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Alteration of myoblast phenotype by dimethyl sulfoxide*

(differentiation/creatine phosphokinase/collagen)

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ABSTRACT **Application of dimethyl sulfoxide to proliferating L8 myoblasts (an established cell line of rat skeletal muscle) for 72 hr completely prevented fusion and induction of creatine phosphokinase (EC 2.7.3.2) activity (an indicator of muscle differentiation). The growth pattern changed from the usual sheets of randomly oriented cells to flattened, whorled monolayers of elongated fibroblast-like cells. By electron microscopy, rough endoplasmic reticulum increased and extracellular material appeared that had the morphologic and staining characteristics of collagen. After 120 hr in dimethyl sulfoxide-containing medium, the cells secreted about 6 times more collagen than untreated controls. Dimethyl sulfoxide was ineffective when applied to L8 cells just prior to fusion, and effects of dimethyl sulfoxide were not readily reversible unless treated cells were subcultured at low density.**

Murine erythroleukemia and neuroblastoma cells in culture differentiate in the presence of dimethyl sulfoxide (Me_2SO) (1, 2) or polar compounds of the polymethylene bisacetamide series (3, 4). We examined the effects of Me_2SO on differentiation of L8 myoblasts, an established cell line of rat skeletal muscle (5). At confluency, these cells fuse to form multinucleate myotubes with concomitant accumulation of muscle-specific proteins, including creatine phosphokinase (EC 2.7.3.2) (CPK) (5, 6). Exposure of logarithmically growing L8 cells to 2% (vol/vol) Me_2SO for 48 hr completely prevented both fusion of myoblasts and accumulation of CPK. However, fusion and increase in enzyme activity were not prevented when Me_2SO was applied to high-density (prefusion stage) cultures. Even though fusion and rise in CPK activity did not occur, our data showed that Me_2SO did not *prevent* cell differentiation, but, rather, induced a phenotypic conversion from myoblast to fibroblast-like cell. Hexamethylene bisacetamide, a potent inducer of erythroleukemia cell differentiation, had similar effects (unpublished data).

MATERIALS AND METHODS

Cell Cultivation. A subline of L8 myoblast (5), stored in liquified N_2, was cultivated in Eagle's minimal essential medium (Earle's base) supplemented with 15% fetal bovine serum, nonessential amino acids, vitamins, pyruvate, and 50 μg of streptomycin per ml, 50 units of penicillin per ml, and 0.25 μg of amphotericin B per ml (Grand Island Biological Company, Grand Island, NY) in a humidified atmosphere of 5% CO_2 and air at 37°. For experiments, cells from subconfluent stock cultures, grown in 250-ml flasks (Falcon Plastics) were detached by incubation in 0.125% trypsin and 0.15% EDTA in Earle's balanced salt solution. For growth curves and cytological examination, 0.5×10^5 or 0.75×10^5 cells were planted in 35-mm Costar cluster dishes (Cambridge, MA) with or without 22-mm coverslip inserts. For cloning studies, 250 or 300 cells were planted in 100-mm dishes. Clonal cultures were left undisturbed for 2 or 3 weeks; all other cultures were refed every third day. In all studies, Me_2SO-containing medium was applied to duplicate cultures either 24 hr after initial planting or at the time when the first multinucleate myotubes became visible.

Cytology. For determination of mitotic indices, cultures grown on coverslips were fixed in 100% methanol and stained for 10 min with 2% aceto-orcein. The percent of mitotic cells was determined in at least 2000 cells in each preparation. Fusion indices were determined by counting the percent of nuclei incorporated into myotubes in 2000–4000 cells in each preparation, which was fixed in 100% methanol and stained with 0.1% crystal violet in 20% ethanol for 15 sec. Thymidine-labeling indices were measured in autoradiograms of coverslip-grown cultures that were pulse-labeled for 15 min with 10 μCi of tritiated thymidine per ml (specific activity 29 Ci/mmol, New England Corp., Boston, MA). In addition, paired cultures were labeled for 24 hr with 0.05 μCi of tritiated thymidine per ml in the presence or absence of 2% Me_2SO. Autoradiograms were processed and stained as described (7). Cell viability was determined in paired cultures by exclusion of trypan blue dye (8). To demonstrate matrix collagen by light microscopy, we fixed coverslip-grown cultures at 3, 6, 10, and 14 days and stained them by Gomori reticulin stain or by a Masson and Gomori trichrome procedure (9).

