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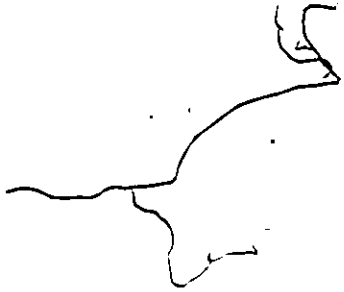
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LA THÈSE A ÉTÉ  
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INDUCED DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS



by

Mary K.S. Edwards

A thesis  
presented to University of Ottawa  
in partial fulfillment of the  
requirements for the degree of  
Master of Science  
in  
Biology

OTTAWA, Ontario, 1983

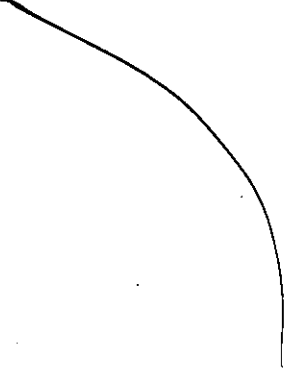
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smoothly, Jose Campione-Piccardo gave me advice on arranging my data, and a supply of delipidized serum, and Steve Smith and Luc Aujame helped with translation. Thanks, gang.

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To Mark

## ABSTRACT

Embryonal carcinoma (EC) cells, the stem cells of malignant teratocarcinomas, can differentiate in culture into a wide range of tissue types, and are considered a model system for early embryonic events. Using the P19 line of EC cells, which differentiates minimally in culture, we have attempted to influence the differentiation repertoire by the addition of various compounds to the culture medium. This thesis describes the response of a subclone of P19 (O1A1) to dimethyl sulfoxide (DMSO), butyric acid, 6-thioguanine (6-TG), and retinoic acid (RA). In the presence of DMSO, butyric acid or 6-TG, O1A1 cells differentiated into a limited range of cell types, most obviously cardiac and skeletal muscle. The response of O1A1 to DMSO was dependant on cell aggregation, but not on continuous exposure to the drug. Skeletal muscle appeared at higher DMSO concentrations and later in the experiment than cardiac muscle. With O1A1, as previously shown with the parental P19 cells, high doses of RA ( $10^{-7}$  M or more) induced the formation of neurons and glial cells. Lower concentrations of RA induced O1A1 aggregates to develop muscle; predominantly cardiac muscle at  $10^{-9}$  M, and skeletal muscle at  $10^{-6}$  M. There is a similarity between the responses of O1A1 cells to graded doses of the

various compounds tested: cardiac muscle at low doses, skeletal muscle at higher ones. Extensive neural differentiation was only seen with RA, probably because the other drugs were toxic at the concentrations that would be required. Thus with these drugs, the differentiation of O1A1 cells can be induced and directed in culture, giving a new level of control to the EC cell system.

## RESUME

Les cellules carcinomes embryonnaires (CE) sont les cellules souches des tératocarcinomes et peuvent se différencier en plusieurs types de tissus. On les considère ainsi comme un modèle des événements embryonnaires. En utilisant la lignée cellulaire P19, qui se différencie minimalement en culture, nous avons essayé d'influencer le répertoire de différenciation par l'addition de divers produits au milieu de culture. Cette thèse décrit les effets du sulfoxide diméthylé (DMSO), de l'acide butyrique, de la 6-thioguanine et de l'acide rétinoïque (AR) sur une sous-lignée de P19 (O1A1). En présence de DMSO, de l'acide butyrique ou du 6-thioguanine, les cellules O1A1 se différencient en un nombre limité de types cellulaires, les muscles cardiaques et squelettiques étant les plus visibles. L'effet de DMSO sur O1A1 dépend de l'agrégation des cellules mais pas de la présence continue du produit. Du muscle squelettique apparaît à des concentrations de DMSO plus élevées et plus tard dans l'expérience que le muscle cardiaque. Avec O1A1, comme il l'a déjà été démontré pour la lignée parente P19, les concentrations élevées d'AR ( $10^{-7}$  M ou plus) donnent lieu à des neurones et des cellules gliales. En présence des concentrations plus basses d'AR, les agrégats d'O1A1 se différen-

cie en cellules musculaires, surtout du muscle cardiaque à  $10^{-9}$  M, et du muscle squelettique à  $10^{-8}$  M. Il y a une similitude entre les réponses de O1A1 à des concentrations différentes de produits testés; du muscle cardiaque aux concentrations les plus basses; du muscle squelettiques aux concentrations plus élevées. Une forte différenciation en neurons n'a été observé qu'avec l'AR, probablement du fait de la toxicité des autres produits aux concentrations requises. Ainsi, avec ces produits, la différenciation des cellules O1A1 peut-être dirigé au milieu de culture, introduisant de cette manière un nouveau niveau de contrôle du système cellulaire du CE.

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List of Abbreviations

CPK	creatine phosphokinase
DMSO	dimethyl sulfoxide
EC	embryonal carcinoma
EDTA	ethylene diamine tetraacetic acid
FITC	fluorescein isothiocyanate conjugated
GFAP	glial fibrillar acidic protein
HMBA	hexamethylene bisacetamide
HPRT	hypoxanthine phosphoribosyl transferase
MEL	murine erythroleukemia
FBS	phosphate buffered saline
RA	retinoic acid
6-TG	6-thioguanine

## Chapter I

### INTRODUCTION

The field of developmental biology has itself developed from the elegant descriptive studies of the classical embryologists to the more recent aetiological emphasis on developmental events at the biochemical and genetic level. Many developmental systems, myogenesis (Buckingham, 1977) and erythropoiesis (Harrison, 1976) in particular, have been carefully studied and much is known about their biochemical sequence of events. These studies, however, have as their starting point cells which are already restricted to the formation of only one mature cell type. Thus they do not touch on the determination events that commit pluripotent cells in the embryo to follow one, and not any other, developmental route. Embryonal carcinoma (EC) cells, the stem cells of malignant teratocarcinomas, provide a culture system that may allow the study of these events (Graham, 1977).

#### DIFFERENTIATION OF TERATOCARCINOMAS IN VIVO

Teratocarcinomas are malignant tumors that usually originate in the gonads, in a wide range of species including humans, and are made up of disorganized masses of various differentiated cell types such as epithelia, muscle, neurons, bone

and cartilage, plus a stem cell population of undifferentiated EC cells (Stevens, 1967a). Tumors with the differentiated cell types, but without the EC stem cells are benign and are called teratomas (Stevens and Pierce, 1975). Strains of mice have been isolated which are particularly susceptible to spontaneous testicular (line 129, Stevens, 1958), or ovarian (line IT, Stevens and Varnum, 1974) teratomas and teratocarcinomas. The tumors can also be produced experimentally by transplanting either early (up to eight days) mouse embryos (Stevens, 1968, 1970, Solter et al 1975, Iles, 1977), or genital ridges from twelve-day embryos (Stevens 1967b, 1970, Pierce, 1967) to extrauterine sites in syngenic host mice. These experimentally derived tumors contain the same range of cell types as the spontaneous ones, including the EC stem cells.

Some of these tumors are transplantable; the EC cells but not the differentiated cell types from teratocarcinomas can give rise to a new tumor if they are injected subcutaneously into a host mouse (Pierce and Dixon, 1959, Pierce et al, 1960). When they are injected into the peritoneal cavity, EC cells multiply as solid tumors on the mesentery and as numerous floating aggregates. These aggregates are known as embryoid bodies because of their resemblance to early mouse embryos: they often become cystic and contain cells resembling cells of the yolk sac, mesenchyme and embryonal epithelia, as well as EC cells (Stevens, 1960, Pierce et al, 1960).

EC cells are pluripotent, since even a single EC cell injected into a host mouse can form a tumor containing a wide range of differentiated cell types (Kleinsmith and Pierce, 1964). EC cells share other morphological (Damjanov et al, 1971), biochemical (Martin et al, 1978), and immunochemical (Damjanov and Solter 1974) features with pluripotent cells of the early embryo. Thus the differentiation of EC cells can be considered (Martin, 1980, Graham, 1977) to be analogous to the differentiation of cells in the early post-implantation embryo. This analogy is strongly supported by the demonstration that EC cells from tumors can respond to normal developmental signals if they are injected into a blastocyst which is then implanted into the uterus of a foster mother (Brinster, 1974, Mintz and Illmensee, 1975). The mice that develop from these blastocysts contain normal cells derived from both the host blastocyst and the injected tumor cells, in varying proportions. The EC cells, instead of forming tumors as they would if injected into an adult mouse, can be controlled by the embryonic environment and participate in the normal development of a wide range of tissues. In one of these mice, nicknamed 'Terry Tom', enzyme analysis showed that the EC derived cells were present in all the tissues tested, and breeding experiments showed that the EC cells had formed functional sperm.

## DIFFERENTIATION OF EC CELLS IN VITRO

EC cell lines have been established in vitro and can be subjected to the usual range of cell culture techniques: cloning, subculturing, storage in biological freezers, etc. They retain their undifferentiated morphology (Martin, 1975), their high alkaline phosphatase levels (Bernstine et al., 1973), and usually a normal or near-normal karyotype over a great number of cell generations, provided they are maintained in exponential growth. For many of these cell lines, this requires culture with a nondividing feeder cell layer, or on collagen coated dishes. EC cells in culture retain their pluripotential nature, both in the ability to form teratocarcinomas with a variety of cell types (reviewed, Martin, 1975), and the ability to contribute to normal tissue formation after injection into a blastocyst (Papaioannou et al., 1975).

EC cells in vitro differentiate under conditions of dense multilayered culture. This can be accomplished by allowing cultures to grow without subculturing until they are very dense (Nicolas et al., 1975). Colonies of EC cells that form when sparsely plated cultures are left undisturbed also provide the closely packed conditions that allow differentiation (McBurney, 1976). Embryoid bodies can also be formed by EC cells in culture if they are plated under conditions that do not allow them to adhere to the culture dish. This can be done, as first described by Martin and Evans (1975a), by

plating feeder-dependant EC cells to dishes without feeder cells, or by transferring the EC cells to bacteriological grade Petri dishes. The embryo bodies are similar to those formed by EC cells in vivo. They can be simple, containing only EC cells, or somewhat more complex; usually with a layer of endoderm and Reichert's membrane-like material around the outside. The latter often develop fluid-filled cysts after four to five days in suspension. Little further differentiation occurs while these cell aggregates are kept in suspension (Martin and Evans, 1975a). When they are allowed to reattach to tissue culture dishes, however, the aggregates of most EC cell lines develop areas of many different tissue types, including muscle, neurons, cartilage and epithelium (Martin and Evans, 1975b).

Various biochemical markers have been used to follow the differentiation of EC cells in culture: alpha-fetoprotein, hemoglobin, creatine phosphokinase, and acetylcholinesterase are examples of markers for differentiated cell types that have been found by various authors in differentiating EC cultures (reviewed Graham, 1977). Also, as EC cells differentiate, they lose the expression of other markers such as surface antigens (Artz et al, 1973, Stern et al, 1976, Jacob, 1977) which they share with cells of early (up to day nine) mouse embryos.

EC cells are thought to be equivalent to cells in the early post-implantation embryo. The embryonic cells at this

stage are going through critical commitment events, but are inaccessible for manipulation except via surgery, the number of cells is very small, and a great number of events are occurring simultaneously. In the EC culture system, the number of cells is limited only by incubator space (and graduate student man-hours!), and they are easily manipulated in culture. However, the differentiation patterns of multipotential EC cells in culture are complex, so it is difficult to study any one determinative event without interference from other simultaneous ones.

SIMPLIFYING THE EC CELL SYSTEM

A few EC cell lines do show less complex differentiation patterns in culture. There are some EC lines, those that produce only the very simple aggregates, which show no differentiation in vitro (Martin and Evans, 1975b). One EC cell line has been described that differentiates into a wide range of tissues in vivo but preferentially forms neurons in culture (Pfeiffer et al, 1981). Another, F9 (Bernstine et al, 1973), spontaneously develops endoderm but not other cell types, in culture or in tumors (Sherman and Miller, 1978).

