSO.

Attempts to passage the cells serially in DMSO were also made. The cells could be maintained in medium containing 1% DMSO, but not in 2%, where few viable cells remained after two consecutive passages.

Bioassay for malignancy

To determine whether any change in the malignant properties of the differentiating cells had occurred, various concentrations of control or 2% DMSO-treated cells were inoculated subcutaneously into groups of syngeneic DBA/2J mice. Five mice were used for each concentration. There was little variation in the time of appearance of tumors in the mice receiving untreated cells or cells treated with DMSO for up to 48 hr. Mice inoculated with cells exposed to DMSO

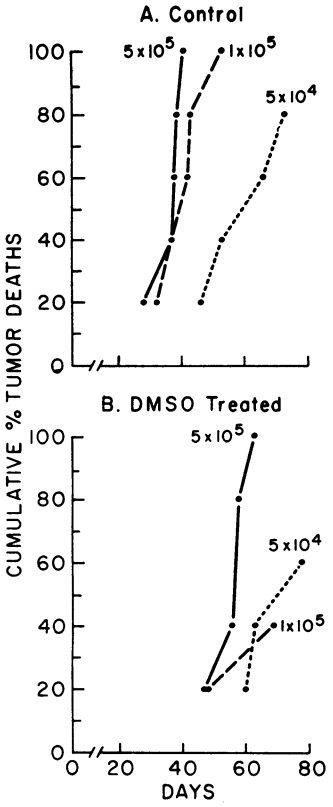

FIG. 3. Comparison of the survival times of mice inoculated with 5×10^5, 1×10^5, or 5×10^4 cells of clone 707 from control (*A*) and 2% DMSO-treated (*B*) cultures grown for 96 hr. Cumulative percentage of deaths is plotted against number of days after inoculation.

for 72 hr, however, lived longer and had slower-growing tumors than mice injected with untreated cells. This was even more evident with cells treated for 96 hr, as shown in Fig. 3. The groups of mice receiving the treated cells survived significantly longer than those inoculated with the control cells. Among the mice receiving the highest concentration of cells (5×10^5), the mean survival time of the control group was 38 days as compared to 56 days for those that received the treated cells. This difference is being further investigated with inocula containing smaller numbers of cells, in the hope of determining the number of tumorigenic cells that resist the effect of DMSO.

Iron metabolism

The effects of DMSO on the rates of cellular iron accumulation and of iron incorporation into heme and hemoglobin are shown in Fig. 4. As compared to the control cells, the accumulation of iron in the DMSO-treated cells was lower than in the controls for the first 72 hr, after which it was greatly and reproducibly stimulated. The stimulatory effect on heme and hemoglobin synthesis occurred earlier: it was apparent by the 48th hour for heme and by the 72nd hour for hemoglobin. By the 72nd and 96th hours respectively, there was a 20- and 60-fold stimulation in heme and a 12- and 40-fold stimulation of hemoglobin synthesis in the treated cells as compared to the untreated cells.

Fig. 5 illustrates the absorption spectra of the hemoglobin extracted from cells that had been grown in DMSO-supplemented medium. The spectra obtained from these cell lysates were identical with those of hemolysates of normal adult DBA/2J mouse cells.

Influence of other factors

While DMSO has thus far proved to be the most efficient inducer of erythroid differentiation in our leukemic cell lines, other factors that might influence erythropoiesis have been explored. Some effect was observed by adding steroids to the medium or by altering the conditions of growth. For example, in a series of 2-step experiments in which the cells were first grown in serumless medium (13) for 4 days and then transferred to the complete medium with 15% fetal calf serum for an additional 4 days of growth, differentiation was induced. Heme production was stimulated 2–3 times and hemoglobin synthesis 10 times over the control cells during the second step.