Electron Microscopy. Cultures grown in the presence or absence of 2% Me_2SO were processed for electron microscopy at 3, 6, and 14 days as described (7).

Biochemistry. For biochemical studies 0.5, 1.0, or 1.5×10^6 cells were grown in 100-mm Falcon dishes. CPK activity was measured by the Rosalki method (10) with Calbiochem CPK reagents (La Jolla, CA). The cultures were rinsed three times with phosphate-buffered saline (pH 7.3) containing 0.25% sucrose, scraped with a rubber policeman, resuspended in phosphate-buffered saline at 4°, freeze-thawed three times, and centrifuged for 15 min at $12,000 \times g$. Enzyme activity was measured over a 5-min period at 30°.

Collagen synthesis and hydroxylation were measured in cultures grown in 100-mm dishes exposed to 10 μCi of [2,3-^{3}H]proline per ml (specific activity 11.1 Ci/mmol; New England Nuclear Corp.) in Dulbecco proline-free medium for 18 hr, as described (11). After dialysis the samples were hydrolyzed in 6 M HCl for 36 hr at 120°. The hydrolysates were lyophilized, redissolved in distilled water with added unlabeled carrier proline (20 μg) and hydroxyproline (200 μg), and applied to Whatman no. 1 chromatography paper (reference spots were unlabeled proline and hydroxyproline alone). Ascending chromatography was carried out for 18 hr at room temperature with butanol/acetic acid/water (4:1:1.6 vol/vol). The dried

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Abbreviations: Me_2SO, dimethyl sulfoxide; CPK, creatine phosphokinase.

* A preliminary report of this study was read before the Society for Neuroscience, Anaheim, CA, November, 1977.

