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Dimethylsulfoxide-induced differentiation and hemoglobin synthesis in tissue cultures of rat erythroleukemia cells transformed by 7,12-dimethylbenz(*a*)anthracene

(erythropoiesis/cell size analysis/Friend virus/Friend cells)

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ABSTRACT **Permanent cell lines from transplantable tumors from 7,12-dimethylbenz(*a*)anthracene-induced erythroleukemia of the rat were established. These cell lines maintain their erythroid nature. Erythroid differentiation can be induced by dimethylsulfoxide. This is shown by decrease in cell size, appearance of red and benzidine positive cells, and induced synthesis of four out of six adult globin chains.**

Erythroid differentiation and hemoglobin synthesis can be induced by dimethylsulfoxide (Me_2SO) in permanent cell cultures of mouse spleen cells transformed by Friend virus (spleen focus forming virus) (1–3). The availability of such cells allows studies of the factors involved in erythroid differentiation. The possibility exists that the Friend virus is both necessary for the specific block in differentiation and also for the specific release of this block by Me_2SO (4–6). It is important to know whether cells of chemically induced, or those of spontaneously occurring, erythroleukemias are equally responsive to highly polar compounds (1, 7) or to fatty acids (8, 9). In addition, we would like to know whether Me_2SO is an erythroid differentiation-inducing agent in species other than mice.

In this paper we present data on the establishment of permanent cell lines from two independently 7,12-dimethylbenz(*a*)anthracene-induced erythroleukemias of the rat (10, 11). These cells can be induced to differentiate and to synthesize adult rat hemoglobin on exposure to Me_2SO. This rat erythroleukemia system obviously can also be used to study the cause of the nonuniform biosynthesis of the six to eight hemoglobins of the adult rat (12–14).

MATERIALS AND METHODS

Source of Cells. Erythroleukemias have been induced by pulse doses of 7,12-dimethylbenz(*a*)anthracene in random bred, weanling Long-Evans rats (10, 11). Blood of these erythroleukemic rats was injected subcutaneously to other newborn rats of the same strain (15). Tumors developed which histologically resemble erythrosarcomas (15). Two tumors of different origin of male animals, one exhibiting a normal karyotype and one with a C-1 trisomy after the 9th and 6th passage, respectively, were used for establishing the cell lines. Cell line RED-2 was derived from the *r*at *e*rythroleukemia with *d*iploid karyotype and REC-1 from a *r*at *e*rythroleukemia with the *C*-1 trisomy.

Establishment of the Cultures. Pieces of tumors were removed and the cells were suspended in cell culture medium by disruption with two forceps. The initial cell density was between 5×10^5 and 2×10^6 cells per ml. Part of the medium was removed daily for the first 3 days. Dense cultures were subdivided.

Medium. Eagle's medium with Earle's balanced salt solution enriched for vitamins and for essential as well as for nonessential amino acids was used. Ten percent fetal calf serum was added (2).

Determination of Cell Size. A Coulter Counter model ZB attached to an Ortec model 9200 multichannel analyzer was used for the determination of cell volume of the cells at various days after Me_2SO treatment. A 50-μm orifice was used, and the conductance of known particles and other cells used as references.

Change in Light Scattering Properties of the Cells. A computer-controlled high-speed cell analyzer/separator (16, 17) was used to observe and record the light scattering signals at 6° in the forward angle of individual living cells as they passed through a focused laser beam. These properties are a function of size, surface structure, and refractive index of the cells.

Benzidine Staining. Benzidine positive cells were identified by routine procedures.

Globin Separation. Adult peripheral erythrocytes of Long-Evans inbred rats were washed in physiological saline. They were lysed by a 10-fold excess of 1% Nonidet P-40 in phosphate-buffered saline pH 8.7 at 4° to avoid crystal formation of the hemoglobin. Labeled tissue culture cells (2) were treated similarly. The lysate of both preparations was pelleted at $10,000 \times g$ for 10 min and the hemoglobin-containing supernatant was precipitated in acetone/HCl (2). The acetone-washed precipitate was dissolved in starting buffer (5 mM Na_2HPO_4, pH 6.5). The globin was separated on a carboxymethylcellulose-urea column with a linear gradient comprised of 200 ml each of starting buffer and 0.06 M Na_2HPO_4, pH 6.7, column dimension 1×12 cm.

Induction of Differentiation. Me_2SO (Spectroscopy grade, Merck, Darmstadt) was added to the culture medium to a final concentration of 1 or 1.2%. Cells in logarithmic growth at a density of 5×10^5 to 2×10^6 per ml were used for induction. Part of the medium and cells were removed and new Me_2SO-containing medium was added to keep the cells at a density between 0.5×10^6 and 1.5×10^6 cells per ml.

