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Induction of δ-Aminolevulinic Acid Synthetase During Erythroid Differentiation of Cultured Leukemia Cells (38155)

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Proerythroblastoid tissue culture cells transformed with Friend leukemia virus (1, 2) can be stimulated to undergo erythroid differentiation in the presence of dimethyl sulfoxide (DMSO) (3). Differentiating erythroid cells have been shown to develop specific erythrocyte-membrane antigen, heme, hemoglobin, and globin mRNA (3–5). Since the formation of globin is dependent upon the production of heme (6), it follows that one of the earliest events initiating differentiation should be observable at the control point of the heme biosynthetic pathway. The control enzyme for the heme pathway is δ-aminolevulinic acid (ALA) synthetase, an inducible mitochondrial enzyme (7). This communication examines the induction of this enzyme in DMSO-treated T-3-Cl-2 cells, a cloned line of murine proerythroblastoid cells transformed by Friend leukemia virus.

Materials and Methods. The Friend leukemia cell line T-3-Cl-2 was originally derived from a spleen focus of Friend virus-induced leukemia. The spleen focus-derived ascites tumor was converted to an *in vitro* line and cloned in soft agar medium (2, 8). Upon treatment with 1%–2% DMSO (3–5), the cells develop a red color characteristic of hemoglobin and become benzidine-positive within 4–7 days. The cells were grown in 250 ml Falcon plastic flasks containing 50 ml of HAM F-12 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated calf serum in 5% CO_2 at 37°. Cells were passed in fresh media at 1×10^5 cells/ml, 1%–2% DMSO added, and the incubation continued for up to 5 days. The cells were harvested by centrifugation at 1000 *g* and the cell pellet was suspended in 10 m*M* Tris (pH 7.4)-0.9% NaCl. A total cell count was determined in a hemacytometer in the presence of trypan blue. The suspension was quick-frozen at −50° and thawed to lyse the cells. The equivalent of 5×10^6 total cells was incubated with 1 μCi 2-ketoglutarate-5-^{14}C (14.3 mCi/mmole), 75 m*M* Tris–HCl, 10 m*M* EDTA, 100 m*M* glycine, 0.2 m*M* pyridoxal phosphate, 0.25 m*M* CoA, pH 7.2, in a final volume of 2.0 ml for 20 min. The reaction was stopped with 0.5 ml of 25% trichloroacetic acid (TCA) and the ^{14}C-ALA production was determined as described previously (9, 10). Hemoglobin production was monitored by a modification of the benzidine-staining technique of Ralph (11).

Results. 2-Ketoglutarate-5-^{14}C was found to be the most efficient substrate for ^{14}C-ALA production with this cell line. Although succinate-2,3-^{14}C produced qualitatively similar results, the nonincubated samples produced higher "zero time" values when passed through Dowex 50 columns (9). Addition of partially-purified succinic thiokinase from rat liver to the incubation mixtures did not appreciably stimulate ALA synthetase production.

Table I shows that the effect of varied concentrations of DMSO upon ALA synthetase activity in cultured T-3-Cl-2 cells. Two percent DMSO was observed to be the optimal concentration for ALA synthetase induction with enzyme activity about 5 times the control level. In cells treated with 1 and 3% DMSO enzyme activity was elevated, but not

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TABLE I. Effect of DMSO Concentration upon ALA Synthetase Activity.[a]

Concentration of DMSO (%)	pmoles ALA produced/ 10^7 cells/hr
—	166
1	205
2	812
3	325

[a] Stock T-3-Cl-2 cells were resuspended in fresh media, and DMSO was added to a final concentration of 1%–3%. Cells were incubated at 37° for 3 days and harvested by centrifugation. ALA synthetase activity was measured as described in Materials and Methods.

even double that of the control, suggesting that ALA synthetase is inducible only over a very limited range of DMSO concentrations. Moreover, at the concentration of ≧ 3% DMSO the cell viability and growth rate decreased markedly.

Table II shows the time course response of T-3-Cl-2 cells incubated with and without DMSO. In Experiment A cells were incubated with and without 1.8% DMSO. Increased levels of ALA synthetase were first observed at 28 hr in the DMSO-treated cells and the specific activity with respect to the controls steadily increased up to the 93rd hr. By 100 hr ALA synthetase activity decreased almost to control levels possibly because of feedback repression of the enzyme by heme (12). Three percent of the DMSO-treated cells were benzidine-positive by day 4 and the level increased to 57% positive cells by day 7. No benzidine-positive cells were ever observed in untreated cultures. In a similar time course experiment (Experiment B), but with 2% DMSO, ALA synthetase activity was not appreciably elevated even by the 49th hr. By the 76th hr the activity increased to 210% of the untreated level and then decreased to below the untreated level by the 121st hr. DMSO-treated cells were 2% benzidine-positive by day 4 and this level increased to 33% positive by day 7. A comparison of the 2 experiments shows biological variability in the time of induction and repression of the enzyme.

Since allylisopropylacetamide (AIA) is a potent inducer of hepatic ALA synthetase in rats (13), its ability to induce the enzyme in T-3-Cl-2 cells was compared to that of DMSO. The effects of AIA and DMSO upon

TABLE II. Time Course of ALA Synthetase Activity in DMSO-treated and Untreated T-3-Cl-2 Cells.[a]

	Hours after DMSO addition	ALA synthetase specific activity (% increase)	% Benzidine-+ cells
Experiment A	22	— 23	
	28	36	
	52	95	
	76	177	
	93	490	
	100	10	3
	168		57
Experiment B	25	— 28	
	49	9	
	76	210	
	100	39	2
	121	— 62	
	170		33

[a] DMSO was added to culture flasks at a concentration of 1.8% (Experiment A) or 2% (Experiment B) and the cells were incubated for intervals up to 7 days. The cells were assayed for ALA synthetase activity and benzidine-positive cells as described in Materials and Methods. ALA synthetase activity is expressed as the % increase in the specific activity of the enzyme in DMSO-treated cells compared to that of the untreated cells.

