MOLECULAR AND CELLULAR BIOLOGY, Dec. 1983, p. 2280–2286
0270-7306/83/122280-07$02.00/0

Vol. 3, No. 12

Induced Muscle Differentiation in an Embryonal Carcinoma Cell Line

MARY K. S. EDWARDS,[1] JOHN F. HARRIS,[2] AND MICHAEL W. McBURNEY[1]*

Departments of Medicine and Biology, University of Ottawa, Ottawa, Canada K1H 8M5,[1] and Department of Surgery, University of Toronto, The Wellesley Hospital, Toronto, Canada M4Y 1J3[2]

Received 29 March 1983/Accepted 13 September 1983

Cells of the teratocarcinoma-derived line P19S1801A1 (01A1) are pluripotent embryonal carcinoma cells and can be induced to differentiate when aggregated and exposed to dimethyl sulfoxide. Many nonneural cell types appear in dimethyl sulfoxide-treated cultures, cardiac and skeletal muscle being the most easily identified. We have used immunofluorescence procedures with monoclonal antibodies directed against muscle myosin to confirm and quantitate the number of muscle cells formed. A monoclonal antibody reactive with an embryonal carcinoma-specific surface antigen was used to confirm the disappearance of undifferentiated cells after dimethyl sulfoxide treatment. Cardiac muscle cells developed within 4 to 5 days of drug exposure, but skeletal muscle cells did not become evident until 7 to 8 days. We have isolated a mutant cell line (D3) which appears to be incapable of muscle development but which does form neurons and glial cells when exposed to high retinoic acid concentrations. We propose that this system will be useful for investigation of the means by which pluripotent cells become committed to development along the striated muscle lineages.

Embryonal carcinoma (EC) cell lines can differentiate in culture into a wide variety of cell types with specialized functions (9, 18). When injected into normal blastocysts, some of these cultured EC cells can participate in embryogenesis and contribute normal tissues to the developing fetus (22, 23, 27). Thus, it seems likely that EC cells differentiating in culture make use of normal embryonic developmental signals and that the system may be treated as a reasonable alternative to the embryo for studies concerned with early developmental events (17). Of particular interest are questions concerning the means by which pluripotent cells become committed to acquire particular differentiated characteristics, such as those of a neuron or a muscle. Our previous studies have demonstrated that cultures of P19 EC cells (21) can be induced to differentiate by cell aggregation in the presence of certain drugs and that the cell types formed depend on the drug used as well as on its concentration. In particular, we have shown that P19 cells treated with high, but nontoxic, doses of retinoic acid (RA) develop into neurons and astrocytes (12), whereas cells from the same population treated with dimethyl sulfoxide (DMSO) (20) or lower RA concentrations (5) differentiate primarily into mesodermal tissues, including muscle. Our ability to manipulate the development of these P19 cells should allow us to address experimentally questions of determination of cell fate.

The present experiments concern the further characterization of the muscle differentiation observed in these cultures. Although muscle is not the only cell type formed, our initial work concerns this cell type since it is easily recognized by light microscopy of living cultures and since a number of muscle-specific gene products are well characterized and available for the analysis of this cell type.

MATERIALS AND METHODS

Cell lines and culture conditions. Cell cultures were grown in alpha minimal essential medium (26) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2.5% fetal calf serum (GIBCO Laboratories) and 7.5% bovine serum (Canadian Veterinary Supplies, Perth, Ontario, Canada). Differentiation experiments were initiated by subculturing the cells into bacteriological-grade petri dishes as previously described (12). Under these conditions, the cells spontaneously form floating aggregates (19). The medium was replaced after 2 days, and the aggregates were plated onto tissue culture dishes after 4 days in suspension. In treated cultures, drugs were usually present in the medium only for the first 2 days of suspension culture, after which the aggregates were washed and transferred to fresh medium with no drugs. At various times after plating, aggregates were scored for the presence of morphologically recognizable cell types by using phase-contrast optics.