One approach towards study of simpler differentiation patterns in EC cell cultures is to try to identify drugs or culture conditions that either restrict the formation of certain differentiated cell types in multipotential EC lines, or allow some specific differentiation in cells that



would not otherwise express those functions. Some success has been obtained with the F9 cell line, which differentiates into extraembryonic endoderm in monolayer culture in response to retinoic acid (RA) (Strickland and Mahdavi, 1978). With further treatment of these cultures with dibutyryl cAMP, these cells change into parietal endoderm (Strickland et al, 1980, Kuff and Fewell, 1980). Visceral endoderm is produced by F9 cells when they are treated with RA as aggregates (Hogan et al, 1981). Hexamethylene bisacetamide (HMBA) has also been reported to induce differentiation; in the multipotent PCCA/1 line (Jakob et al, 1978) and in the PCC4azal line, which differentiates poorly in culture (Speers et al, 1979), but not in F9 (Jakob et al, 1978). The cell types produced by HMBA treatment were not precisely characterized in these reports. Speers et al (1979) described the cells morphologically as "epithelial" in monolayer experiments and as "fibroblast-like" if aggregates were treated.

Previous work in this laboratory has shown that RA induces aggregates of the EC cell line P19 to form neurons, glial, and fibroblast-like cells (Jones-Villeneuve et al, 1982). P19 is a karyotypically normal male cell line that was derived directly from a solid teratocarcinoma obtained by embryo transplantation (McBurney and Rogers, 1982). It does not require feeder cells for growth in culture. The only differentiated cell type produced spontaneously by P19

in culture is extraembryonic endoderm, observed in patches around the periphery of untreated aggregates. These cells have been shown to be pluripotent *in vivo* since they participate in the formation of a range of normal tissues when they are injected into blastocysts (Rossant and McBurney, 1982). Thus RA induces the expression of some but not all of the differentiative potential of P19, which would not otherwise be expressed in culture.

P19S1801A1 (O1A1), is a 6-thioguanine (6-TG) and ouabain resistant subclone of P19, isolated without mutagenesis (McBurney et al, 1982). In the process of isolating this clone, it was observed that some of the colonies in medium with 6TG contained areas of beating muscle. 6TG, like HMBa, is an inducer of erythroid differentiation in Friend cells, or murine erythroleukemia (MEL) cells (Gusella and Houseman, 1976). This suggested that other drugs active in the MEL system might also have an effect on the differentiation of O1A1 cells. Of these, we found that 6-TG, butyric acid, and dimethyl sulfoxide (DMSO), but not hemin or ouabain, induced the formation of cardiac and skeletal muscle, but not neurons or glia, in aggregates of O1A1 cells (McBurney et al, 1982). Thus these compounds induce O1A1 cells to differentiate into a limited range of cell types, different from those induced by RA.

MEL cells have been used as a model system for the study of the regulation of differentiation. Some of the observa-

tions made in these studies, which are summarized in the next section, may also be applicable to the response of C1A1 cells to some of the same inductive compounds.

#### MEL CELLS: CHEMICALLY INDUCED DIFFERENTIATION IN VITRO

MEL cell lines, derived from leukemic mice infected with the Friend virus complex, were first established by Charlotte Friend and her coworkers in 1966. The transforming Friend virus (Friend 1957) is a complex of a defective spleen focus forming virus and a murine leukemia helper virus (Mirand et al, 1968). In susceptible mice, infection with Friend virus produces hepatosplenomegaly and erythroblastosis (Friend, 1957). The target cell for infection appears to be one of the early erythropoietin-responsive red blood cell precursors (Mirand, 1967). In culture, MEL cells appear to represent a population of chronically infected erythroid precursor cells arrested at the proerythroblast stage of development (Friend et al, 1966, Singer et al, 1974). Most of these cell lines display a low level of spontaneous differentiation in culture, as judged by benzidine staining for hemoglobin production (Singer et al 1974, Orkin et al, 1975, Rovera and Bonaiuto, 1976, Preisler et al, 1976), though a few differentiate at a fairly high rate (Rovera and Bonaiuto, 1976). In the presence of 1-2% DMSO, approximately 90% of the cells differentiate to the normoblast level (Friend et al, 1971). During this differentiation, the cells undergo

a series of changes that parallel normal red blood cell maturation (Harrison, 1976). These include chromatin condensation, withdrawal from the cell cycle, and heme synthesis (Friend et al, 1971), accumulation of globin mRNAs, globin and hemoglobin (Gusella et al, 1980, Boyer et al, 1972), increased activity of the enzymes delta-aminolevulinic acid synthetase (Ebert and Ikawa, 1974, Takahashi et al, 1974), and carbonic anhydrase (Kabat et al, 1975), as well as production of the membrane protein spectrin (Eisen et al, 1977, Friend, 1971). Thus even though MEL cells are transformed, and grow and differentiate independently of erythropoietin (Mirand et al, 1967, Hankins and Krantz, 1975, Liac and Axelrad, 1975), they are considered to follow essentially a normal pattern of erythroid differentiation.

MEL cells can be induced to differentiate by a number of different compounds (reviewed by Marks and Rifkind, 1978). These include DMSO, and other polar low molecular weight compounds such as HMEA (Tanaka et al, 1975, Fibach et al, 1977) and N-methyl formamide (Reuben et al, 1976). Some normal cellular components such as purines and their derivatives (Gusella and Houseman, 1976) and short chain fatty acids (Takahashi et al, 1975) are also effective inducers, as are ouabain (Bernstein et al, 1976) and other metabolic inhibitors (Ebert et al, 1976). The diversity in chemical structure of these inducers seems to indicate that they do not all act by the same mechanism, though many of these compounds have effects on cell membranes.

DMSO has been shown to have a number of effects on cellular systems which could account for its inductive ability (Friend and Friedman, 1978). Recently, the membrane effects of DMSO; a decrease in membrane fluidity and an increase in  $Ca^{++}$  transport, have been the focus of a number of studies. Local anaesthetics such as procaine and tetracaine inhibit DMSO induction of MEL cells (Bernstein et al, 1976b, Tsiftsoğlu et al, 1980), and also increase the fluidity of the cell membrane, an effect opposite to that of DMSO (Lyman et al, 1976). An increase in  $Ca^{++}$  transport has also been implicated as a factor in DMSO induction (Levenson et al, 1971, Bridges et al, 1981).

Genetic studies have also been used to investigate the action of inducers on MEL cells. Variant MEL lines have been established that are responsive to some inducers, but not to others, strengthening the argument that different agents may act by different mechanisms (Rovera and Surrey, 1978). In cell hybridization experiments using inducible MEL cell lines and these non-responsive variants, the hybrids produced are usually also non-responsive (Harrison, 1977), indicating in most cases a <sup>4</sup>trans-dominant genetic arrangement for the non-inducible mutation.

### OUTLINE OF THE THESIS PROJECT

The work reported in this thesis primarily expands on the initial observations of differentiation of O1A1 cells in response to DMSO and RA. The first stage of this project, reported in Chapter 3, was a more detailed investigation of the DMSC response kinetics, using morphological and immunological criteria. The response of O1A1 to varying doses of RA was also studied, using a similar approach but a wider range of markers, since the differentiation pattern was more complex than expected. This, plus further studies using other MEL inducers, is reported in Chapter 4. The third phase of this project was some preliminary investigation into the mechanism of the O1A1 response to DMSO, and its relationship with the response to RA. This involved some genetic studies similar in approach to those done with MEL cells, and treatment of the cells with DMSO under varying culture conditions.

## Chapter II

### MATERIALS AND METHODS

#### CULTURE CONDITIONS

Cell cultures were grown in alpha medium (Stanners et al, 1971) (Gibco Laboratories, Grand Island, N.Y.), supplemented with 2.5% fetal calf serum (Gibco) and 7.5% bovine serum (Canadian Veterinary Supplies, Perth, Ont.) at 37 degrees C., in a humidified 5% carbon dioxide atmosphere. Cells were routinely subcultured every two days using  $Ca^{++}$  and  $Mg^{++}$  -free phosphate buffered saline (PBS) containing 0.25% trypsin and 1 mM ethylene diamine tetracetic acid (EDTA) to remove them from the tissue culture surfaces. Cultures were routinely replaced from frozen stocks every six weeks.

#### DIFFERENTIATION EXPERIMENTS

The routine protocol used for differentiation experiments is as follows: cells in exponential growth were subcultured into bacteriological grade petri dishes, at a final concentration of about  $10^5$  cells/ml. The cells spontaneously formed floating aggregates under these conditions (Martin and Evans, 1975b). The medium was replaced after two days and the aggregates were plated into tissue culture dishes

after four days in suspension. In treated cultures, drugs were usually present in the medium only for the first two days of aggregation. Then the aggregates were washed and transferred to fresh medium without drugs (Fig.1). Aggregates were scored using phase contrast optics for the presence of morphologically recognizable cell types at various times after plating, as indicated in the text.

#### CELL DENSITY AND CELL VOLUME

Measurements were made on a Coulter counter with channelizer and X-Y plotter attachments (Coulter Electronics, Hialeah, Fla.). The channel number that was the average between the two channel numbers containing 50% of the maximum number of counts was used to give an indication of the cell size. These values were then used to calculate the relative cell volumes (Chapter 3, Fig.7).

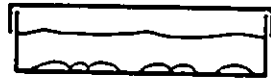
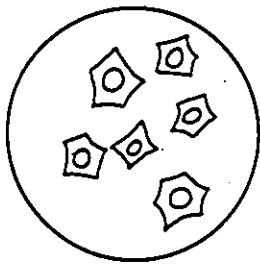
#### RETINOIC ACID PREPARATION

Retinoic acid (Sigma Chemical Co., St.Louis, MO) was prepared as a stock solution at  $10^{-2}$  M in 95% ethanol, and stored at 4 degrees C. in the dark. The stock solution was diluted directly into the culture medium to obtain the final desired concentration (Jones-Villeneuve et al, 1982).

Figure 1

The usual protocol for differentiation experiments with O1A1 cells involves cell aggregation and a two day exposure to DMSO or other drugs. Undifferentiated O1A1 cells (upper left) are transferred to bacterial grade Petri dishes where they form aggregates. These aggregates are exposed to DMSO or other compounds for two days, then plated after four days in suspension into tissue culture dishes. They adhere to these dishes, and the differentiated cell types grow out onto the culture surface where they can be observed under phase contrast optics (lower left).





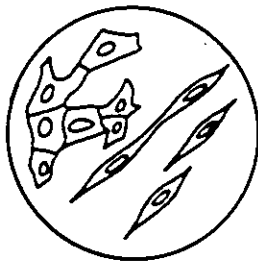
Day 0



Day 2



Day 4



7

TABLE 1

Antibodies used in immunofluorescence staining experiments.

antigen	antibody	source	dilution
muscle myosin	MF20H2H8 monoclonal mouse IgG tissue culture supernatant	D.A. Fischman	undiluted
	M-7 monoclonal mouse IgG concentrated tissue culture supernatant	P. Merrifield	1:20
	FITC goat anti-mouse IgG	Boehringer	1:20
E.C. surface antigen	AEC3A1-9 monoclonal mouse IgM ascitic fluid	J. Harris	1:50
	FITC rabbit anti- mouse IgM	Cedarlane Laboratories, Hornby, Ont.	1:5
glial fibrillar acidic protein	polyclonal (rabbit)	V. Kalnins	1:35
	FITC goat anti- rabbit IgG	Hyland, Costa Mesa, CA	1:5
cytokeratin- associated protein	TROMA-1 monoclonal rat IgG tissue culture supernatant	R. Kemler	1:1
	FITC goat anti-rat IgG	Cedarlane	1:5

FITC:fluorescein isothiocyanate conjugated

## IMMUNOFLUORESCENCE ASSAYS

Staining of cells in intact plated aggregates was accomplished by plating aggregates onto glass cover slips and fixing and staining them *in situ*. The cells were rinsed once in PBS, pH 7.0, then fixed for four minutes in methanol and for two minutes in acetone, both at -20 degrees C. The cover slips were rinsed again in PBS, then covered with the first antibody at the concentrations indicated in Table 1, for 45 minutes. Unbound first antibody was removed by three five-minute washes in PBS, then the aggregates were treated with the appropriate fluorescein conjugated second antibody (Table 1), again for 45 minutes, and again washed three times with PBS. The cover slips were mounted cell side down on glass microscope slides, in a drop of 50% glycerol in PBS, and examined under fluorescence optics.

The procedure for staining with AEC3A1-9 was slightly different, since this antibody reacts with a surface antigen. Fixation (15 min. in -20 degree methanol) was done after the staining procedure instead of preceding it. Since the aggregates were not fixed during staining and washing, these manipulations were done on ice, and a 1:1 mixture of alpha medium and PBS was used for dilutions and washes. These cells were also counterstained with 1 ug/ml ethidium bromide.