DISCUSSION

The murine virus-induced leukemia under study is always associated with an erythropoietic response *in vivo* and has been thought to resemble the erythremic myelosis seen in diGuglielmo's disease in man (3). The cells of the mass culture derived from the leukemic tissues, as well as the cells of cloned lines, are capable of performing specific functions characteristic of their normal erythrocytic counterparts, i.e., they synthesize heme, which is utilized to produce measurable amounts of hemoglobin. The hemoglobin present in these cells has been identified by its histochemical staining properties, peroxidase activity, incorporation of ^{59}Fe, absorption spectrum, and characteristic migration pattern during discontinuous electrophoresis on polyacrylamide gels. The electrophoretic pattern of hemoglobin bands appears to be

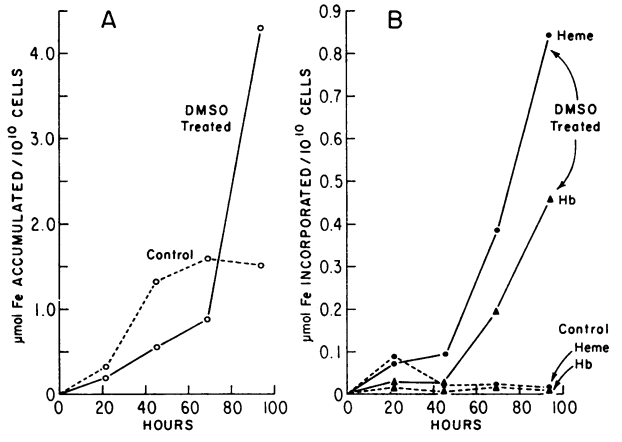

FIG. 4. Effect of DMSO on the rate of cellular iron accumulation (*A*) and the rates of incorporation of iron into heme and hemoglobin (*B*). Clone 707 cells were grown in the absence or presence of 2% DMSO in medium supplemented with ferrous (^{59}Fe) citrate (Squibb) to yield a final concentration of 1.2×10^4 cpm/μmol Fe. The ^{59}Fe solution was first incubated with fetal calf serum. Cells harvested on the first or second day of incubation had been seeded at 2.0×10^5 cells/ml and on the third or fourth day at 1.0×10^5 cells/ml. The cells from 20 ml of medium were harvested for each determination of cellular iron accumulation and iron incorporation into heme. The cells from 80 ml were harvested for each duplicate hemoglobin determination. The methods for the harvesting of the cells and for these determinations are described in a forthcoming paper (5) and briefly in *Methods*.

similar, if not identical, to that of hemolysates of adult DBA/2J mice, the strain from which the cells originated (4, 5).

In the present report, DMSO, at concentrations that did not appreciably inhibit cell replication, has been shown to induce a high percentage of the cells to differentiate along the erythroid pathway *in vitro*. Exposure of the cells to DMSO for 1 hr followed by transfer to DMSO-free medium was sufficient to "trigger" the synthesis of hemoglobin. In the treated cultures, the increase in the number of benzidine-positive cells was always correlated with an increase in the amount of hemoglobin synthesis. The effect—as measured by morphologic and histochemical alteration, by increased synthesis of heme and hemoglobin, and by a change in the growth pattern when assayed in syngeneic mice—was clearly discernible after 96 hr and correlates with data on the kinetics of maturation of erythroid cells (14, 15).

Electron-microscopic examination of the cells revealed that both control and DMSO-treated cells had viruses budding from the membrane surfaces. Whether the properties of virus from the treated cells are altered remains to be determined. Single ribosomes appear to predominate in the control cells and clustered ribosomes in the form of triads and tetrads are abundant in the treated cells. Similar differences between ribosomes of maturing erythroid cells before and during hemoglobin synthesis have been described (16, 17).

The survival curves of mice receiving treated or untreated cells suggest that the normoblasts in the DMSO-treated cultures may have lost their malignancy. Approximately 15% of the population of the DMSO-treated cells bioassayed remained at the blastic benzidine-negative stage and these are presumed to have given rise to the observed tumors. This is indicated by the finding that the survival time of the mice inoculated with 5×10^4 control cells was similar to that of the mice inoculated with 5×10^5 treated cells.

Dimethyl sulfoxide may stimulate differentiation of the leukemic cells directly or indirectly through one or several of its known biological activities by affecting synthesis of nucleic acids and proteins (18–26). It could presumably act also by altering the intracellular concentration of low molecular weight compounds (27–30) and by affecting the secondary and tertiary structure of macromolecules (31–35).