The cell lines used were P19 and a subclone resistant to 6-thioguanine and ouabain, P19S1801A1 (01A1), which have both been described (12, 20, 21). We also isolated a variant cell line, P19S1801A1D3 (D3), which

was nonresponsive to induction of differentiation by DMSO. An unmutagenized culture of 01A1 cells was aggregated in medium containing 1% DMSO and was plated out after 4 days in suspension. The proportion of undifferentiated EC cells was enriched by dispersing the culture and plating for 60 min at 37°C. The EC cells were recovered in the unattached fraction and were grown up. The culture was once again aggregated in DMSO, and the cycle was repeated three more times over a period of 10 weeks. After the final cycle, no differentiation occurred, and cells were cloned. One clone, D3, was examined in detail.

Immunofluorescence assays. Immunofluorescent staining of intermediate filaments in cells of intact plated aggregates was done as previously described (12). Muscle cells were stained by treatment first with a monoclonal antibody directed against muscle-specific myosin, then with fluorescein-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim [Canada] Ltd., Dorval, Quebec Canada) diluted 1:5. The anti-muscle myosin monoclonal antibody, MF20 (2), was generously provided by D. A. Fischman. It reacts with light meromyosin and was used as undiluted tissue culture supernatant. Immunofluorescent staining with the monoclonal antibody AEC3A1-9 (J. F. Harris, J. Chin, M. A. S. Jewett, M. Kennedy, and R. M. Gorczynski, manuscript in preparation) was done as described (13).

The proportion of cells staining with a particular antibody was determined by staining dissociated cells attached to poly-L-lysine cover slips as previously described (5).

CPK assays. Creatine phosphokinase (CPK) isozymes were separated by DEAE-Sephadex ion-exchange column chromatography (15) with 25 mM Tris-hydrochloride (pH 7.5) containing 50, 100, and 300 mM NaCl used as stepwise elution buffers. Cell extracts and column fractions were assayed for CPK activity by standard spectrophotometric techniques (25), and protein measurements were done using the modified Lowry procedure of Hartree (11).

RESULTS

Rhythmically contracting cardiac muscle and skeletal muscle (myoblasts and myotubes) are very easy to distinguish by visual inspection of live cultures. Therefore, the extent of muscle differentiation was evaluated in preliminary experiments by simply assessing the proportion of plated aggregates which contained muscle cells of each type. The visual scoring of muscle cells in this way may not accurately reflect the proportion of cells which were muscle, but it was useful in allowing us to establish conditions appropriate for muscle development. Most aggregates contained several hundred cells, and in this range, muscle seemed to develop equally well in aggregates of all sizes.

Kinetics of DMSO-induced muscle differentiation. Previous results (20) indicated that muscle development in 01A1 cultures required cell aggregation and exposure of these aggregates to DMSO. In all experiments reported below, aggregated cells were maintained in suspension for 4 days before being allowed to reattach to a plastic surface.

DMSO was effective in inducing differentiation, even if it was not continously present in the cultures (Fig. 1). Cells were aggregated in medium containing 1% DMSO, and at various times the aggregates were washed and transferred to medium without the drug. The aggregates were plated on day 4 and scored for morphologically recognizable cell types on days 7 through 9 of the experiment. Aggregates exposed to DMSO for 11 h or less contained virtually no muscle and abundant EC cells, whereas cultures exposed for 40 to 50 h contained abundant beating muscle and virtually no EC cells. The areas of beating muscle appeared to be more extensive than those in cultures maintained continuously in DMSO, perhaps because terminal muscle differentiation is inhibited by DMSO, as has been shown for L8 myoblasts (3). Subsequent experiments were routinely done with a 48-h exposure to DMSO.

The effect of DMSO concentration is shown in Fig. 2. With increasing concentrations between 0.25 and 1% DMSO, more and larger areas of muscle were observed, along with fewer and smaller areas of morphologically undifferentiated EC cells. The majority of aggregates contained large areas of beating muscle at DMSO concentrations above 0.5%. Skeletal muscle became apparent only at higher DMSO concentrations, 0.75% or more. Neurons were not seen except in small numbers in cultures treated with 1.25 or 1.5% DMSO.