Cloning of Cells. Cells were cloned in medium with 0.35% GIBCO agar and 20% fetal calf serum. The clones

Abbreviation: Me_2SO, dimethylsulfoxide.

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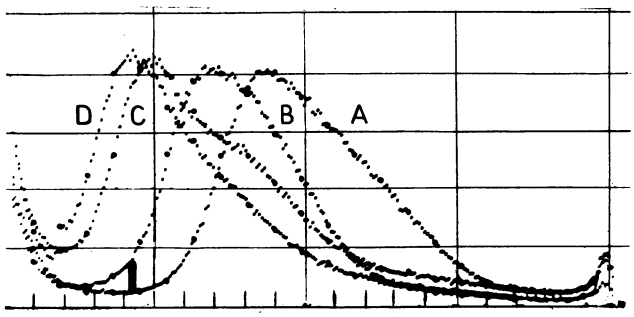

FIG. 1. Volume measurements of erythroleukemic rat cells induced by 1.2% Me_2SO. The different frequency distributions are superimposed with increasing volume on the abscissa and number of cells on the ordinate as determined by Coulter conductance measurements using a 50-μm orifice. A, uninduced cells; B, 1 day of Me_2SO induction, 1.2%; C, 3 days of induction; D, 4 days of induction.

were picked up after 12 days of growth in a moist chamber. Cell clones D1–10 are derived from RED-2 and clones C1–10 from REC-1.

RESULTS

Establishment of rat erythroleukemic cells in culture

Cells of both tumors adapted to growth in tissue culture at once. REC-1, which was derived from a tumor with C-1 trisomy, is heteroploid. RED-2, which is derived from a diploid tumor, did not change in karyotype. The doubling time of cell clone D5 is 12–17 hr.

Me_2SO-induced erythroid differentiation

The rat erythroleukemia cells, upon induction with Me_2SO, undergo changes in their cell size that indicate that they are dividing without fully reproducing their normal cytoplasmic contents. In Fig. 1 is shown the progression of the cells to smaller volume with increasing duration of Me_2SO treatment. The progression is exactly parallel to that observed for mouse cells transformed by Friend virus and induced to differentiate with Me_2SO treatment, although the final size reached is somewhat larger for the rat cells (17). The time correlation of size change with globin production and benzidine positive cells suggests that the size reduction is a consequence of the differentiation process in which normal protein synthesis is shut off in favor of the specialized production of globin. In Fig. 2 is shown the change in light scattering properties of the cells as they undergo differentiation in Me_2SO. The cells become more refractile and smaller, as observed by phase contrast microscopy, and these changes are shown by the smaller light scattering signal observed for the cells after Me_2SO induction. Some cells during maximal induction morphologically resemble reticulocytes or erythrocytes and have lost their nuclei. The cell pellets are slightly reddish at the second to third day of treatment and are maximally red at the fourth to sixth day of exposure to Me_2SO. The color intensity is less than in induced B8 or F4 mouse erythroleukemia cells (2, 5). A pronounced increase in benzidine staining for heme is observed parallel to the color change (Fig. 3). The benzidine positive cells (B^+) can be divided into two categories: those with very strong staining (about 10% of all cells of D5 at day 6) and those with weaker staining (about 10–20%). Cells with strong staining are usually smaller in size, but most of them still show a prominent condensed nucleus. The uncloned cell lines are more heterogeneous in their response to Me_2SO and have at most 10–20% B^+ cells, whereas D5 or D4 shows above 50% B^+ cells at maximal induction. Other clones are less inducible. Less than 0.1% of the uninduced D5 cells are B^+ (Fig. 3).

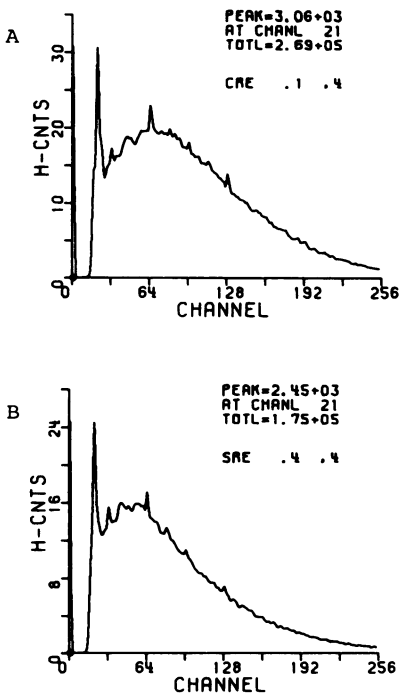

FIG. 2. Size measurements of erythroleukemia rat cells obtained with the cell separator. Light scattered in the forward angle was collected at 6° with an aperture of 0.5° using 488 nm laser light incident at 53° to the fluid stream. (A) Frequency distribution of the light scattering intensities from uninduced rat erythroleukemic cells, and (B) from the same cell line 3 days after induction with 1.2% Me_2SO are plotted with increasing signal size on the abscissa and number of cells in the ordinate. CRE and SRE are computer file names. H-CNTS = hectocounts.