TABLE III. Effect of DMSO and AIA upon ALA Synthetase in T-3-Cl-2 Cells.[a]

Concentration of DMSO (%)	Concentration of AIA (μg/ml)	pmoles ALA produced/ 10^7 cells/hr	Stimulation Factor
Experiment A			
—	—	71	0.0
1	—	150	2.1
—	400	167	2.3
1	100	206	2.8
1	200	220	3.1
1	400	316	4.5
Experiment B			
—	—	71	0.0
1	—	116	1.6
1	400	136	1.9
1	800	242	3.4
1	1600	154	2.2

[a] Twenty-four hours after cell split and addition of DMSO, AIA, dissolved in saline, was added to the appropriate flask. On the third day of culture the cells were harvested and assayed for ALA synthetase activity as described in Materials and Methods except that 1 μCi 2-ketoglutarate-^{14}C (uniformly labeled; 95 mCi/mmole) was used as substrate.

ALA synthetase induction in these cells is shown in Table III. In Experiment A 1% DMSO doubled ALA synthetase activity compared to that in the untreated cells. AIA at 400 μg/ml was approximately equivalent to 1% DMSO in its ability to stimulate the induction of ALA synthetase activity. The enzyme activity of cells incubated with AIA (400 μg/ml) and DMSO appeared to equal the sum of the activities of cells incubated with these compounds individually. In Experiment B, despite a generally depressed enzymatic response, the maximum stimulation of ALA synthetase by AIA in the presence of DMSO was obtained at an AIA concentration of 800 μg/ml. At doses over 800 μg/ml decreased cell viability and growth were observed.

Discussion. Proerythroblastoid cells can be induced to synthesize hemoglobin within 7 days *in vitro* by the addition of 1%–2% DMSO to the tissue culture media. The cells incorporate ^{59}Fe, can be stained by benzidine, show the typical absorption spectrum of hemoglobin, and exhibit a characteristic migration pattern during discontinuous electrophoresis (14). In addition, the α– and β–globins produced are identical to those synthesized by reticulocytes of the mouse strain from which the cells were derived (15).

The mechanism by which DMSO initiates red cell differentiation is unknown (16). DMSO could trigger differentiation in this virus-infected proerythroblastoid cell line by combinations of the following mechanisms: (a) by modifying the cell morphology to more nearly resemble normal cells (16), (b) by making the cell membrane more permeable to certain intra- or extracellular compounds, (c) by directly inducing the control enzyme(s) responsible for hemoglobin formation, and (d) by stimulating the production of Friend virus (4) or budding virus (17) which then triggers the induction of control enzymes of the heme pathway.

The possibility exists that DMSO may induce ALA synthetase (mechanism c) in these cells, since a known inducer of the enzyme is also effective in T-3-Cl-2 cells. However, these two agents do not "induce" the heme pathway similarly. AIA induces ALA synthetase to about the same extent and in about the same time as did 1% DMSO, but inducing levels of AIA did not stimulate hemoglobin production. At high doses of AIA detectable amounts of hemoglobin sometimes could be observed using a sensitive colorimetric procedure (experiments in progress). A primary effect of AIA in rat liver is the breakdown of the heme moiety of hemoproteins facilitating the induction of ALA synthetase at the translational level (18). The absence

of detectable hemoglobin production at low doses of AIA despite the induction of ALA synthetase may reflect the breakdown of existing and newly formed hemoproteins.

Another possibility is that DMSO-stimulated production of virus or certain viral components may indirectly stimulate heme synthesis (mechanism d). Sato *et al.* (17) have observed increased budding of virus particles, but no enhanced virus production following DMSO treatment. Others have found that DMSO stimulated virus production in murine and human cells when combined with 5-iodo-2′-deoxyuridine, but not alone (19). In contrast to the finding of Sato *et al.* (17), Ikawa *et al.* (4) have reported a 200-fold increase in virus production as a result of DMSO treatment of T-3-Cl-2 cells. Ebert and Pearson have observed a 16-fold elevation of ALA synthetase activity in virus-producing-transformed rat embryo fibroblasts as compared to normal embryo fibroblasts, suggesting that virus production can influence heme synthetic activity (10).

This tissue culture line of differentiating cells offers a unique opportunity to study the control of differentiation, enzyme induction, oncogenesis, and their interrelationships. Since several different biological compounds have shown enzyme-inducing activity in this cell line, this model system, free from the complex biological interactions of animal tissues, should permit detailed investigations of their mechanism of action. The potential for DMSO to induce ALA synthetase in rat liver and spleen also is being investigated.

Summary. A cloned line of Friend erythroleukemia cells (T-3-Cl-2) can be induced to produce hemoglobin when grown in the presence of 1%–2% DMSO. Such DMSO-treated cells become 30%–60% benzidine-positive in 5–7 days. The control enzyme for hemoglobin synthesis, δ-aminolevulinic acid (ALA) synthetase becomes elevated after 28 hr in DMSO-treated cells. Furthermore, this synthetase activity increased 2–6 times the level of control cells during the following 3 days. Maximal ALA synthetase activity was found when the culture media contained 2% DMSO. Allylisopropylacetamide alone was found to be as effective an inducer of ALA synthetase as 1% DMSO, and could augment enzyme activity when present with DMSO.

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