Monoclonal antibodies which react with mus-

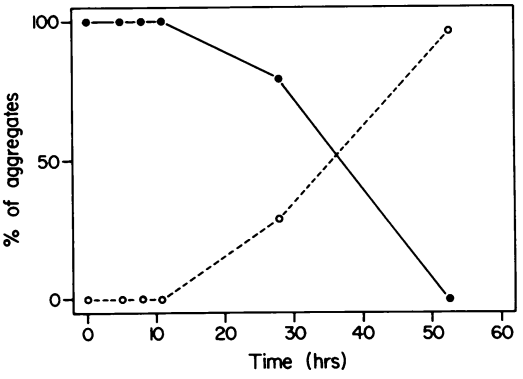

FIG. 1. Evidence that differentiation of 01A1 cells does not require continuous exposure to DMSO. DMSO (1%) was added at the initiation of cell aggregation (time 0), and at various times afterward aggregates were washed and transferred to fresh medium without the drug. Aggregates were plated at day 4 and were scored for the presence of morphologically undifferentiated EC cells (●) and beating muscle (○) on days 7 through 9 of the experiment. Between 25 and 40 aggregates were scored for each point.

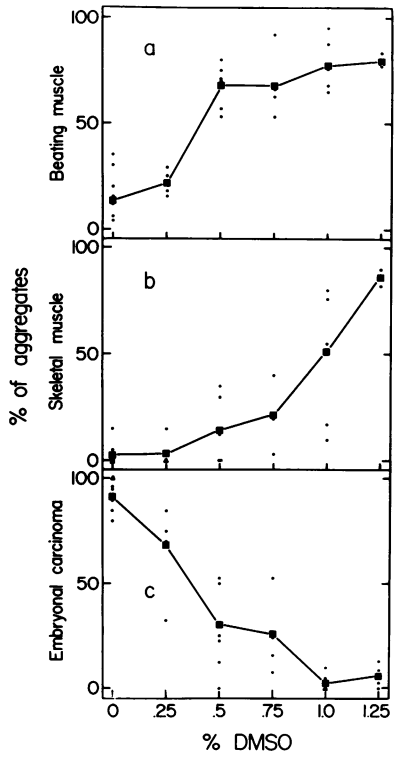

FIG. 2. Response of aggregates of 01A1 cells to various concentrations of DMSO. 01A1 aggregates were exposed to DMSO for 2 days in suspension, plated on day 4, and scored for the presence of beating muscle (a) and morphologically undifferentiated EC cells (c) on days 8 through 10 of the experiment. Skeletal muscle (b) was scored on days 10 through 12. Between 20 and 40 aggregates were scored for each point. The small circles represent the results of individual experiments, and the squares represent the overall weighted averages.

cle-specific myosin were used in immunofluorescence experiments to confirm the identity of the muscle cells in these cultures. Staining was observed in mononucleate cardiac muscle as well as in multinucleate skeletal muscle cells and unfused skeletal myoblasts (Fig. 3).

We also tested cell extracts from day 11 DMSO-treated cultures for the presence of the muscle-specific isozyme of CPK, CPK-M (1, 25, 30). Only a small amount (<5%) of the CPK activity was eluted off DEAE-Sephadex columns at salt concentrations indicative of the CPK-MB heterodimer. All other activity was of the BB form, the form present in nonmuscle tissue and in embryonic muscle cells (1, 25, 30).

The rate of muscle cell development in DMSO-treated cultures was followed by using morphological and immunofluorescent staining criteria. Beating muscle was evident at day 5, 1 day after the aggregates were plated (Fig. 4a). Many of the treated aggregates at days 5 or 6 contained areas that were morphologically recognizable as cardiac muscle but which did not start beating until 1 or 2 days later. Skeletal myoblasts appeared later than cardiac muscle and were first observed on day 7. The myoblasts increased in number and began to fuse into myotubes during the following 3 days of the experiment.

The proportion of muscle-myosin-positive cells was determined for parallel cultures in the same experiments (Fig. 4b). Muscle-myosin-positive cells were first observed in DMSO-treated cultures at day 4, while the aggregates were still in suspension, and increased during the rest of the experiment to 10 to 15% of the cells by day 10. Some of the positive cells were clearly multinucleate towards the end of the experiment, so the number of muscle-myosin-