To determine the proportion of cells staining with a particular antibody, aggregates were dissociated with PBS con-

taining 1 mM EDTA. The cell suspension was washed twice with PBS, then diluted with FBS to  $2 \times 10^6$  cells/ml. Cover slips were coated with poly-L-lysine by dipping them into a 1 mg/ml solution of poly-L-lysine in water for one minute. These cover slips were then covered with the cell suspension for 10 minutes to allow the cells to settle, rinsed with PBS, and stained as above. 500 cells were scored for each culture.

#### CHROMOSOME PREPARATION

Cultures in exponential growth were exposed to 0.06 ug/ml colcemid for 1-2 hours, suspended in PBS containing trypsin and EDTA, washed in PBS, then incubated at 37 degrees C in 0.56 M KCl. The cells were fixed in a cold 3:1 mixture of methanol:acetic acid, and the drops of the fixed cell suspension were dropped onto cold microscope slides. The chromosome spreads were air dried and G-banded by dipping the slides into a solution of 0.025% trypsin in 0.9% NaCl, for 30-60 seconds, then staining them with Giemsa stain. 20-30 metaphase spreads were examined for each cell line.

#### PLATING EFFICIENCY

Six 60-mm culture dishes were set up for each culture tested, and incubated for 7-8 days. These were duplicate cultures, with  $10^2$ ,  $10^3$ , or  $10^4$  cells, in medium containing  $10^{-4}$  M mercaptoethanol. The proportion of viable cells was det~~er~~

etermined by trypan blue exclusion. Colonies were fixed with Bouin's fixative and counted. Plating efficiency results were calculated from average counts of dishes containing less than 150 colonies, and corrected for the proportion of viable cells.

#### SOURCES OF CHEMICALS

All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., or Fisher Scientific, Missisauga, Ont., with the exceptions of the following; trypan blue (Flow Laboratories, Missisauga, Ont.), DEAE-Sephadex A-50 beads (Pharmacia Fine Chemicals, Uppsala, Sweden), CPK electrophoresis buffer (Gelman Instrument Co., Ann Arbor, MI).

### Chapter III

#### THE EFFECTS OF DMSO ON P19S1801A1 CELLS

Two methods were used to study DMSO induction of myogenesis in 01A1 cells: visual scoring of plated aggregates, and immunofluorescent staining with specific antibodies. Visual scoring was used alone to follow the response of 01A1 to different lengths of exposure to DMSO, and different concentrations of the drug. The most important question in these experiments was whether or not conditions had been established within any one aggregate that would induce myogenesis. In the third section of this chapter, dealing with the kinetics of the appearance of muscle cells and the disappearance of undifferentiated EC cells in DMSO treated cultures, the relative proportion of these cell types becomes important, so immunofluorescence staining was also used.

#### **RESULTS**

##### **Length of exposure to DMSO**

Our initial experiments indicated that differentiation of 01A1 cells did not require continuous exposure to DMSO. To determine the length of exposure required to induce cardiac muscle differentiation, cells were aggregated in medium con-

taining 1% DMSO, and at various times afterwards the aggregates were transferred to medium without the drug. The aggregates were plated on day 4 and scored for morphologically recognizable cell types on days 7-9 of the experiment (Fig. 1). Aggregates exposed to DMSO for eleven hours or less contained virtually no muscle, whereas cultures exposed for 40-50 hours contained beating muscle in 90% of the aggregates. These areas of beating muscle appeared to be more extensive than those in cultures maintained continuously in DMSO, possibly because DMSO inhibits myogenesis (Blau and Epstein, 1979). Subsequent experiments were therefore routinely done using a 48-hour exposure to DMSO.

After aggregate formation, shorter exposure times appeared to be sufficient for the DMSC response. Aggregates were formed in the absence of drug, then treated with DMSO for 28-29 hours, beginning on day 2 or 3 of the experiment. Virtually all of these aggregates contained beating muscle when they were scored on day 9.

#### Concentration of DMSO

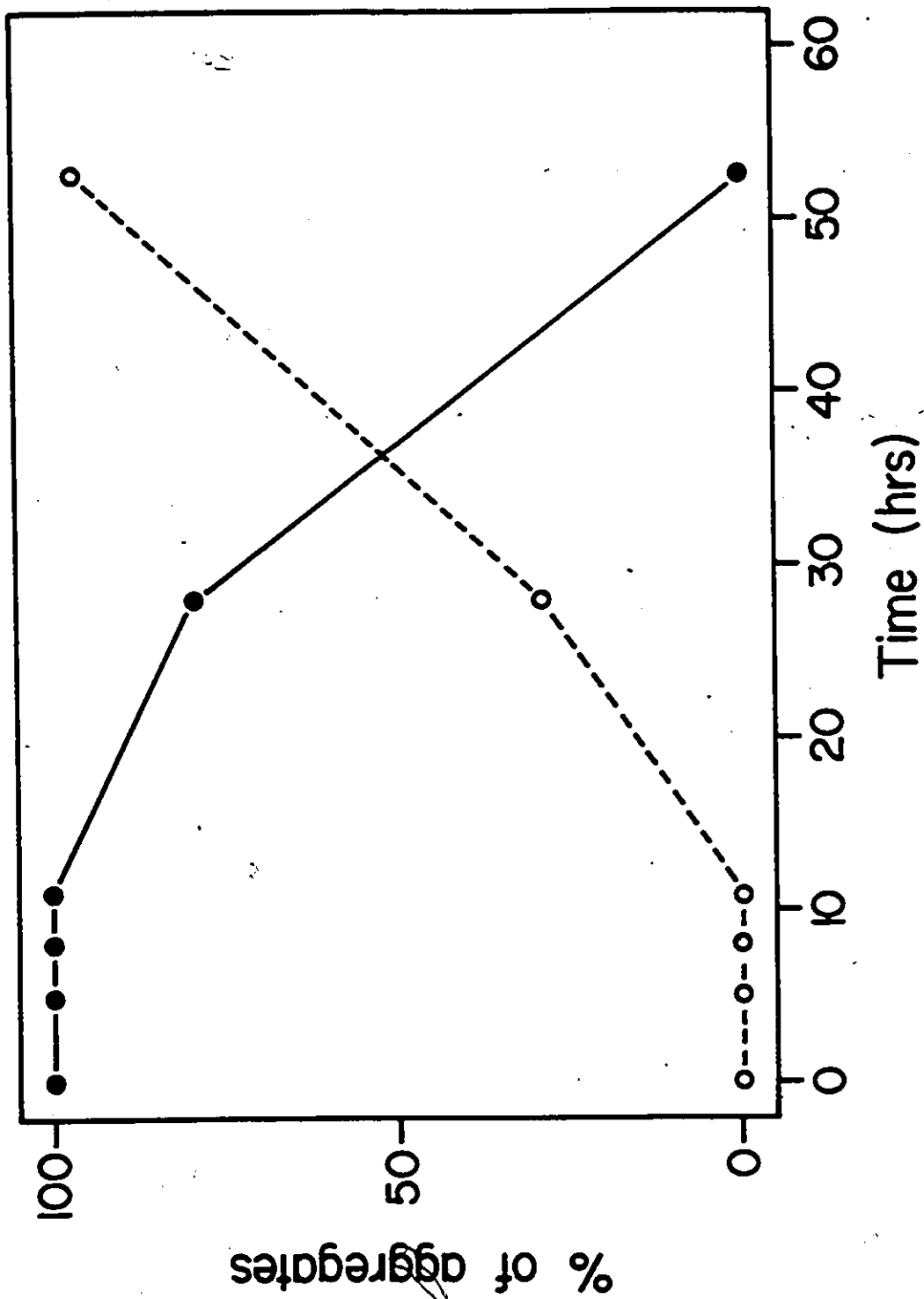
Another aspect of our initial experiments that has been explored in more detail is the response of O1A1 aggregates to various concentrations of DMSO. With increasing concentrations between 0.25% and 1% DMSO, more and larger areas of muscle were observed, along with fewer and smaller areas of morphologically undifferentiated EC cells (Fig. 2). The ma-

majority of aggregates contained large areas of beating muscle at DMSO concentrations above 0.5%, and those which did not, commonly contained areas that appeared to be quiescent cardiac muscle. Skeletal muscle became apparent at higher DMSO concentrations, 0.75% or more. Neurons were not seen except for a small number of cells in cultures treated with 1.25 or 1.5% DMSO. Monoclonal antibodies which react with muscle-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 multinucleate skeletal muscle cells and unfused skeletal myoblasts (Fig.3)

A small proportion of O1A1 aggregates not exposed to DMSO developed areas of beating muscle. This proportion increased slightly with the time aggregates remained in suspension before plating. In one experiment, 7.5 % of the untreated aggregates developed beating muscle when plated after 4 days in suspension. This did not increase significantly when the suspension time was increased to 10 days: after this time only 9% of the aggregates showed beating muscle. No skeletal muscle or neurons were observed in untreated O1A1 aggregates. This was considered to represent a consistent but low level of spontaneous differentiation in the untreated aggregates.

**Figure 1**

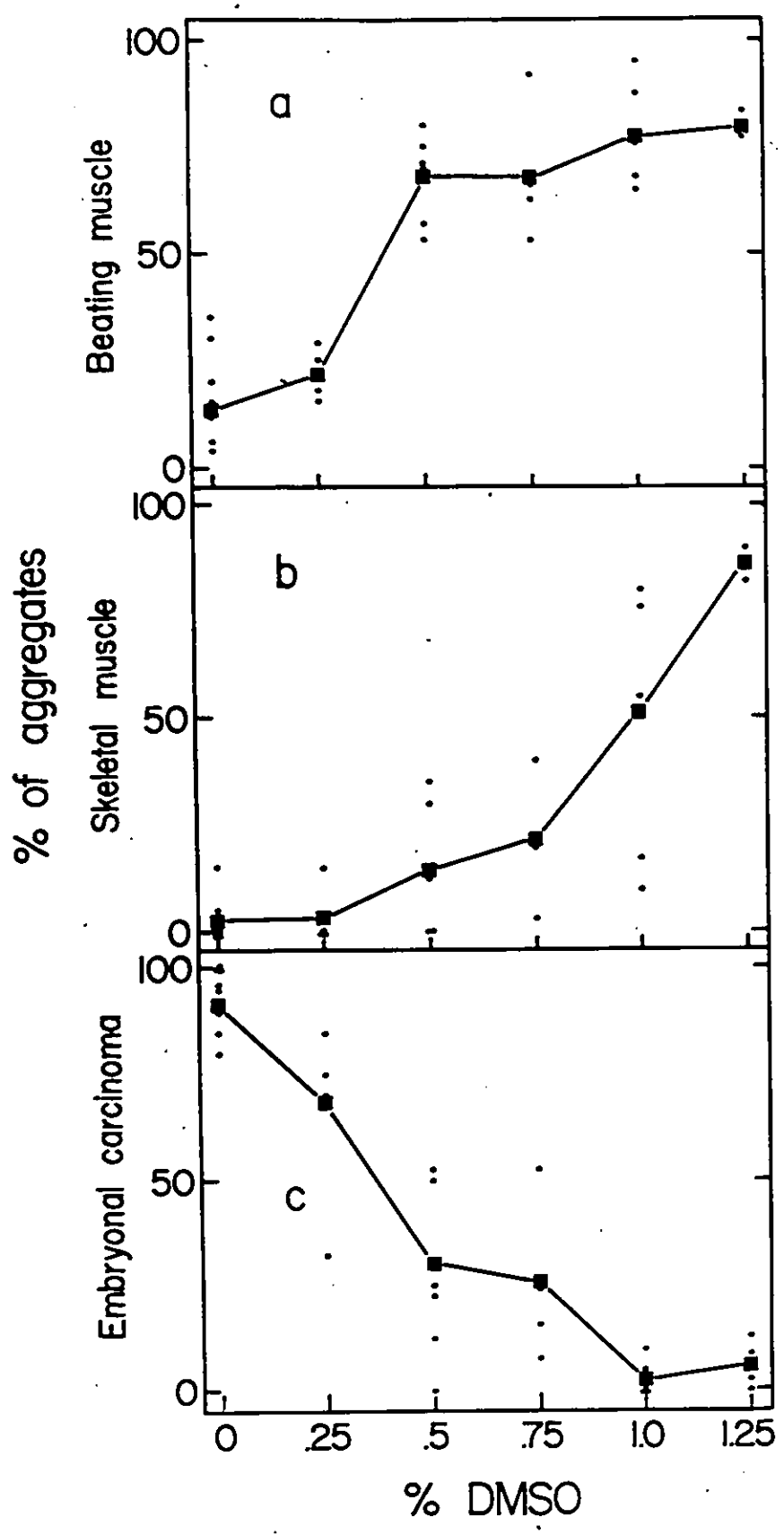
The response of O1A1 cells to DMSO does not depend on continuous exposure to the drug. 1% DMSO was added, at the initiation of aggregation (time 0), and at various times afterward aggregates were washed and transferred to fresh medium without the drug. Aggregates were plated at day 4, and scored for the presence of morphologically undifferentiated EC cells (closed circles) and beating muscle (open circles) on days 7-9 of the experiment. 25-40 aggregates were scored for each point. This was repeated four times, and this figure shows the results from one representative experiment.