Hemoglobin synthesis

Six adult rat hemoglobins can be separated on acrylamide gels with isoelectric focusing in Wistar rats (12). We find eight hemoglobins in Long-Evans rats with four distinct β

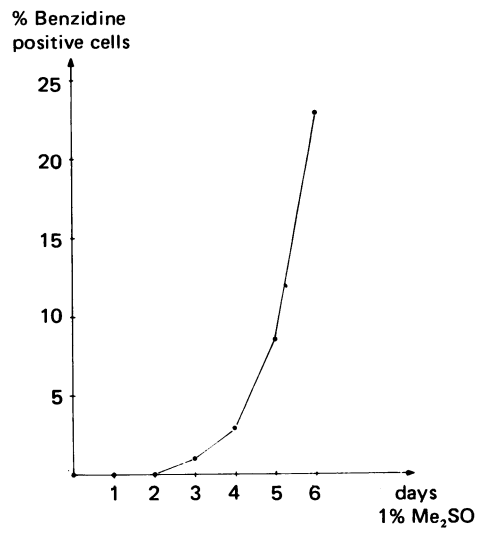

FIG. 3. Increase in benzidine positive cells in cell clone D5 exposed to 1.2% Me_2SO.

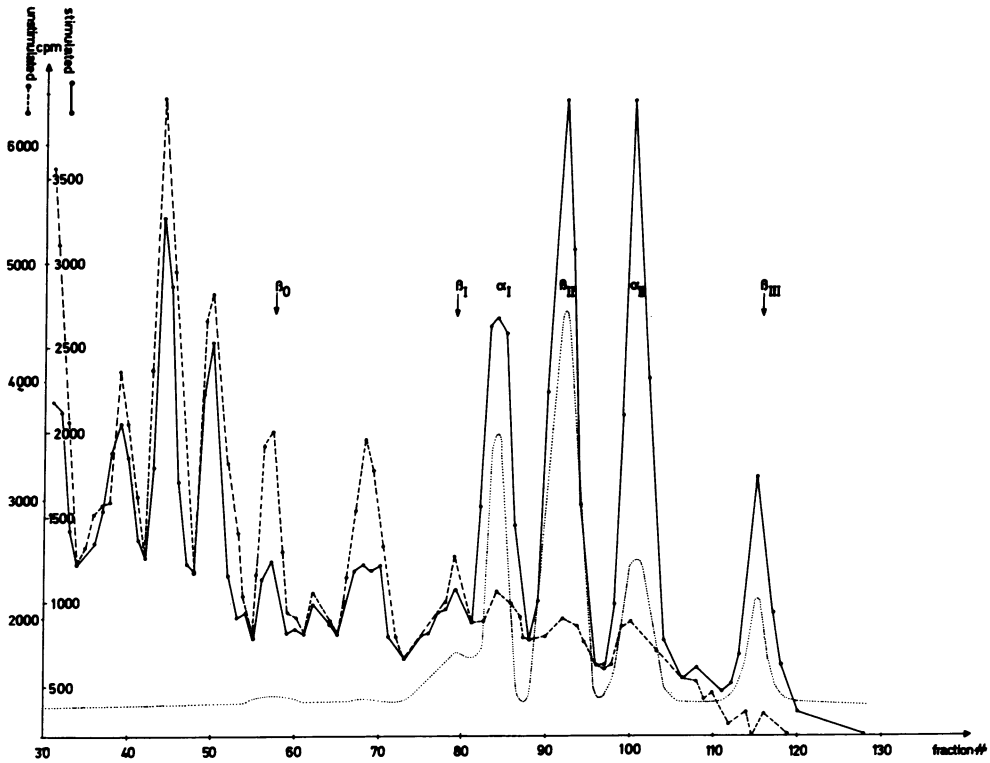

FIG. 4. Induction of adult rat globin synthesis in uncloned rat cell line REC; carboxymethylcellulose-urea chromatography (see *Materials and Methods*). The early eluting fractions have not been monitored. The globin chains are identified to correspond with the nomenclature of Garrick *et al.* (13), using our own data on the globin chain composition of Long-Evans rat hemoglobins (unpublished). (●---●) Unstimulated; (●—●) stimulated, 1% Me_2SO, 5 days; (· · · ·) OD.