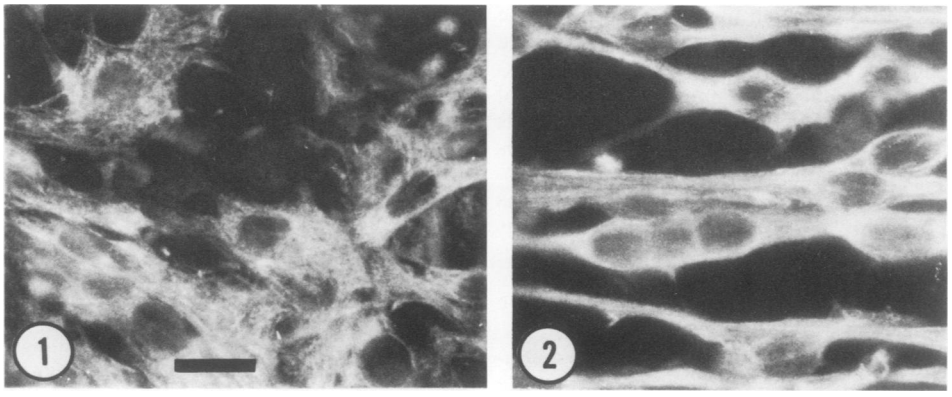

FIG. 3. Cardiac muscle (panel 1) and skeletal muscle (panel 2) in DMSO-treated 01A1 cultures. Both types of muscle cells stained brightly in immunofluorescence experiments with antibody against muscle-specific myosin. This staining was dependent on the presence of the first antibody, and only cells with presumptive muscle form were stained.

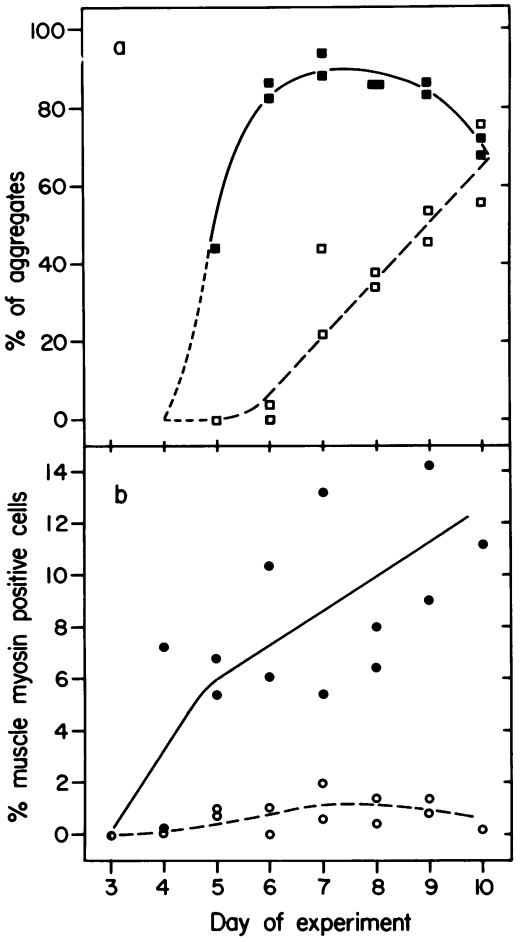

FIG. 4. Rate of muscle development in DMSO-treated 01A1 aggregates. (a) Aggregates were exposed to 1% DMSO for 2 days, plated at day 4, and then scored daily for the presence of beating (■) and skeletal (□) muscle. Fifty aggregates were scored for each point. (b) Replicate 01A1 cultures were dispersed at daily intervals during the differentiation protocol and stained with antibody against muscle-specific myosin. The proportion of muscle-myosin-positive cells is indicated for untreated (○) and 1% DMSO-treated (●) cultures. Five hundred cells were scored for each point. Both panels in this figure represent the results of two separate experiments.

containing cells represents an underestimate of the number of muscle nuclei.

In addition to inducing the appearance of muscle and other unidentified differentiated cell types, DMSO also induced the disappearance of undifferentiated EC cells from aggregated cultures. The disappearance of cells with the EC cell-specific AEC3A1-9 antigen was monitored in DMSO-treated cultures by using immunofluorescence, immunoabsorbance, and Western blot analyses with results similar to those reported in of the accompanying article (13, Fig. 5 and 6).

Effects of other drugs on muscle development. Since DMSO is a potent inducer of murine erythroleukemia (MEL) cells (6, 7), a number of other MEL cell inducers (10, 16) were tested for their effect on 01A1 cells. Neither hemin nor ouabain induced differentiation, but both 6-thioguanine and butyric acid had the same effect as DMSO (Table 1), that is, cardiac muscle was abundant at low concentrations, and skeletal muscle was abundant at higher drug concentrations. At the highest drug concentrations (slightly toxic doses), some aggregates developed neurons. Thus, the relationship between drug dose and cell type formed is similar for all of the differentiation-inducing drugs, RA (5), DMSO, 6-thioguanine, and butyrate. Of these four drugs, only RA is not toxic at doses high enough to induce efficient neural differentiation.