**Figure 2**

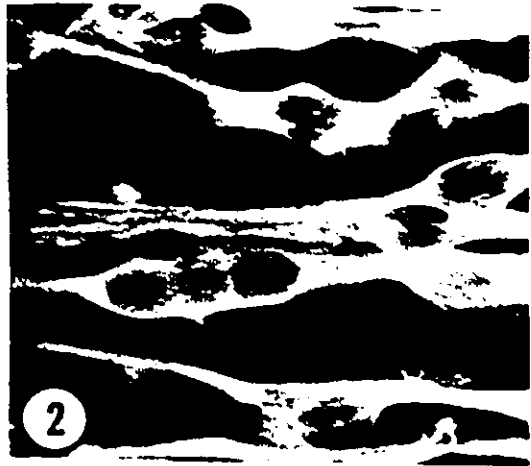
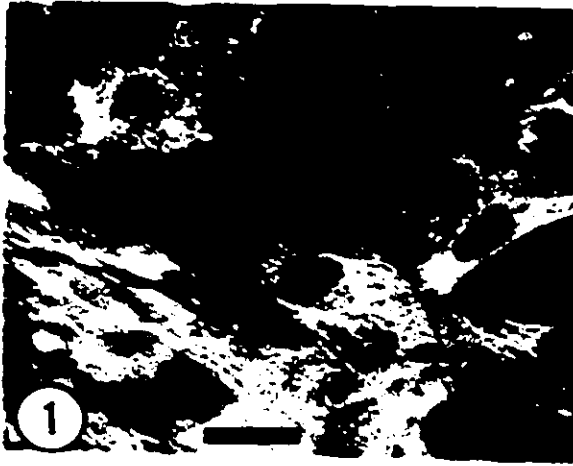
The response of O1A1 aggregates to various concentrations of DMSO. O1A1 aggregates were exposed to DMSO for 2 days in suspension, plated on day 4, and scored for the presence of beating muscle (panel a) and morphologically undifferentiated EC cells (panel c) on days 8-10 of the experiment. Skeletal muscle (panel b) was scored on days 10-12. 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.

4



**Figure 3**

Both cardiac (1) and skeletal (2) muscle in DMSO-treated O1A1 cultures stained brightly in immunofluorescence experiments with antibody against muscle specific myosin. This staining was done with MF20H2H8, and similar staining patterns were observed with M-7. Bar, 25  $\mu$ m.



### Time course

The appearance of muscle cells in DMSO treated cultures was followed using morphological and immunofluorescent staining criteria. Cardiac muscle was evident at day 5, one 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 one or two days later. Skeletal myoblasts appeared later than cardiac muscle, and were first observed on day 7. They increased in number and began to fuse into myotubes during the following three 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 10-15% of the cells by day 10. Some of the positive cells were clearly multinucleate towards the end of the experiment, so the cell number represents an underestimate of the number of muscle nuclei. The muscle myosin antibody used in these experiments was MF20H2B8, obtained from Drs. D.A. Fischman and D. Morgenstern. This antibody apparently reacts with light meromyosin (D.A.Fischman and D.Morgenstern, Down State Medical Centre, NY, personal communication) Similar results were obtained with M-7, obtained

from Dr. P. Merrifield, University of Virginia. Aggregates treated with 0.5% DMSO gave a similar pattern of muscle development over time, except that the proportion of aggregates with skeletal muscle, and the proportion of muscle myosin containing cells were consistently lower.

The aggregation phase of the experiment could be shortened to two days without affecting the proportion of aggregates which produced beating muscle after DMSO treatment. Plating the aggregates at this early stage, however, did seem to delay the appearance of muscle by about two days, so that the beating muscle was not observed until days 7-8. Development of beating muscle was not entirely dependant on plating, since a few DMSO treated aggregates, left in suspension 6-7 days, contained some beating cells.

The same cell suspensions used for the muscle myosin staining experiments above were also used in three assays to monitor the disappearance of undifferentiated EC cells in DMSO treated cultures (Fig.5). The relative plating efficiency of DMSO treated cultures dropped to about 1%. The proportion of cells staining with AEC3A1-9, a monoclonal antibody against an EC surface antigen (J.Harris, Dept. of Surgery, University of Toronto, in preparation) dropped from 90-100% to about 25%. The staining of the positive cells was much less intense in the treated cultures than in the untreated ones. Thirdly, immunabsorbtion experiments with AEC3A1-9, done by J.Harris in Toronto, showed that the am-

ount of the EC antigen per cell decreased to about 1% of control values.

Staining of intact aggregates plated onto cover slips showed that some of the cells in DMSO treated aggregates stained weakly in a spotted pattern on the cell surface, particularly where the cells were adhering to the glass (Fig.6.2). In contrast, EC cells in monolayer or in untreated aggregates showed a more intense pattern of staining on virtually every cell. This change in staining pattern suggests that the weakly positive cells in DMSO treated cultures did not represent a residual population of undifferentiated EC cells.

In P19 aggregates treated with RA, the cell size decreased dramatically compared with the untreated controls during the first six days of the experiment (Jones-Villeneuve et al, Department of Biology, University of Ottawa, in preparation). In contrast, cells in DMSO treated O1A1 aggregates (Fig.7) were the same size as untreated controls throughout the suspension phase of the experiment. Both the treated and untreated cells showed some decrease in volume, presumably due to accumulation of cells in the G1 phase of the cell cycle.

Procaine, a local anaesthetic, has been shown (Bernstein et al, 1976, Tsiftsoglu et al, 1981) to inhibit the DMSO-induced differentiation of MEL cells. This inhibition is particularly effective if the cells are pretreated with pro-

caine before DMSO induction. Procaine also had an inhibitory effect on DMSO-induced muscle differentiation of O1A1 cells. This inhibition was dependant on the concentration of procaine, but did not change if the cells were pre-treated with the anaesthetic for 24 hours before the addition of DMSO. Procaine had no effect on the differentiation of O1A1 cells into neurons in response to  $10^{-7}$  M RA (data not shown).

**Figure 4**

Muscle appears in DMSO-treated O1A1 aggregates. Panel a: Aggregates were exposed to 1% DMSO for 2 days and plated at day 4, then scored daily for the presence of beating (closed circles) and skeletal (open circles) muscle. 50 aggregates were scored for each point. Panel b: Replicate O1A1 cultures were dispersed at daily intervals during the differentiation protocol, and stained with antibody against muscle specific myosin. The closed circles represent the myosin positive cells in 1% DMSO treated aggregates, and the open circles, untreated control aggregates. 500 cells were scored for each point. This figure represents the results of two separate experiments.

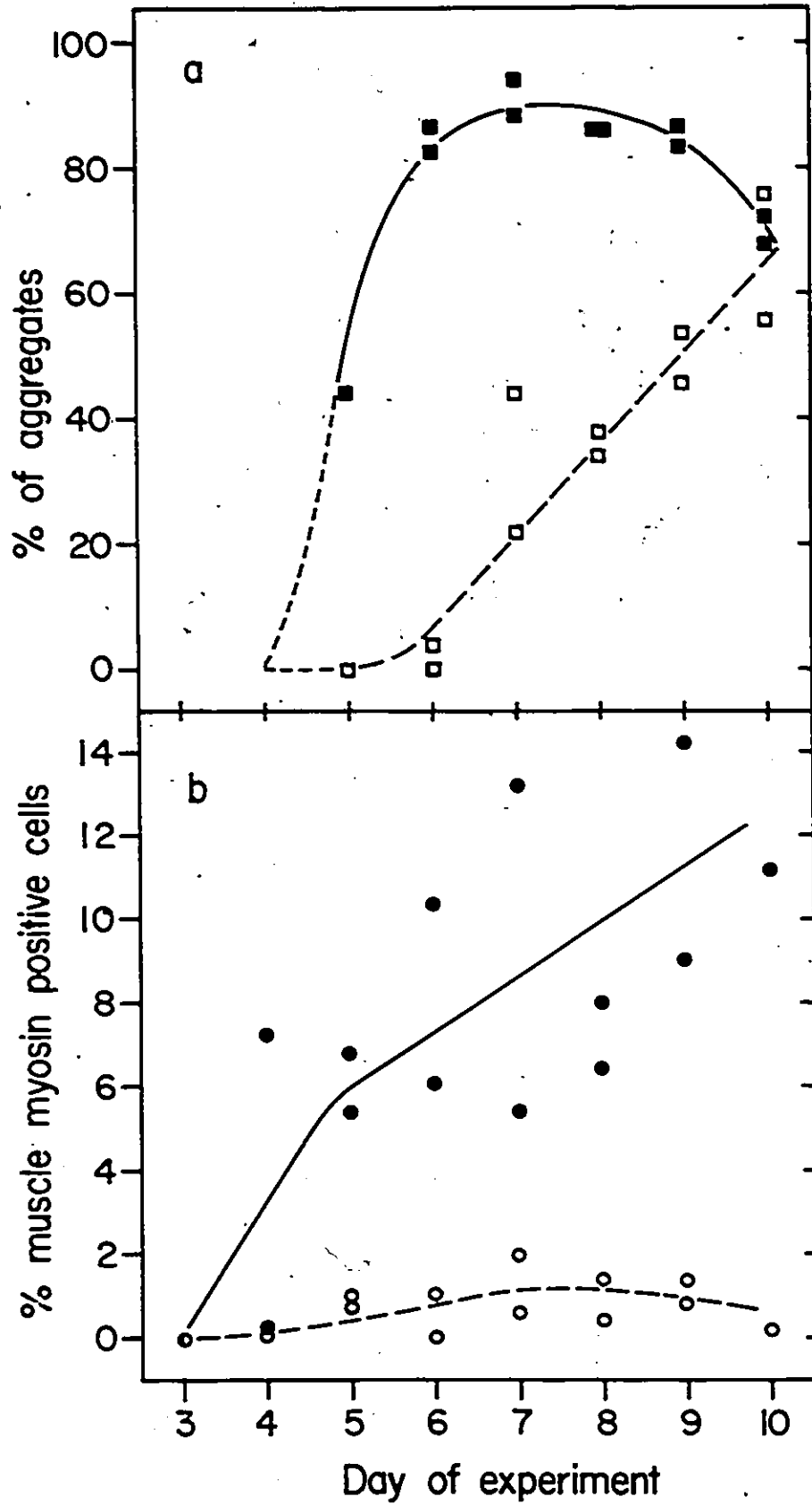
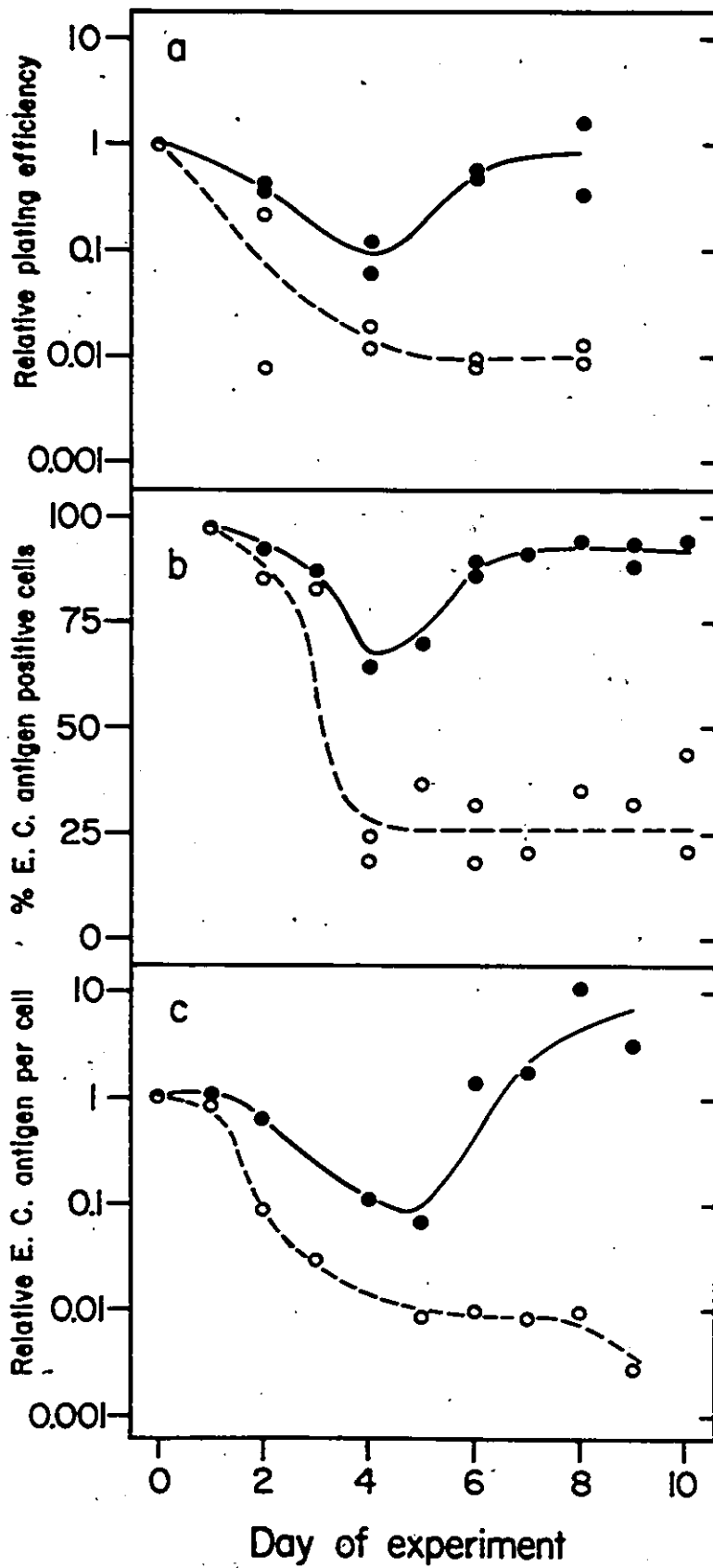