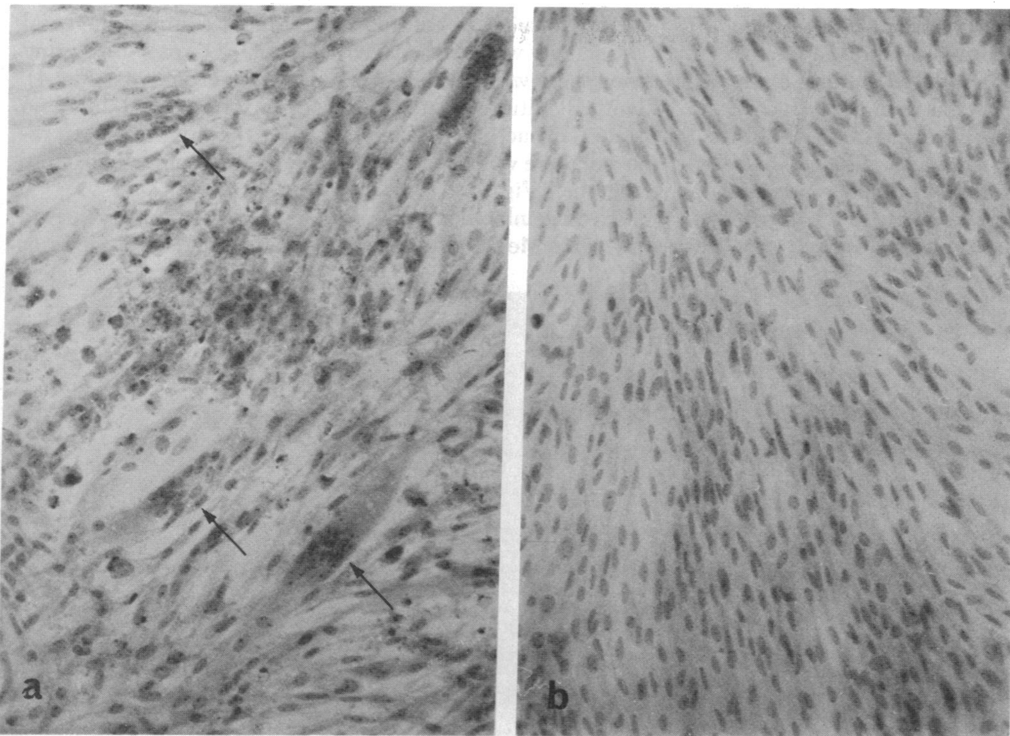

FIG. 1. (*a*) Culture of L8 cells in control medium for 96 hr photographed at low magnification to demonstrate typical growth pattern. Some myotubes have already formed (arrows). (*b*) Replicate culture grown in control medium for 24 hr then refed with medium containing 2% (vol/vol) Me_2SO. The flattened cells are aligned in parallel. Fusion does not occur. (Crystal violet stain, ×190.)

chromatograms were stained with 2% isatin in acetone (proline and hydroxyproline spots were separated by 2.5 cm). The spots were eluted and radioactivity was measured in a Packard Tricarb liquid scintillation counter, with appropriate controls to test for quenching and counting efficiency. The recovery of radioactivity in the proline and hydroxyproline spots was around 87% of the total radioactivity in the hydrolysate. Protein was determined by the method of Lowry *et al.* (12) before and after extraction of the collagen with 0.5 M acetic acid.

RESULTS

Alterations in Growth and Cell Morphology. Under control conditions, L8 cells exhibited an irregular growth pattern, resembling transformed cells. Individual cells were often hemispherical and tri- or multipolar, with refractile cytoplasm and pleomorphic nuclei (Fig. 1*a*). At 96 hr, the first multinucleate myotubes were detectable in the cultures, and at 144 hr, 85% or more nuclei were incorporated into myotubes. After 48 hr of exposure to 2% Me_2SO, the population doubling time increased (Fig. 2) while mitotic- and thymidine-labeling indices declined (from 7.1 to 4.3% and 43.3 to 23.2%, respectively) without a decrease in cell viability. After 3 days in Me_2SO, most cells were elongated, flattened, and aligned in parallel, forming a confluent, wavy monolayer (Fig. 1*b*). The cytoplasm of Me_2SO-treated cells was no longer refractile and nuclei were more homogeneous in form and size (Fig. 1*b*). No multinucleate myotubes were detected, not even after 14 days of cultivation with Me_2SO. Lower Me_2SO levels (0.25 and 0.50%) were ineffective, while 0.75 and 1.0% had only marginal effects on cell fusion after 72 hr of exposure.

Cells grown at cloning density under control conditions for 2–3 weeks exhibited multinucleated myotubes in 73–83% of primary colonies. In the presence of Me_2SO, no fusing colonies were found after 3 weeks. The remarkable alteration in growth pattern of Me_2SO-treated cells was accompanied by an increase in adhesiveness of the cells to the substratum; it took 5 min to remove 50% of the control cells with 0.06% trypsin at 37°, but 12 min of trypsinization was required to remove half of the cells treated with Me_2SO for 72 hr. At 10 and 14 days of Me_2SO treatment, the cultures revealed typical collagen staining with Masson trichrome (blue) and Gomori trichrome (green). Little or no staining of matrix components was detected in control cultures. Neither control nor Me_2SO-treated cultures showed any significant argyrophilia with the Gomori reticulin stain, indicating that neither control nor Me_2SO-treated cells produced detectable amounts of reticulin-type collagen [i.e., type III collagen found in almost all organs (13)].

Electron Microscopy. Control and Me_2SO-treated cultures were examined 3, 6, and 14 days after planting. The cytoplasm of control cells at 3 days of cultivation contained moderate numbers of polyribosome clusters and a few profiles of rough endoplasmic reticulum. At 6 days the cells had mostly nonmembrane-bound polyribosomes and very little rough endoplasmic reticulum (Fig. 3*a*). In Me_2SO-treated cells only few nonmembrane-bound polysomes were noted, but there was a