Retinoids are present in the bovine serum used to supplement the culture medium. Medium supplemented with 10% fetal calf serum contains about 4×10^{-8} M retinol (8; see also product analysis of Hyclone Sterile Systems, Logan, Utah). Since DMSO is known to facilitate the transport of drugs across biological membranes (6, 28), it could conceivably act to induce muscle formation simply by increasing the permeability of 01A1 cells to the serum retinoids. To test this hypothesis, fetal calf serum was delipidized by the method of Rothblat et al. (24), and 01A1 cells were tested for their response to DMSO in medium supplemented only with this delipidized serum. According to Fuchs and Green (8), this procedure removes all detectable vitamin A from the medium, so it should eliminate the DMSO response completely if the above hypothesis is correct. In three experiments with two different batches of delipidized serum, DMSO remained effective in inducing muscle differentiation, suggesting that

TABLE 1. Differentiation of 01A1 aggregates exposed to 6-thioguanine and butyrate

Drug	Concn (mM)	% of aggregates containing cell types:			
			Muscle		
		EC	Beating	Skeletal	Neurons
6-Thioguanine	20	70	5	0	0
	60	21	42	0	0
	180	0	50	67	11
Butyrate	0.5	30	45	10	0
	1	15	50	40	15
	2	20	0	10	40

DMSO does not act indirectly via the serum retinoids.

In another test of the above hypothesis, 01A1 aggregates were exposed simultaneously to 0.5% DMSO and to graded concentrations of RA to determine whether DMSO would potentiate the action of RA. The DMSO did not alter the relationship between RA concentration and the cell types formed (Fig. 5) except at low RA doses, at which the effect was due to the DMSO itself. In the experiment shown in Fig. 5, the proportion of aggregates scored with neurons was low because the aggregates were scored on day 11, when many of the distinctive neural processes had receded, making identification of neurons more difficult.

In P19 cell aggregates treated with high concentrations of RA, the cell size decreased dramatically compared with the untreated controls during the first 6 days of the experiment (13). In contrast, cells in DMSO-treated 01A1 aggregates remained similar in size to cells of untreated controls (data not shown).

Isolation and characterization of mutant cells. One way to elucidate the intracellular mechanism of DMSO action might be to isolate mutant cells no longer responsive to the inducing effects of the drug and to attempt to identify the defective cellular component. With this in mind, we isolated a cloned subline of 01A1 cells called P19S1801A1D3 (D3), which no longer differentiated when aggregated in the presence of DMSO. D3 cells have a model chromosome number of 77.

Figure 6 shows the response of D3 and of the parental lines to DMSO. Both P19 and 01A1 responded to DMSO by a decrease in the proportion of aggregates containing EC cells and by an increase in the proportion containing muscle. P19 consistently produced less cardiac and skeletal muscle than 01A1, although DMSO caused the disappearance of EC cells from both cultures with equal efficiency. Cells of the D3 line did not differentiate at any of the DMSO concentrations tested. Thus, DMSO-treated D3 cells did not develop beating muscle (Fig. 6a) or muscle-myosin-positive cells, but they remained morphologically undifferentiated (Fig. 6b), and 95 to 100% were stained with the EC-specific monoclonal antibody AEC3A1-9. D3 cells did not differentiate in the presence of butyrate (1 mM) or 6-thioguanine (180 mM), nor did muscle develop in response to low doses of RA (10^{-9} to 10^{-8} M). At higher RA concentrations (10^{-8} to 10^{-6} M), neurons did develop efficiently, with the same dose response as in the parental 01A1 cells. Glial cells were also present in D3 cultures treated with high RA concentrations, as judged by immunofluorescent staining with antibodies against glial fibrillar protein.