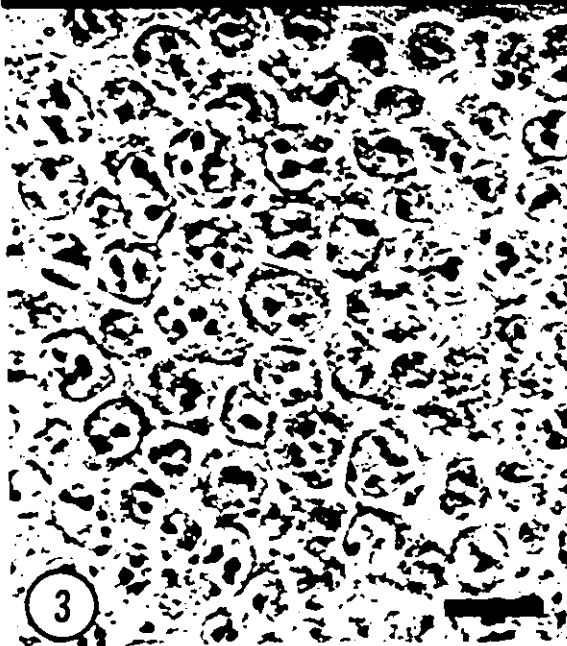
Figure 5

EC cells disappear from DMSO treated cultures. The plating efficiency (panel a) and proportion of cells staining with AEC3A1-9 (panel b) was determined at various times for replicate cultures of O1A1 aggregates, either untreated (closed circles) or treated with 1% DMSO (open circles). These panels represent the results of two separate experiments. Cells from these cultures were also analysed by immunabsorbtion for the relative amount of the EC surface antigen (recognized by AEC3A1-9) present per cell. (panel c, data from J.Harris)



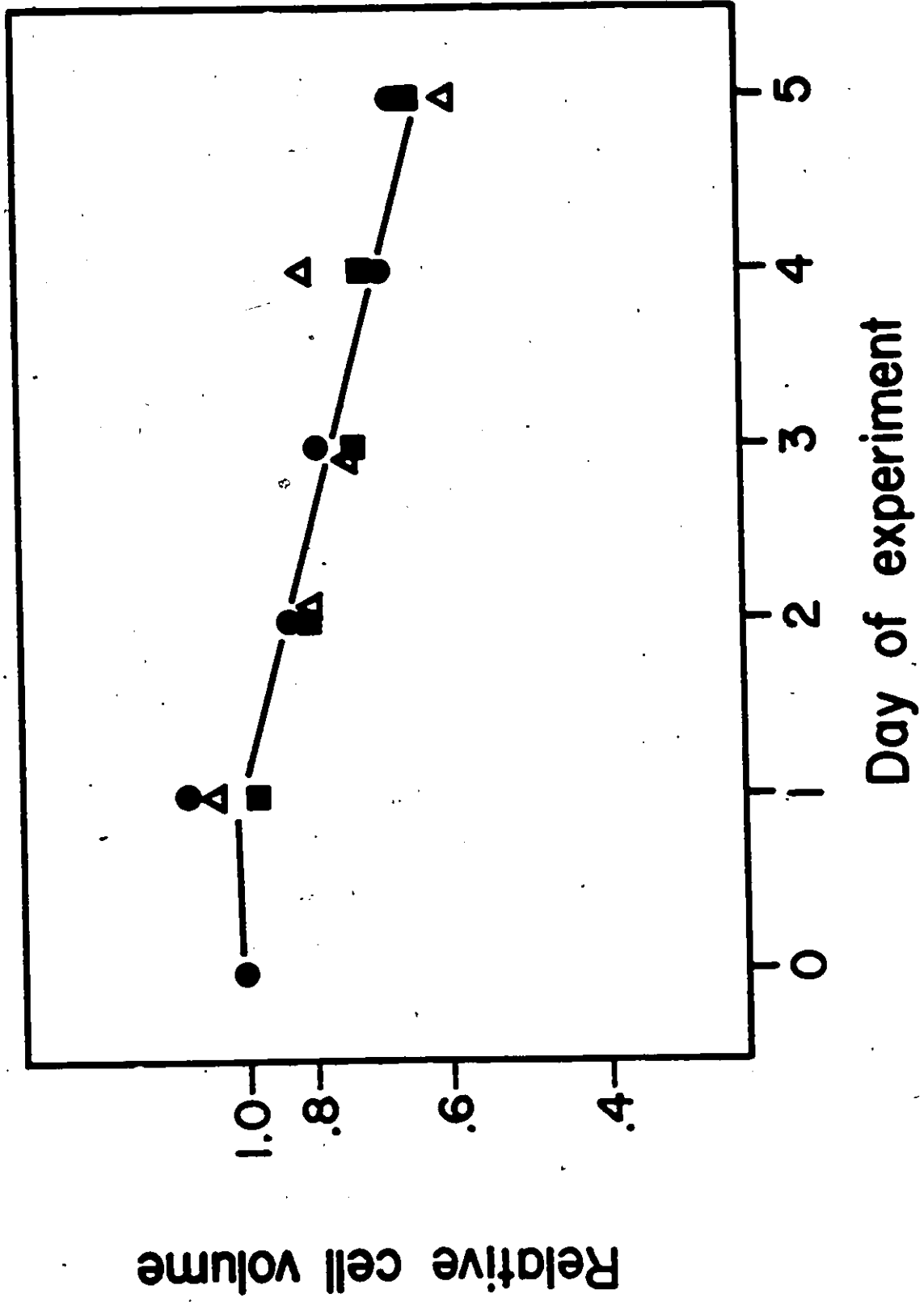
**Figure 6**

Changes in immunofluorescence staining pattern with AEC3A1-9 after differentiation of O1A1 cells. The same field of cells in a 1% DMSO treated aggregate was photographed on day 9 using phase contrast (1) and fluorescence (2) optics after staining with AEC3A1-9. Phase (3) and fluorescence (4) photomicrographs of untreated O1A1 cells in monolayer culture show that these undifferentiated cells stain much more intensely with this antibody. Bar, 25um.



**Figure 7**

The size of O1A1 cells in DMSO-treated aggregates is the same as untreated controls. Duplicate cultures of O1A1 aggregates that were untreated (circles), or treated with 0.5% (squares) or 1% (triangles) DMSO, were dispersed at various times, and the cell size determined using a Coulter counter channelizer.



## DISCUSSION

Our initial results (McBurney et al, 1982, appendix 1) indicated that DMSO can induce the O1A1 line of EC cells to differentiate into cardiac and skeletal muscle in vitro. The results reported in this chapter expand on these observations, giving further details of the response to different concentrations and lengths of exposure, as well as the time course of the response.

The muscle cells in DMSO treated cultures were easily identified by their morphology and contractility. The cultures did contain alpha-actin (McBurney et al, 1982), and muscle myosin, but the thick and thin filaments observed in the electron microscope were not organized into mature myofibrils, and there was evidence for only a small amount of the muscle specific isozyme of creatine phosphokinase (CPK) (Appendix A). This immaturity may be simply due to the limited time span of the experiments, or to the inhibitory effect of DMSO, which produces a lag in myogenesis even after the drug is removed (Blau and Epstein, 1979).

The concentration of DMSO, the duration of exposure to the drug, and DMSO treatment while the cells were in the closely packed arrangement of the aggregate were all important factors in the differentiation response observed. Between 0.25% and 1% DMSO, increasing concentrations resulted in higher proportions of muscle cells and lower proportions of undifferentiated EC cells in the cultures. At 1% DMSO,

nearly all the cells were differentiated, and there were muscle cells in virtually all the aggregates. The concentration of DMSO also had some effect on the type of differentiation observed, since skeletal muscle was common only at DMSO concentrations above 0.75%. DMSO has no inductive effect on monolayer O1A1 cultures (McBurney et al, 1982). By visual examination, aggregates of O1A1 cells undergo compaction during the first 10-12 hours of suspension culture. The length-of-exposure data presented here suggests that exposure to DMSO did not induce differentiation until after compaction had taken place. This is probably because the cell contact or cell density conditions required, which are also not present in the monolayer cultures, had not been established. After compaction, longer exposures result in higher proportions of aggregates with beating muscle, and lower proportions with areas of EC cells. This gradual rise suggests that the commitment of O1A1 cells may involve a cell cycle component as has been postulated for MEL cells (Levy et al, 1975). Exposure to DMSO for about 30 hours after compaction appears to be required for the maximum inductive effect. The response of O1A1 cells to various lengths of DMSO exposure is nearly the same as that found for the response of P19 cells to SA (Jones-Villeneuve et al, 1982).

About 25% of the cells in DMSO treated cultures stained with AEC3A1-9, but in most of these the staining was much less intense than that seen in monolayer cultures or un-

treated aggregates. This change in intensity was also seen in aggregates stained in situ, and these weakly positive cells were not considered to represent a residual population of undifferentiated EC cells. Speers et al (1979), using a polyclonal antiserum against EC surface antigens, showed a similar decrease in the intensity of staining in the differentiated cells in their cultures. In DMSO treated C1A1 aggregates, no cells with muscle or endoderm morphology were observed to stain with AEC3A1-9. This is consistent with observations on P10 cells treated with RA in monolayer culture. These cells differentiate extensively into parietal endoderm, and the proportion of cells staining with AEC3A1-9 is reduced to near zero (G.D.Paterno and M.W.McBurney, Dept. of Medicine, University of Ottawa, in preparation).

A small and variable percentage of the untreated aggregates did contain areas of beating muscle. Staining with antibodies against muscle specific myosin showed that less than 2% of the cells were muscle. C1A1 is deficient in hypoxanthine phosphoribosyl transferase (HPRT), therefore the cells probably overproduce and excrete purines (Seegmiller et al, 1967). Purines and purine analogs, including 6-TG have been shown to induce differentiation of MEL cells (Gusella and Houseman, 1976). Since three of the MEL cell inducers, including the purine analog 6-TG, also act to induce C1A1 cells, it seems likely that other MEL cell inducers, specifically other purines, could have the same effect. If the

01A1 cells are producing excess purines and these do have an inductive effect, this could account for the presence of some muscle cells in the untreated aggregates. No muscle is observed in untreated aggregates of P19 cells, which have normal HPRT activity (E.M.Jones-Villeneuve, Dept. of Biology, University of Ottawa, personal communication).

Three models could explain the observation of a limited range of differentiated cell types in DMSO-treated cultures:

- 1) The drug could act by selectively killing undifferentiated cells and precursors to other cell types not found in DMSO-treated cultures.
- 2) The drug could simply initiate differentiation along pathways that the cells were already committed or preprogrammed to follow.
- 3) The drug could act by inducing pluripotent, uncommitted EC cells to differentiate along a limited number of developmental routes.