Of particular interest was the report of Schrek *et al.* on the difference in cytocidal effect of DMSO on normal and leukemic lymphocytes (36). DMSO at a concentration of 2% was more cytotoxic *in vitro* to human lymphocytes from patients with chronic lymphocytic leukemia and from AKR leukemic mice than to normal control lymphocytes.

In studies with fibroblasts of strain L-929 exposed to DMSO, Berliner and Ruhman (37) observed a sensitivity pattern somewhat analogous to that described here. The fibroblasts in 1% DMSO grew at a rate similar to that of the controls, but proliferation was suppressed at 3 or 5%. Cells transferred from medium containing growth-inhibiting concentrations of DMSO to control medium grew normally and showed no evidence of increased resistance to a second exposure to DMSO. This reversibility of inhibition parallels the findings in the leukemic cell system in the present study.

Dimethyl sulfoxide has also been observed to affect maturation and pigmentation of fungi. Sproston and Setlow (38) reported that the UV requirement for sporulation of fungi could be replaced by DMSO (1–10%). They suggested that DMSO acted by releasing ergosterol, the sterol necessary for

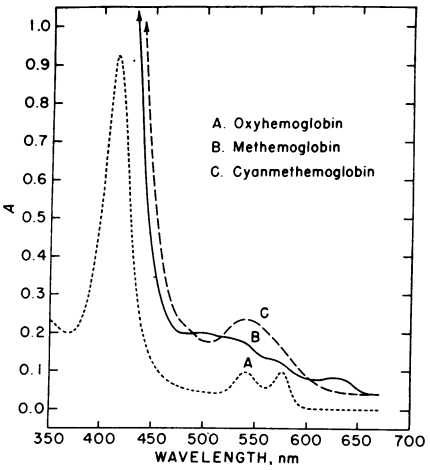

FIG. 5. Absorption spectra of extracts obtained from cells of clone 707 grown in medium containing 2% DMSO for 70 hr. A hemoglobin-rich fraction obtained by $(NH_4)_2SO_4$ precipitation (see *Methods*) was diluted with 0.1 M KPO_4 buffer pH 6.6. The readings were taken on a Cary model 14 recording spectrophotometer with a 1.0-cm light path. *A*, Cell extract, oxyhemoglobin. *B*, Oxidation of the extract (1.0 ml) with 5 μl of 5% $K_3Fe(CN)_6$ to form methemoglobin. *C*, Solution corresponding to Curve *B* treated with 5 μl of 1% KCN to form cyanmethemoglobin.

conidial production. Other investigators (39–41) described the blockage of pigment formation in various species of fungi by concentrations over 1%. Growth rate and sporulation were unaffected until concentrations of DMSO well in excess of those required for pigment inhibition were used.

The observation that differentiation can be induced among the leukemic cells by transfer to complete medium after an initial period of "starvation" in serumless medium cannot be interpreted at present. It is interesting to note that axon outgrowth on neuroblastoma cells occurs after the removal of serum from the culture (42) and differentiation of slime molds can be induced by starvation of the plasmodium (43).

The possibility that the regulatory mechanisms that control differentiation and malignancy are similar has been considered (44–46). Differentiation of human as well as murine neuroblastoma cells *in vitro* has been observed by a number of investigators (47–49). Kleinsmith and Pierce have shown that not only are mouse teratoma cells multipotential but a clone derived from a single cell can differentiate into as many as 11 different tissues (50). Other reports suggest that human leukemia cells may also possess the ability to differentiate (51–53). Finally, 5-bromodeoxyuridine has been shown to effect the differentiation of malignant cells (54, 55) and of embryonic cells (56).

It is evident that established lines of leukemic cells can be used for the investigation of problems related to the molecular control of differentiation and oncogenesis. Perhaps an interference in the normal flow of information from DNA into cell proteins results in malignancy. It may now be possible to ascertain if agents such as DMSO influence part(s) of the genome of the malignant cell to permit it to mature fully to the stage of its normal counterpart.

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