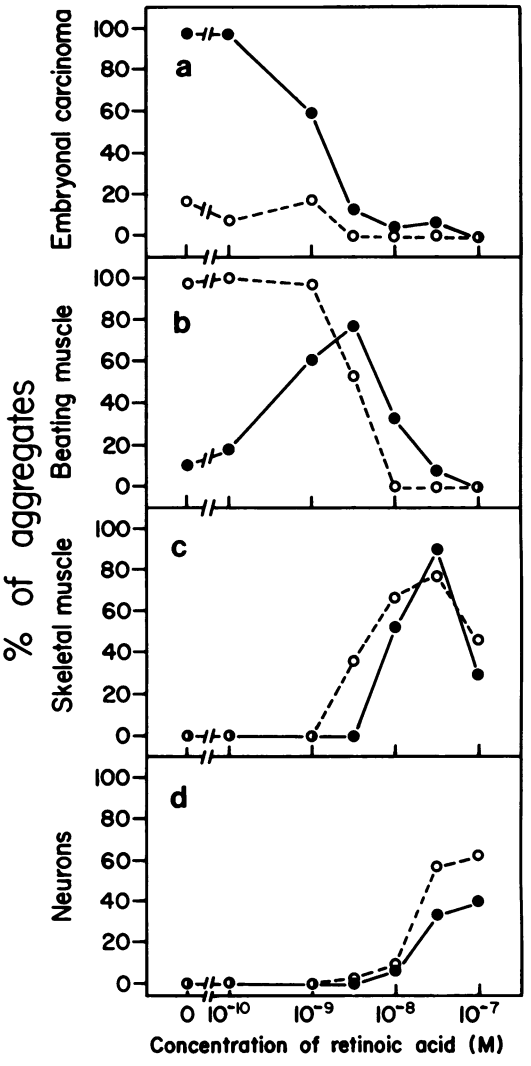

FIG. 5. Differentiation patterns produced by RA in the presence and absence of DMSO. O1A1 aggregates were exposed to various concentrations of RA in the presence (○) or absence (●) of 0.5% DMSO. These aggregates were plated on day 4 and scored on day 11 for the presence of morphologically recognizable cell types. This figure shows the proportion of aggregates scored as containing EC cells (a), beating muscle (b), skeletal muscle (c), and neurons (d).

DISCUSSION

The results reported above extend our previous observations (20) and elaborate on the conditions required for the induction of muscle differentiation in 01A1 cells.

The muscle cells in DMSO-treated 01A1 cultures contain muscle-specific myosin as judged by immunofluorescent staining. However, only

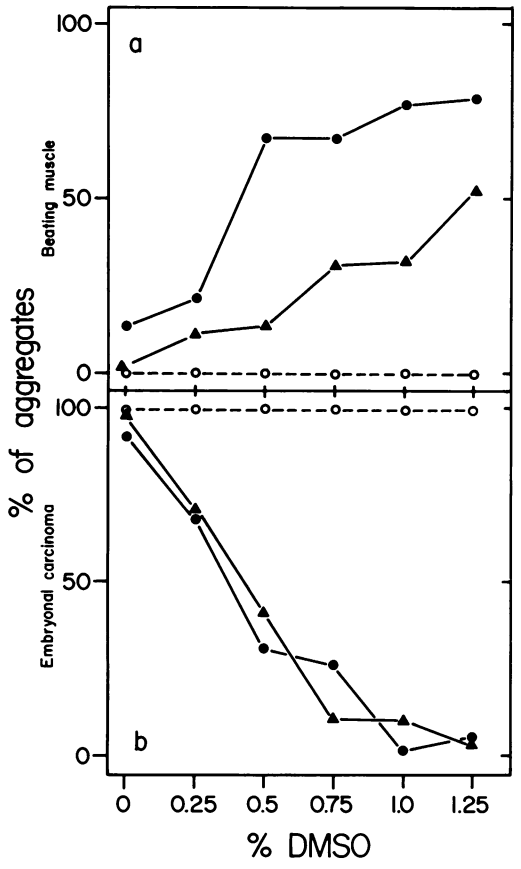

FIG. 6. Failure of D3 aggregates to respond to DMSO. Aggregates of 01A1 (●), P19 (▲), and D3 (○) were exposed to various concentrations of DMSO for 2 days, plated on day 4, and scored for the presence of beating muscle (a) and undifferentiated EC cells (b) on days 8 through 10 of the experiment. The data shown are averaged from seven experiments for 01A1 and D3 and from three experiments for P19.

small amounts of the muscle-specific CPK-M isozyme were present in cell extracts from DMSO-treated cultures. The M isomer of CPK is not expressed in embryonic mouse muscle until the last few days of gestation (1). Thus, the very small amount of the CPK-M form in DMSO-induced muscle, taken together with earlier electron microscopic observations (20) indicating the presence of only immature myofibrils, suggests that the muscle tissue in 01A1 cultures is equivalent to immature embryonic muscle.