Even though the observation of some muscle differentiation in the untreated controls makes models 1 and 2 seem plausible, other results make them appear less likely. There is no evidence for cytotoxicity at the DMSO concentrations used for differentiation experiments (McBurney et al, 1982), which argues against model 1. Parallel cultures treated with  $10^{-7}$  RA developed a very different range of cell types, which argues against model 2. Furthermore, both RA and DMSO are effective if the cells are treated for only the first 48 hours of the experiment though the differentiated cell types appear days later. After DMSO treated cultures are plated,

very few undifferentiated cells remain in the culture, even though they are faster growing than the differentiated cell types. Thus the most likely model appears to be action of the drug to induce uncommitted EC cells to differentiate along a limited number of developmental pathways.

The mechanism of action of DMSO on MEL cells, and on C1A1 cells, is unknown, though recent work with MEL cells has shown that an increase in the rate of  $Ca^{++}$  influx may be one of the important effects of DMSO in that system. Levenson et al (1981) described an increase in  $Ca^{++}$  uptake and a consequent increase in  $Ca^{++}$  content in DMSO treated MEL cells. Cells treated with DMSO plus amiloride did not show this increase, and were also blocked from differentiation while amiloride was present. The calcium chelator EGTA also blocks MEL differentiation (Levenson et al, 1980, Bridges et al, 1981), as do local anaesthetics such as procaine and tetracaine (Bernstein et al, 1976, Tsiftoglou et al, 1981). These anaesthetics have been shown to disrupt  $Ca^{++}$  binding to red blood cell membranes (Low et al, 1979). The inhibitory effects of each of these compounds on DMSO treated MEL cells can be reversed by the addition of either extra calcium chloride or the calcium ionophore A23187 to the medium. This ionophore accelerates calcium transport rates, and when MEL cells are treated with DMSO plus A23187, they initiate commitment without the usual 12 hour time lag observed with DMSO alone (Bridges et al, 1981). There is also no time lag

after the removal of the block caused by anaesthetics (Tsiftsoglu, 1981). This suggests to these authors that the 12 hour lag represents the time required for DMSO to alter the calcium transport rate or calcium content of the cells. This alteration in calcium transport is necessary but not sufficient for MEL cell commitment since A23187 by itself does not induce these cells.

Only one of the experiments analagous to this work with MEL cells has been done with O1A1; the local anaesthetic procaine did inhibit the response of O1A1 cells to DMSO. This suggests that the action of DMSO may involve  $Ca^{++}$  transport in these cells as well. The fluidity of the cell membrane may also be involved, since DMSO stabilizes membranes and procaine renders them more fluid (Lyman et al, 1976).

## Chapter IV.

### EFFECTS OF OTHER DRUGS ON P19S1801A1 CELLS

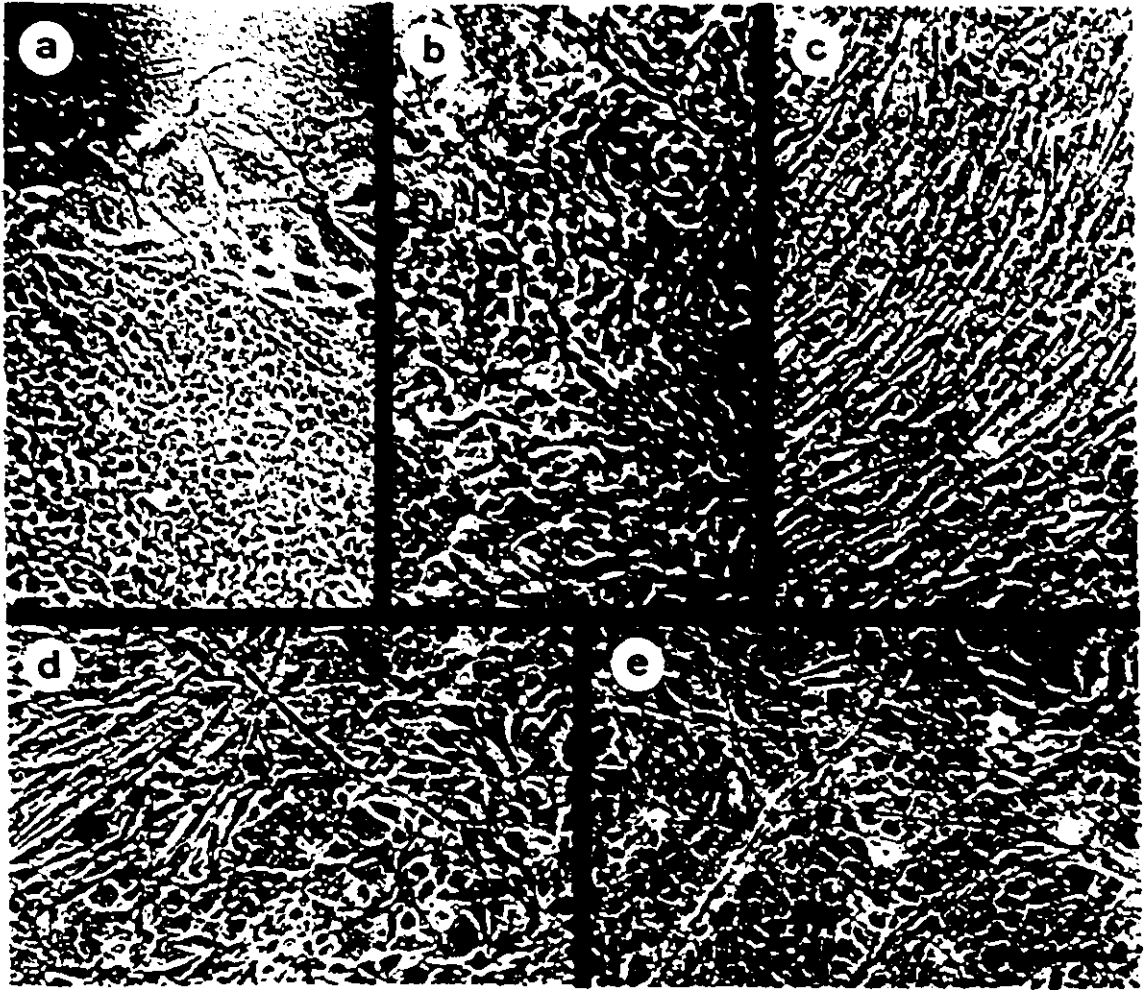
#### RESULTS

##### Retinoic acid

Since aggregates of P19 cells respond to RA by forming neurons and glial cells, the initial characterization of the O1A1 subclone included a test of its response to graded doses of RA. O1A1 produced neurons with the same dose response kinetics as P19. It was also noticed in these experiments that, at RA concentrations less than those required for neuron formation, the O1A1 aggregates formed muscle. Small amounts of cardiac muscle had previously been observed in P19 aggregates treated with low RA concentrations, but these observations were not consistently reproducible. Skeletal muscle was not observed in P19 cultures, but these cultures had been scored before the skeletal myoblasts would have been obvious (E.M. Jones-Villeneuve, Dept. of Biology, University of Ottawa, personal communication). The response of the O1A1 cells to RA was investigated in more detail, using scoring for morphologically recognizable cell types (Figs. 1, 2) and immunofluorescent staining (Fig. 3).

Untreated O1A1 aggregates, and those treated with  $10^{-10}$  RA, were made up almost entirely of undifferentiated EC cells, with some extraembryonic endoderm (Fig. 1a). Treatment

Figure 1 Light photomicrographs of O1A1 cells following aggregation and exposure to various RA concentrations. Panel a, untreated, shows undifferentiated EC cells (thin arrow) and extraembryonic endodermal cells (thick arrow). Panel b,  $10^{-9}$  M RA, shows an area of spontaneously contracting cardiac muscle. Panel c,  $10^{-6}$  M RA, shows an area of bipolar myoblasts and a few myotubes. Panel d,  $3 \times 10^{-6}$  M RA, shows a field containing both myotubes (thick arrow) and neurons (thin arrows). Panel e,  $10^{-7}$  M RA, shows an area containing neurons (thin arrow) growing over an area of astroglia (thick arrow). Bar, 10 $\mu$ m.



with increasing concentrations of RA resulted in a decrease in the proportion of undifferentiated EC cells, judged by visual scoring (Fig.2a). There was a corresponding decrease in the number of positive cells and the intensity of their staining with AEC3A1-9 (Fig.3a). At RA concentrations above  $10^{-6}$  M, the percentage of cells staining with AEC3A1-9 remained at about 25%, comparable to the values obtained with DMSO treated aggregates (Chapter 2, Fig.5), and RA treated P19 aggregates (E.M.V.Jones-Villeneuve et al, Dept. of Biology, University of Ottawa, in preparation).

With exposure to increasing concentrations of RA, reproducible changes in the pattern of differentiation were observed. At  $10^{-9}$  to  $3 \times 10^{-9}$  M RA a large proportion of the aggregates contained rhythmically beating cardiac muscle (Figs.1b,2b). Slightly higher concentrations,  $10^{-6}$  M RA, gave a predominance of skeletal muscle (Figs.1c,2c), with much less beating muscle evident. At  $10^{-6}$  M RA, some neurons were also present, by visual scoring (Fig.1d), plus a small proportion of glial cells, containing glial fibrillar acidic protein (GFAP), by immunofluorescence staining (Fig.3d). At  $10^{-7}$  M RA, or higher concentrations, the most obvious cell types were neurons (Figs.1e,3d), and cells containing GFAP were also present. Very little of either muscle type was evident by visual scoring or by staining for muscle myosin (Fig.3c).

Immunofluorescent staining with TROMA-1 (Kessler et al, 1981) was used in these experiments to follow the proportion of cells of epithelial origin. TROMA-1 reacts with a cytokeratin-related protein. The extraembryonic endoderm cells seen in the untreated aggregates (Fig. 1a) were TROMA-1 positive, and probably account for most of the broad peak of positively staining cells observed at low doses of RA (Fig. 3b). At  $10^{-7}$  M RA, very few TROMA-1 positive cells were observed. This is consistent with our previous observations using P19 (Jones-Villeneuve et al, 1982), in which neither extraembryonic endoderm nor other cells containing cytokeratins were observed at high RA concentrations.

Neurons and beating muscle were first observed at days 5-6 of the experiment and the number of these cells increased during the following 2-3 days. Skeletal muscle and astroglia appeared somewhat later, about days 7-9. This sequence in time meant that the proportion of aggregates showing any particular cell type varied depending on when the culture was scored. The overall pattern of response, however, did not change with time during the experiment, but depended on the concentration of RA that was used in the treatment of the cells. The spectrum of response illustrated in Figures 2 and 3 is representative of the pattern seen in each of 6 experiments.

RA and other retinoids are present in the bovine serum used as a culture medium supplement. Fuchs and Green (1981)

Figure 2 Differential appearance of cell types as assessed by visual scoring of live cultures. Each aggregate was scored for the presence of cells with EC morphology (panel a), cardiac muscle (panel b), skeletal muscle (panel c), and neurons (panel d). This scoring was carried out 11 days following the initiation of the experiment, although the pattern observed was the same no matter when the scoring was done.