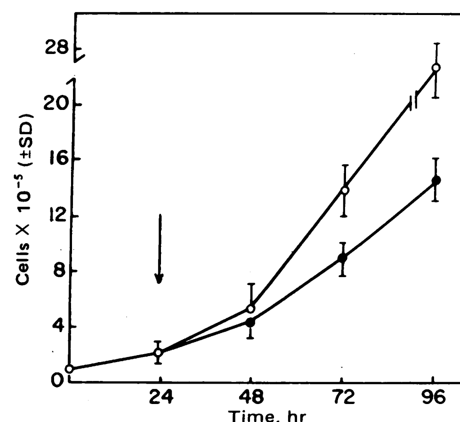

FIG. 2. Growth curves of control (O) and Me_2SO-treated L8 cells (●). Me_2SO (2%) was added 24 hr after planting. At 24 hr after treatment there is no significant change in cell growth, but an increase in population doubling time is noted at 48 and 72 hr after application of Me_2SO.

massive increase in membrane-bound ribosomes. Developing myofibrils were commonly seen in myotubes of 14-day-old cultures. None were found in Me_2SO-treated cultures at any time. Extracellular fibrillar material was commonly seen in Me_2SO-treated cells at 6 days (Fig. 3*b*) and was quite plentiful at 14 days, sometimes with distinct periodicity (Fig. 3*c*). None was observed in association with control cells. Neither in control nor in Me_2SO-treated cells was there evidence of a basement membrane [which contains collagen type IV (13)].

CPK Activity. In control, subconfluent cultures, the basal CPK activity was less than 0.05 milliunit/μg of protein. During fusion there was a 5- to 6-fold rise in enzyme activity (Fig. 4). Identical cultures treated with Me_2SO for 72 hr exhibited no rise in CPK activity. Even 96 hr after the drug had been removed, there was still no rise in CPK and the cells failed to fuse altogether (Fig. 4).

Effective inhibition of fusion and CPK activity was achieved only when Me_2SO was applied for extended periods (48 hr or