The length of drug treatment, the concentration of the drug, and cell aggregation are all important factors in the efficient induction of 01A1 cell differentiation. Continuous exposure to the inducing drug was not required for differentiation. The drug could be removed from the aggregated cultures 2 or 3 days before the first muscle-myosin-containing cells were observed. This observation supports an induction model for the drug action (20) in which the drug serves only as a trigger for a series of cellular events culminating in the differentiation of the cell types observed.

The mutant D3 cells do not differentiate under any of the conditions, including treatment with low concentrations of RA, which would normally induce the parental 01A1 cells to form muscle. D3 cells do, however, respond to higher concentrations of RA by differentiating into neurons and glial cells. Thus, this cell line may be a mutant specifically blocked in the ability to develop into certain cell types or in the ability to respond to a particular set of developmental signals.

The four chemicals (DMSO, RA, 6-thioguanine, and butyrate) active in inducing 01A1 differentiation all show a similar relationship between dose and differentiation: as the dose of drug increases, the proportion of cells remaining as undifferentiated EC decreases, but different dose levels result in the production of different specialized cell types. The pattern of cell types produced over the range of concentrations tested is the same for all of these drugs: cardiac muscle is most abundant at low doses, and higher doses are required for skeletal muscle. Still higher doses of RA induce the abundant formation of neurons and glia (12). The highest doses of the other three drugs also produced some neurons, but these concentrations approached toxic levels, so the extensive neural differentiation seen with RA was not observed with these drugs.

The similarity of response of 01A1 cells to the four inducing drugs reported here, and the block in differentiation in response to these drugs in the RAC65 cell line (13), suggest that there are some common intracellular effects produced by these chemically unrelated inducing agents. The action of DMSO did not seem to be mediated by serum retinoids because DMSO was effective in inducing differentiation in medium containing lipid-free serum and because DMSO did not potentiate the response of 01A1 cell aggregates to high RA doses. DMSO has effects on a large number of other biological systems and has been extensively studied as an inducer of differentiation of MEL cells (6, 7). Recent evidence with the MEL cells suggests that a rise in cytoplasmic Ca^{2+} is a consequence of DMSO treatment and is a necessary but insufficient condition for MEL differentiation (4, 14, 29). Calcium metabolism may be important in 01A1 differentiation, and effects on Ca^{2+} metabolism or Ca^{2+}-mediated processes may be the common consequence of the action of the four differentiation-inducing drugs.

ACKNOWLEDGMENTS

We thank D. A. Fischman for the generous gift of monoclonal antibodies to muscle myosin. We also thank John Bell for immunofluorescent staining of glial cells in D3 cultures, José Campione-Piccardo for preparation of delipidized serum and for helpful discussions, and J. Chin for expert technical assistance.

This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada.