chains and two α chains (unpublished data). They are the same as those described by other authors for other strains of rats (13, 14). The exception is β_0 (Fig. 4), which moves earliest during chromatographic separation. An attempt has not yet been made using peptide or amino-acid analysis to show β_0 to be different from the other β chains. The six globin fractions can be separated by carboxymethylcellulose-urea chromatography (Fig. 4). Unstimulated rat cells of RED-2, REC-1, and D5 were labeled with [^{14}C]- or [^{3}H]leucine, and the labeled proteins were separated together with carrier globin on carboxymethylcellulose-urea columns (Fig. 4). Only minor radioactivity peaks eluted with five of the six carrier globin chains. This was not more than 1% of the total labeled cell proteins. However, with optimal stimulation by Me_2SO, e.g., at day 6 with REC or D5 cells, about 15% of the total label is eluted with only four of the carrier globins (Fig. 4). No other new radioactivity peak is observed after day 2 of stimulation. The identity of the induced proteins with that of carrier globin shows that only adult globin is induced and synthesized in rat erythroleukemia cells. Two of the globins (β_0 and β_1) are not induced, or only to a minor degree. The increase in the relative rate of globin synthesis of clone D5 is shown in Fig. 5. The decrease in the rate of synthesis after day 8 reflects outgrowth of nondifferentiating cells.

DISCUSSION

We show here the establishment of rat erythroleukemia cells in culture. The cells are derived from transplantable erythrosarcomas that had been induced by a carcinogen, 7,12-dimethylbenz(*a*)anthracene (10, 18). The cells maintain their erythroid nature under permanent culture conditions.

Addition of Me_2SO to two independently isolated rat erythroleukemia cell lines and to several clones of these lines induced a change to smaller average cell size (Figs. 1 and 2).

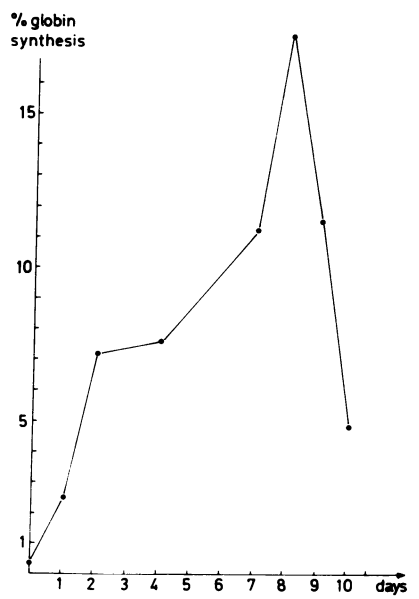

FIG. 5. Increase in the relative rate of globin synthesis of clone D5 after exposure to 1% Me_2SO. Globin chains were separated as described, and the amount of radioactive label ([^{14}C]- or [^{3}H]leucine) was determined after background subtraction, using the total percentage of labeling in cytoplasmic Nonidet P-40-soluble proteins of nonstimulated cells (labeled with [^{3}H]- or [^{14}C]leucine). The decrease in the relative rate of globin synthesis during later days of Me_2SO exposure reflects the outgrowth of Me_2SO-adapted but nondifferentiating erythroleukemia cells.

The cells show heme and globin synthesis, as evidenced by benzidine staining, appearance of red-colored cell pellets, and cochromatography of labeled and induced proteins with adult rat carrier globin. Only four of the six rat globin chains were noticeably induced on stimulation.

Our data show that Me_2SO is not only able to induce erythroid differentiation in cells transformed by Friend virus, but also in this chemically induced erythroleukemia. The action of Me_2SO is not limited to one species, to the mouse, but can also be found with transformed rat cells. A human polycythemia vera cell line (19) with erythroid characteristics, however, does not respond to Me_2SO with erythroid differentiation (refs. 19 and 20, and unpublished data). Chick erythroleukemia cells also do not respond to Me_2SO with erythroid differentiation (T. Graf, personal communication). Fatty acids do not induce globin synthesis in rat cells, although the same substances induce hemoglobin synthesis in mouse erythroleukemia cells (8, 9). We have preliminary data that an endogenous rat virus is induced during Me_2SO-stimulated erythroid differentiation in these rat cell lines. This is again analogous to the induction of an endogenous spleen focus forming virus complex in virus positive and negative mouse cell lines transformed by Friend (spleen focus forming) virus (6). This system can possibly be used to study the function of endogenous viruses in transformed cells and their role during induced differentiation of such cells.

Further work is needed to correlate the synthesis of presumably eight hemoglobins in Long-Evans rats *in vivo* to the four globin chains synthesized in our cell lines. The rat erythroleukemia cells can potentially be used to study the cause of the nonuniform hemoglobin synthesis (12) (switch) in the descendants of cloned erythroleukemia cells.

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