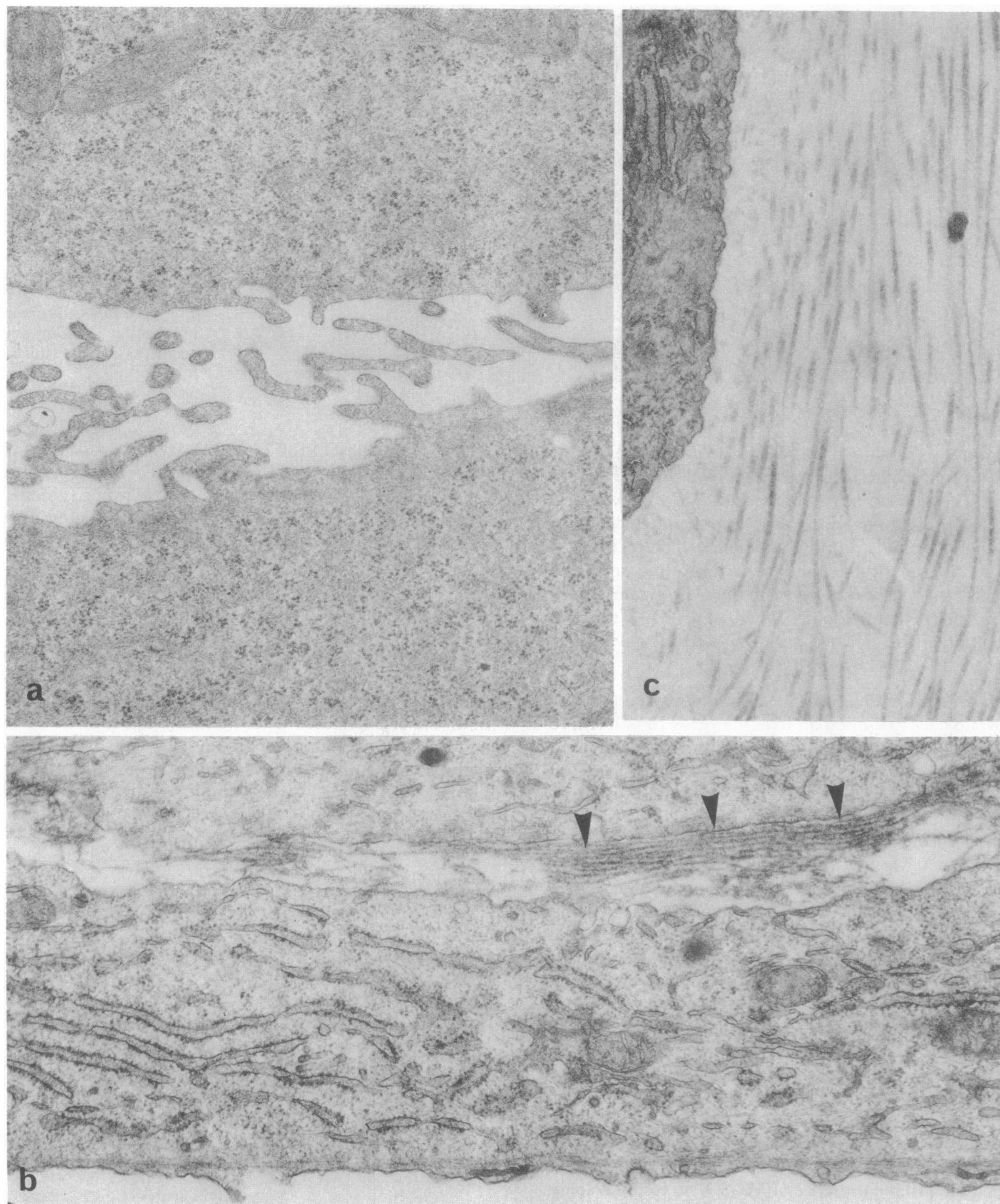

FIG. 3. (*a*) Mononuclear L8 cells in a 6-day-old control culture. The cell surfaces exhibit microvilli. The cytoplasm contains numerous non-membrane-bound ribosome clusters. (×24,425.) (*b*) Me_2SO-treated L8 cells grown in medium containing 2% Me_2SO for 6 days. Cell surfaces are smooth. The cells are aligned in parallel and within intercellular spaces there are bundles of fibrils (arrowheads). The cytoplasm contains numerous profiles of rough endoplasmic reticulum. (×24,425.) (*c*) Culture of L8 cells, exposed to Me_2SO for 14 days exhibit a matrix of typical collagen fibrils. (×28,975.)

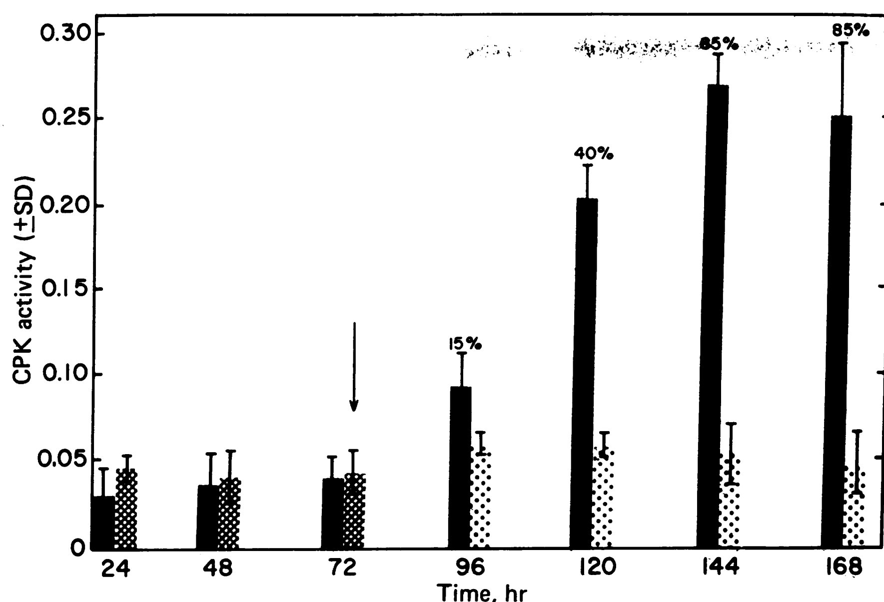

FIG. 4. Replicate cultures grown in the presence or absence of 2% Me_2SO were assayed for CPK activity (milliunits/μg of protein). Me_2SO was added 24 hr after initial plating and then removed 72 hr later. There is a significant rise in CPK activity during fusion of control L8 cells. Me_2SO-treated cultures fail to fuse and there is no rise in CPK activity. Fusion indices are indicated on top of the bars. ■, Control; ■, Me_2SO; ▣; Me_2SO removed.

longer) to logarithmically growing cultures at subconfluent density. Briefer application (24 hr or less) was only partially effective. Removal of Me_2SO after 24 hr resulted in a fusion index of 32% at 144 hr and CPK activity did not rise more than 2-fold above basal levels (Table 1).