LITERATURE CITED

1. **Adamson, E.** 1976. Isoenzyme transitions of creatine phosphokinase, aldolase, and phosphoglycerate mutase in differentiating mouse cells. J. Embyol. Exp. Morphol. **35**:355–367.
2. **Bader, D., T. Masaki, and D. A. Fischman.** 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. J. Cell Biol. **95**:763–770.
3. **Blau, H. M., and C. J. Epstein.** 1979. Manipulation of myogenesis *in vitro*: reversible inhibition by DMSO. Cell **17**:95–108.
4. **Bridges, K., R. Levenson, D. Housman, and L. Cantley.** 1981. Calcium regulates the commitment of murine erythroleukemia cells to terminal erythroid differentiation. J. Cell Biol. **90**:542–544.
5. **Edwards, M. K. S., and M. W. McBurney.** 1983. The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. Dev. Biol. **98**:187–191.
6. **Friend, C., and B. A. Freedman.** 1978. Effects and possible mechanism of action of dimethyl sulfoxide on Friend cell differentiation. Biochem. Pharmacol. **27**:1309–1313.
7. **Friend, C., W. Scher, J. G. Holland, and T. Sato.** 1971. Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. U.S.A. **68**:378–382.
8. **Fuchs, E., and H. Green.** 1981. Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. Cell **25**:617–625.
9. **Graham, C. E.** 1977. Teratocarcinoma cells and normal mouse embryogenesis p. 315–397. *In* M. I. Sherman (ed.), Concepts in mammalian embryogenesis. M.I.T. Press, Cambridge, Mass.
10. **Gusella, J. F., and D. Housman.** 1976. Induction of erythroid differentiation *in vitro* by purines and purine analogues. Cell **8**:263–269.
11. **Hartree, E. F.** 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48**:422–427.
12. **Jones-Villeneuve, E. M. V., M. W. McBurney, K. A. Rogers, and V. I. Kalnins.** 1982. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J. Cell Biol. **94**:253–262.
13. **Jones-Villeneuve, E. M. V., M. A. Rudnicki, J. F. Harris, and M. W. McBurney.** 1983. Retinoic acid-induced neural differentiation of embryonal carcinoma cells. Mol. Cell. Biol. **3**:2271–2279.
14. **Levenson, R., D. Housman, and L. Cantley.** 1980. Amiloride inhibits murine erythroleukemia cell differentiation: evidence for a Ca^{--} requirement for commitment. Proc. Natl. Acad. Sci. U.S.A. **77**:5948–5952.
15. **Lough, J., and R. Bischoff.** 1977. Differentiation of creatine phosphokinase during myogenesis: quantitative fractionation of isozymes. Dev. Biol. **57**:330–344.
16. **Marks, P. A., and R. A. Rifkind.** 1978. Erythroleukemic differentiation. Annu. Rev. Biochem. **47**:419–448.
17. **Martin, G. R.** 1975. Teratocarcinomas as a model system for the study of embryogenesis and neoplasia. Cell **5**:229–243.
18. **Martin, G. R.** 1980. Teratocarcinomas and mammalian embryogenesis. Science **209**:768–776.
19. **Martin, G. R., and M. J. Evans.** 1975. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **72**:1441–1447.
20. **McBurney, M. W., E. M. V. Jones-Villeneuve, M. K. S. Edwards, and P. J. Anderson.** 1982. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature (London) **299**:165–167.
21. **McBurney, M. W., and B. J. Rogers.** 1982. Isolation of male embryonal carcinoma cells and their chromosomal replication patterns. Dev. Biol. **89**:503–508.
22. **Papaioannou, V. E., M. W. McBurney, R. L. Gardner, and M. J. Evans.** 1975. Fate of teratocarcinoma cells injected into early mouse embryos. Nature (London) **258**:70–73.
23. **Rossant, J., and M. W. McBurney.** 1982. The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. J. Embyol. Exp. Morphol. **70**:99–112.
24. **Rothblat, G. H., L. Y. Arbogast, L. Ouelette, and B. V. Howard.** 1976. Preparation of delipidized serum protein for use in cell culture systems. In Vitro (Rockville) **12**:554–557.
25. **Shainberg, A., G. Yagil, and D. Yaffe.** 1971. Alterations of enzymatic activities during muscle differentiation *in vitro*. Dev. Biol. **25**:1–29.
26. **Stanners, C. P., G. Eliciera, and H. Green.** 1971. Two types of ribosomes in mouse-hamster hybrid cells. Nature (London) New Biol. **230**:52–54.
27. **Stewart, T. A., and B. Mintz.** 1981. Successive generations of mice produced from an established culture line of euploid teratocarcinoma cells. Proc. Natl. Acad. Sci. U.S.A. **78**:6314–6318.
28. **Stroughton, R. B., and W. Fritsch.** 1964. Influence of dimethyl sulfoxide (DMSO) on human percutaneous absorption. Arch. Dermatol. **90**:512–517.
29. **Tsiftsoglu, A. S., A. A. Mitrani, and D. E. Housman.** 1981. Procaine inhibits the erythroid differentiation of MEL cells by blocking commitment: possible involvement of calcium metabolism. J. Cell. Physiol. **108**:327–335.
30. **Ziter, F. A.** 1974. Creatine kinase in developing skeletal and cardiac muscle of the rat. Exp. Neurol. **43**:539–546.