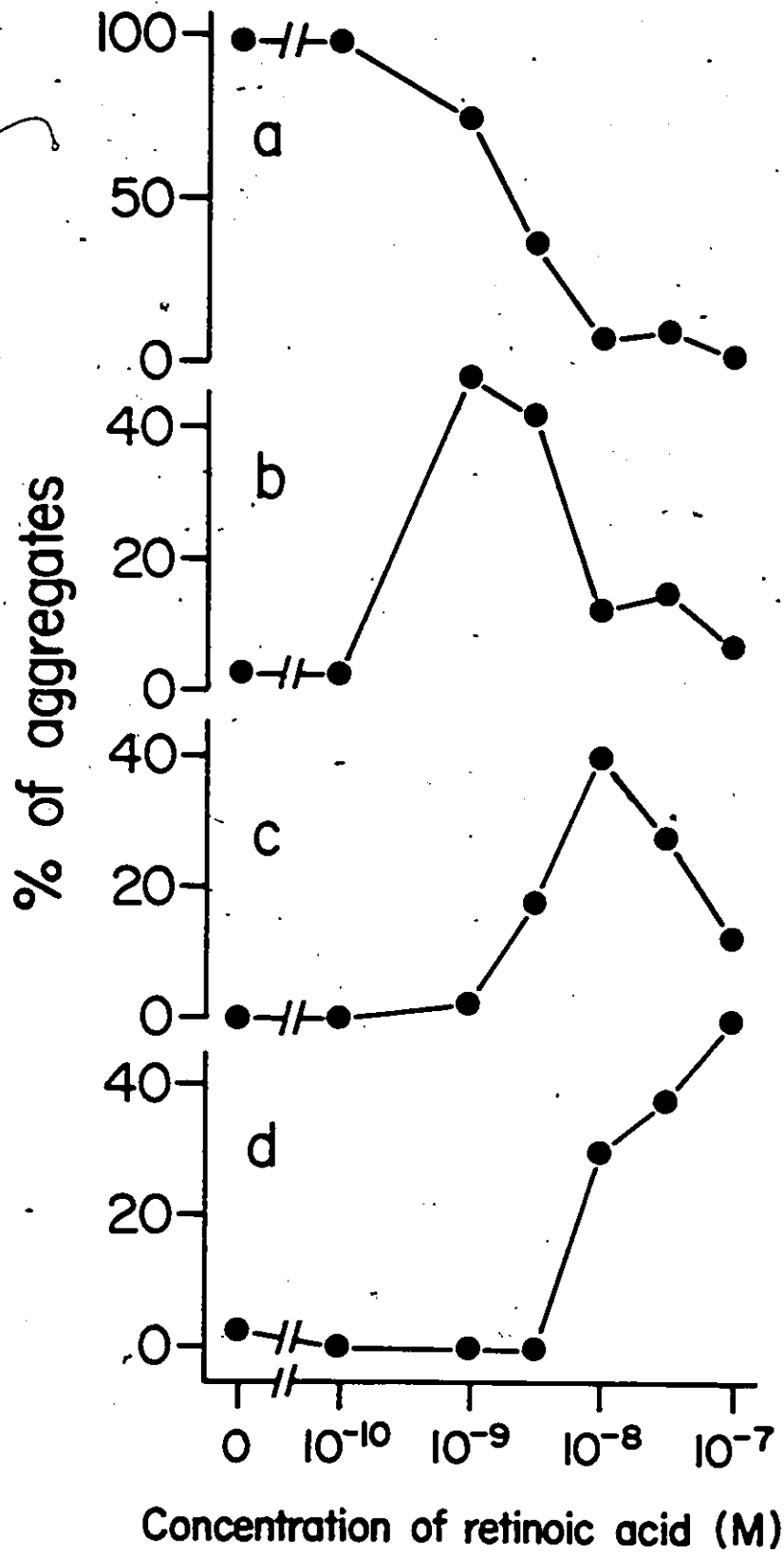
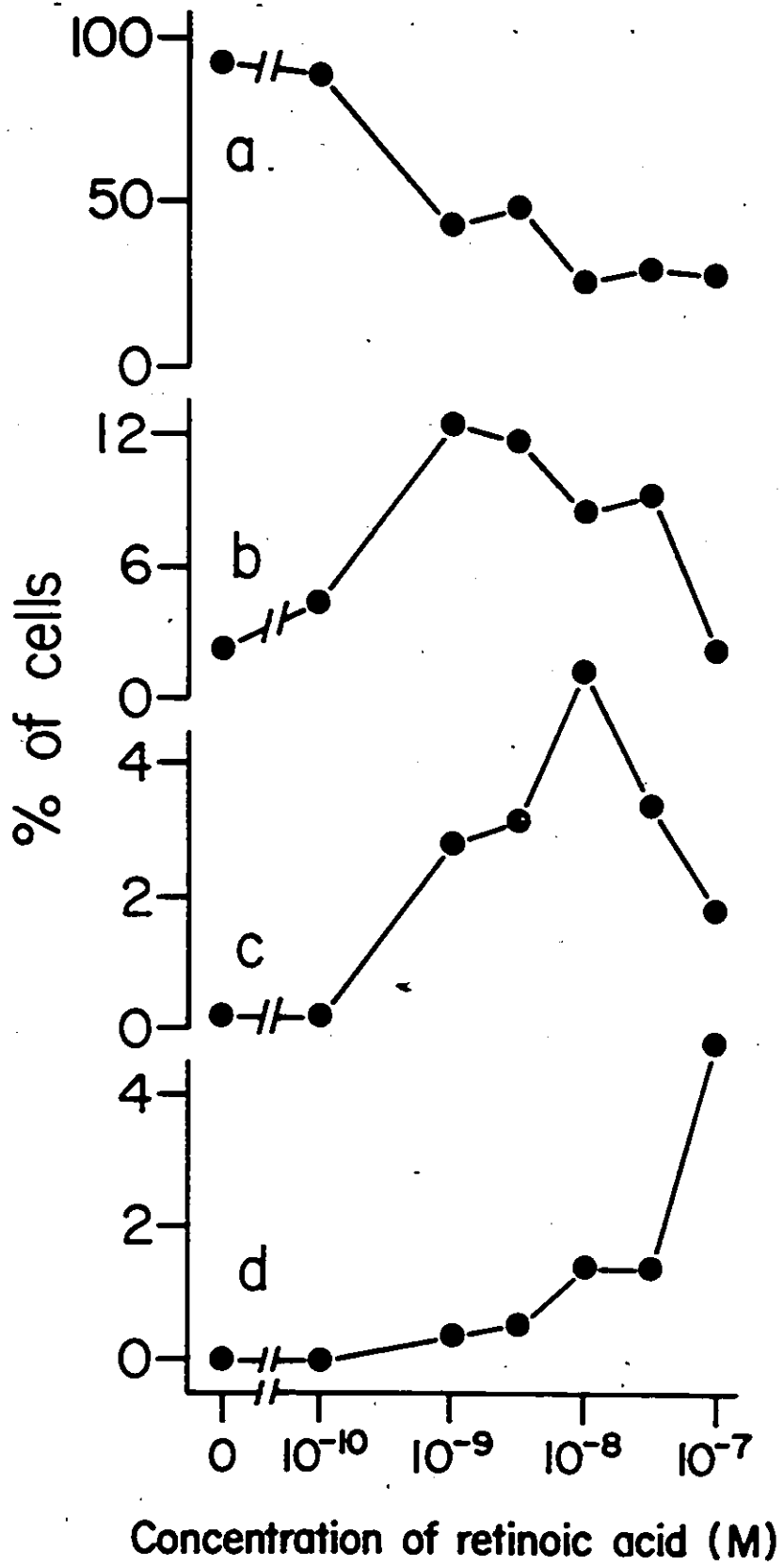


Figure 3 Differential appearance of cell types as assessed by immunofluorescent staining. Following the visual scoring reported in Figure 2, cultures were dispersed and stained with antibodies against cell-type specific antigens : panel a, AEC3A1-9, which recognises a surface antigen on undifferentiated EC cells; panel b, TROMA-1, which reacts with a cytokeratin-associated protein; panel c, MF20H28 a monoclonal antibody directed against muscle-specific myosin. Panel d is the number of mature astroglial cells assessed by their staining with a polyclonal antibody raised against glial fibrillar acidic protein. Five hundred cells were scored for each point.



found that their medium, supplemented as ours is, with 10% fetal bovine serum, contained  $3.6 \times 10^{-8}$  M retinol. Since DMSO is known to facilitate the transport of drugs across biological membranes (Jacob et al, 1964, Stroughton and Fritsch, 1974), it could conceivably act to induce muscle formation simply by increasing the permeability of C1A1 cells to the serum retinoids. To test this hypothesis I used fetal calf serum delipidized by Dr. J. Campicne-Piccardo in our laboratory according to the method of Rothblat et al (1976). C1A1 cells were tested for their response to DMSO in media supplemented only with this delipidized serum. According to Fuchs and Green (1981), this procedure removes all detectable vitamin A from the medium, so it would eliminate the DMSO response completely if the above hypothesis is correct. In three experiments, with two different batches of delipidized serum, the DMSO response was maintained, though in one experiment it was reduced to about 50% (by visual scoring) of the control levels using normal serum (data not shown). Thus DMSO does not appear to act indirectly via the serum retinoids.

To further test the relationship between the cells' response to DMSO and RA, C1A1 aggregates were exposed simultaneously to 0.5% or 1% DMSO and the graded concentrations of RA used above. The same pattern of response to RA was observed in the presence or absence of DMSO (Fig.4). Treatment with  $10^{-10}$  M RA and DMSO together resulted in the same res-

ponse as the DMSO alone: very few areas of undifferentiated EC cells and a large amount of beating muscle. At higher RA concentrations, the addition of DMSO had very little effect on the response already described with RA alone. In the experiment shown in Fig. 4, the proportion of aggregates scored with neurons was low because the aggregates had been scored at day 11, when the distinctive neural processes had largely receded.

#### Other Murine Erythroleukemia Cell Inducers

Since DMSO is a potent inducer of MEL cells, a number of other MEL cell inducers were tested for their effect on C1A1 cells. Hemin (0.5 mM) and ouabain (1.5 mM) were found to have no effect. The response to 6-TG and butyric acid is shown in Table 1. Both of these drugs had the same effect as DMSO: cardiac muscle at low concentrations, and skeletal muscle plus a few neurons at higher ones. The highest concentration of butyric acid tested, 2 mM, was toxic to C1A1 cells, since only some of the aggregates plated successfully onto the tissue culture dishes. 40% of these, however, did develop areas of neurons, which may indicate a response similar to the higher doses of RA.

Figure 4 Simultaneous treatment of O1A1 cells with RA and DMSO produces differentiation patterns similar to those produced by RA alone. O1A1 aggregates were exposed to various concentrations of RA, in the presence (open circles) or absence (closed circles) of 1% DMSO. These aggregates were plated on day 4 and scored on day 11 for the presence of morphologically recognisable cell types.

% of aggregates

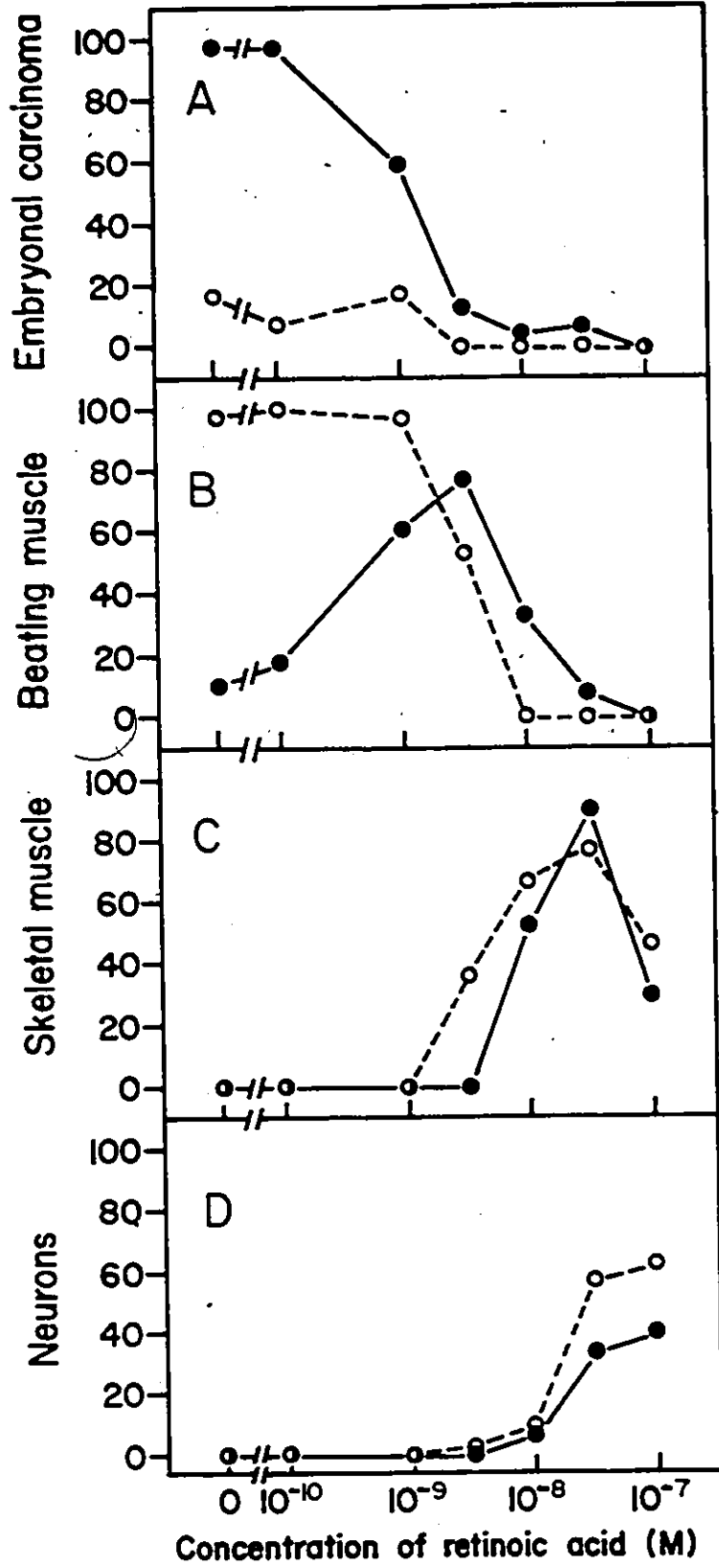


TABLE 1

Response of OL1 aggregates  
to butyrate and 6-thioguanine.