Effective inhibition of fusion and CPK activity was also dependent upon the stage of the culture. When applied to confluent monolayers shortly before or early during the fusion stage, syncytial formation proceeded at an almost normal rate (fusion index 71% ± 8) and CPK activity approached control levels (4- to 5-times basal activity) at 144 hr.

Recovery from Me_2SO. If Me_2SO was removed from *subconfluent* cultures after 72 hr, the inhibitory effect was reversed after a 96-hr lag, with a 2-fold rise in CPK activity and a fusion index of 40% (±9.5). Cultures that reached confluency in the presence of Me_2SO for 72 hr failed to show any fusion and there was no increase in CPK levels after removal of the drug (Table 1). After 192 hr, however, a few myotubes were noted (<10%).

L8 cells did not fuse if they reached confluency after 96 hr in Me_2SO and were then trypsinized and planted at the same density in Me_2SO-free medium. However, if the cells were replanted at one-tenth the initial density (i.e., subconfluent), the fusion index rose to 43% (±8.3) 144 hr after subculture in Me_2SO-free medium.

Collagen Formation. Control and Me_2SO-treated cells were exposed to [^{3}H]proline for 18 hr at 72 and 120 hr after drug treatment. The collagen was extracted with 0.5 M acetic acid; the nondialyzable proline and hydroxyproline were separated by chromatography after acid hydrolysis, and the radioactivity was measured. Since the control cells were fusing, it was not possible to express the data on a per cell basis. Instead, the base line used was noncollagen protein (i.e., after extraction with acetic acid). Since hydroxyproline is found exclusively in collagen and the degree of hydroxylation was not significantly different in control and Me_2SO-treated cultures, the relative amounts of collagen secreted in the medium were based on the amount of nondialyzable hydroxyproline found (Table 2).

After 72 hr of Me_2SO treatment there was a 1.7-fold increase in collagen secreted in the medium, as compared to control cultures. At 120 hr the relative increase in collagen secretion rose to almost 6-fold (Table 2).

Table 1. Relationship between length of Me_2SO exposure and recovery from Me_2SO

Hr of exposure to Me_2SO	% fusion ± SD	CPK activity ± SD*
0	85 ± 8.1	0.28 ± 0.03
24	32 ± 6.3	0.11 ± 0.02
48	7 ± 6.1	0.08 ± 0.05
72	0	0.06 ± 0.02
96	<0.3	0.05 ± 0.03

Cells were planted in 35-mm dishes with or without coverslip inserts (0.75×10^5 cells per dish). At times indicated the Me_2SO-containing medium was removed and replaced with drug-free medium. Fusion indices and CPK activity were measured after 144 hr in drug-free medium.

* Milliunit/μg of protein.

DISCUSSION

In this study, exposure of L8 cells to Me_2SO altered the genetic program from a myogenic pattern to a cell type that phenotypically resembled a differentiated fibroblast.† This cell transition was reversible only after a period of cell proliferation in Me_2SO-free medium. As in the Friend erythroleukemia system (1, 15, 16), the observed effects were time and dose dependent and, for optimal effects, Me_2SO had to be present during the period of rapid cell proliferation. A single cell doubling did not seem to be sufficient to bring about these phenotypic alterations; although continuous exposure of L8 cells (which have a generation time of 12–14 hr) to [^{3}H]thymidine in the presence or absence of Me_2SO for 24 hr resulted in intense nuclear labeling of virtually all cells, treatment with Me_2SO for 24 hr was insufficient to abolish differentiation along myogenic lines. However, this assumption must be put to more rigid experimental test, using synchronized cell cultures. Virtually all cells in this cell line can ultimately fuse to form myotubes under control conditions; the cells do not seem to be absolutely committed to the myogenic lineage until they have acquired the potential to fuse (see also refs. 17 and 18).

Inhibition of fusion by Me_2SO could have been due to a primary effect on the plasmalemma so that intracellular CPK

† In a recent abstract, Blau and Epstein (14) also reported that Me_2SO inhibited cell fusion and that there was no rise in CPK activity in L8 cells.

Table 2. Proline and hydroxyproline determinations and collagen secretion

Culture conditions	Fraction	cpm/mg cell protein		Hyp, Me_2SO/ control
		Pro	Hyp	
Control	Cells	78,116	2,605	
	Medium	20,164	7,113	
Me_2SO (72 hr)	Cells	84,512	5,105	1.9
	Medium	31,172	12,127	1.7
Control	Cells	88,608	2,320	
	Medium	33,321	13,023	
Me_2SO (120 hr)	Cells	76,072	5,185	2.2
	Medium	141,131	74,417	5.7

Twenty-four-hour cultures of L8 cells were grown in the presence or absence of 2% Me_2SO for 72 and 120 hr. Cultures were then exposed to [^{3}H]proline. The cpm were calculated per mg of cell protein (after extraction of collagen). All the recovered dialyzable tritiated hydroxyproline (Hyp) is part of the collagen; since the collagen in control and Me_2SO-treated cultures is hydroxylated to about the same extent, the relative amount of collagen synthesized and secreted into the medium can be calculated.

levels failed to rise because enzyme molecules had leaked into the medium. However, application of Me_2SO to prefusion-stage cultures did not affect the rate of fusion and intracellular CPK activity rose to control levels in the continued presence of Me_2SO. Cell transitions involving myogenic cells have also been noted under other conditions. Abbott *et al.* (18) found that cloned myogenic stem cells could generate progeny indistinguishable from fibroblasts by morphologic and biochemical criteria. Schubert and LaCorbiere (19) noted cell transitions of L6 myoblasts to chondrocyte-like cells under the influence of 6-aminonicotinamide, and to a lesser extent by dibutyryl cyclic AMP. Lipton (20) reported modulation of myogenic cells into collagen-forming fibroblast-like cells after treatment with 5-bromodeoxyuridine.

Another effect of Me_2SO on L8 cells was the striking change in growth pattern from randomly oriented, often multilayered, cell sheets in control cells to flattened, fibroblast-like monolayers of parallel aligned cells, concomitant with a relative increase in adhesivity of the cells to the substratum. Alteration of growth pattern and cell form by Me_2SO has been noted in other cell systems (21, 22), and Rabson *et al.* (11) reported that the morphology of CBT tumor cells reverted to normal on exposure to hexamethylene bisacetamide, concomitant with a large increase in fibroblast-type collagen synthesis. Therefore, selective environmental pressures may alter the differentiation of myogenic stem cells to fibroblast-like cells that synthesize fibrillar collagen. Virtually all cells are capable of synthesizing collagen proteins to some degree, and at least four types of normal collagen have been recognized (based on biochemical and antigenic properties). Type III collagen often exists as a fine, fibrillar network (reticulin; refs. 23, 24) which can be visualized by the Gomori silver impregnation method both *in vivo* and *in vitro* (9, 25). Neither control nor Me_2SO-treated L8 cultures exhibited argyrophilia, so that reticulin-type collagen was either not synthesized in large amounts or was not laid down into fibrillar networks. Type IV collagen is only present in basement membranes (13, 24). By electron microscopy, neither control nor Me_2SO-treated L8 cells showed a detectable basement membrane, not even after staining in bulk with uranyl acetate. Since the Me_2SO-treated cells are morphologically and ultrastructurally indistinguishable from fibroblasts (Fig. 3*b*) and the cells produced collagen fibrils, often with distinct periodicity of collagen (Fig. 3*c*), characteristic of connective tissue (collagen type I; see ref. 23), the Me_2SO-treated cells can be considered to be fibroblasts. The biochemical properties of the collagen produced by these cells should be studied to evaluate the less likely possibility that the L8 cells, converted by Me_2SO, are atypical chondrocytes [which synthesize collagen type II (13, 24)]. The present data, however, suggest that muscle stem cells could participate in the replacement of skeletal muscle by fibrous connective tissue in human diseases such as Duchenne muscular dystrophy.

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