		<u>% of aggregates</u>			
		<u>E.C.</u>	<u>beating</u>	<u>skeletal</u>	<u>neurons</u>
			<u>muscle</u>	<u>muscle</u>	
6-TG	20 mM	70	5	0	0
	60 mM	21	42	0	0
	180 mM	0	50	66.7	11.1
butyrate	0.5 mM	30	45	10	0
	1 mM	15	50	40	15
	2 mM	20	0	10	40

## DISCUSSION

The results reported in this chapter indicate that the cell types formed by RA induction of O1A1 cells are dependant on the concentration of the drug used. The most obvious cell type in aggregates exposed to low RA concentrations ( $<3 \times 10^{-9}$  M) is cardiac muscle. At progressively higher concentrations, skeletal muscle, then neurons and glial cells became the characteristic cell types in the culture. Also, as the concentration of RA is increased, the proportion of undifferentiated EC cells decreases. Two or more of these recognizable cell types were present at most RA concentrations, usually as separate areas within the same aggregate. Where neurons and skeletal muscle developed close together, neural processes could sometimes be seen next to muscle cells, but it was not determined whether neuromuscular junctions were formed. The pattern of differentiation observed varied with the concentration of RA, and was the same no matter when the experiment was scored.

The spectrum of response seen with various doses of RA is also observed with DMSO, butyrate, and 6-TG. However, since these drugs are more toxic than RA, the highest non-toxic dosages resulted in the skeletal muscle differentiation, with a few neurons, found in cultures treated with  $10^{-6}$  M RA. Two other MEL cell inducers, ouabain and hemin, were found to have no effect on O1A1 cells. The response of MEL cells to ouabain has been shown to vary with the concentration of  $K^+$

in the medium (Bernstein et al, 1976b). The effects of  $K^+$  were not tested with O1A1 cells. It is not surprising that hemin is ineffective as an inducer of O1A1 cells, since it acts in MEL cells only to induce the production of globin. (Gusella et al, 1980). A range of MEL cell inducers, also excluding hemin and ouabain, have been shown to induce the differentiation of the human promyelocytic cell line HL-60 (Collins et al, 1980).

Treatment with RA results in differentiation of a number of EC cell lines. Most, like P19, form neurons when aggregated in the presence of  $10^{-7}$  M RA (Jones-Villeneuve et al, 1982). The F9 cell line (Bernstine et al, 1973) produces some visceral endoderm (Hogan et al, 1981) when aggregated in the presence of RA, and only a few neurons at very high RA concentrations ( $10^{-6}$  M) (Jones-Villeneuve, 1982). The effect of RA varies with the cellular environment. Monolayer cultures of P19 differentiate into fibroblast-like cells, with very few if any neurons when exposed to RA (Jones-Villeneuve et al, 1982). F9 in monolayer culture forms extraembryonic endoderm-like cells in the presence of RA (Strickland and Mahdavi, 1978), and P10 forms parietal endoderm under these conditions (Paterno and McBurney, in preparation). Thus, aggregation is a factor in the response of EC cells to RA, though it is not an essential requirement as it appears to be in the O1A1 response to DMSO.

P19, its derivatives, and P10, are the only cell lines found in our laboratory to be responsive to DMSO (McBurney et al, 1982), and P10 aggregates form neurons, not muscle, when treated with 1% DMSO (G.D. Paterno and M.W. McBurney, Dept. of Medicine, University of Ottawa, unpublished observations). This may represent a greater sensitivity to induction by DMSO in P10 cells than in O1A1, with a resulting shift along the dose-response spectrum. However, lower doses of DMSO have not been tested with this cell line, so it is not known if they would induce muscle formation. Other MEL cell inducers; HMB, dimethyl acetamide and polybrene, have been shown to induce the differentiation of the PCC4azal line of EC cells into epithelial-like cells if monolayer cultures are treated, and fibroblast-like cells, plus perhaps some other cell types, if aggregates are treated (Speers et al, 1979).

Recent reports suggest that retinoids may be involved in pattern formation in developing and regenerating limbs (Maden, 1982; Tickle et al, 1982). Pattern formation in limbs is thought to result from differential cellular response to gradients of diffusible morphogens (Wolpert, 1971, Tickle, 1980). Since some EC cell lines, in particular P19, respond to normal embryonic signals when injected into mouse blastocysts (Rossant and McBurney, 1982), our data add weight to the suggestion that gradients of retinoids may be one class of morphogen active in normal development.

## Chapter V

### MUTANTS AND HYBRIDS

#### RESULTS

Part of our approach to studying the response of P19 and O1A1 cells to RA and DMSO has been the isolation and characterization of mutant cell lines no longer responsive to induction by these drugs. Two such non-responsive variant lines have been isolated without mutagenesis in this laboratory.

P19S1801A1D3 (D3) was derived from O1A1 cells by exposing them to DMSO as in the usual protocol, then dispersing the differentiated aggregates with trypsin/EDTA and plating the cells at low density. Undifferentiated colonies that appeared were picked and grown to confluence. These cells were then used to repeat the cycle a total of three times, until the cells were no longer DMSO responsive. D3 cells are sub-tetraploid (Fig.1) with a modal chromosome number of 77, including a long marker chromosome. P19S18RAC6-5 (RAC6-5) was isolated in a similar fashion from P19, after cycles of exposure to RA. This cell line has a modal chromosome number of 42, with three chromosomes #3 and #13 (Fig.2).

Figure 1

Metaphase chromosomes of the D3 cell line. These cells have a modal chromosome number of 77, with a long marker chromosome (arrow).



**Figure 2.**

Preliminary karyotype of the RAC6-5 cell line. These cells have a modal cell number of 42, with trisomy of chromosomes #3 and #13.

P19S18 RAC6-5

1 2 3 4 5 6  
[Illegible marks]

7 8 9 10 11 12  
[Illegible marks]

13 14 15 16 17 18  
[Illegible marks]

19 X Y  
[Illegible marks]



Figure 3 shows the response of D3, and the parental lines, to DMSO. Both F19 and O1A1 respond to DMSO by a decrease in the proportion of aggregates containing EC cells, and an increase in the proportion containing muscle. F19 consistently produced less cardiac and skeletal muscle than O1A1. Neither D3 nor RAC6-5 was responsive to DMSO. The D3 cell line produced neither skeletal nor cardiac muscle at any of the DMSO concentrations tested. The proportion of aggregates containing undifferentiated EC cells also remained high after DMSO treatment for both cell lines (RAC6-5 response, M.W. McBurney, Dept. of Medicine, University of Ottawa, unpublished data). D3 cultures treated with 1% DMSO contained no muscle myosin positive cells (Table 1). Neither D3 nor RAC6-5 differentiated in the presence of butyrate or 6-TG (Table 2). D3 produced no muscle in response to low doses of RA. At higher RA concentrations it did form neurons efficiently (Fig. 2), with the same dose response as the parental O1A1 cells (see Chapter 4, Fig. 2 for comparison). RAC6-5 was non responsive to RA concentrations below  $10^{-6}$  M (E.M.V. Jones-Villeneuve et al, Dept. of Biology, University of Ottawa, in preparation).

Cell-cell hybridization techniques were used as the first approach to studying the nature of the mutations in these non-responsive cell lines. Using polyethylene glycol to promote fusion, and HAT medium (Littlefield, 1964) supplemented with cuabain to select against unfused cells and self-fusion

**Figure 3**

D3 aggregates do not respond to DMSO. Aggregates of 01A1 (closed circles), P19 (triangles) and D3 (open circles) were exposed to various concentrations of DMSO for two days, plated on day 4, and scored for the presence of beating muscle (panel a) and undifferentiated EC cells (panel b) on days 8-10 of the experiment. The data for 01A1 is shown for comparison, and is the same as the weighted average for the experiments represented in Fig.2a, Chapter 3. The data for P19 is averaged from three experiments. D3 did not show any beating muscle in seven experiments.

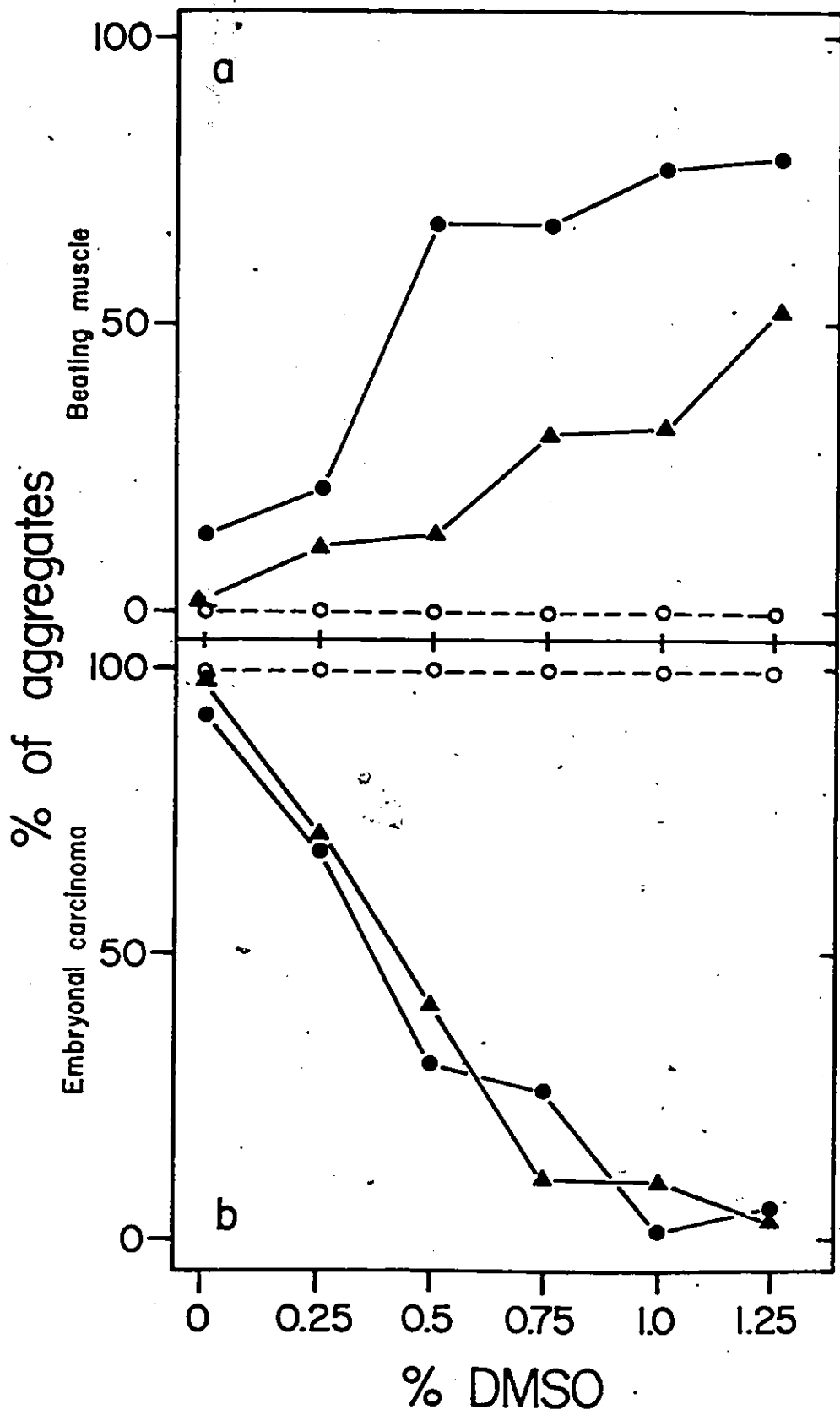


TABLE 1

Response of OL1 and D3 to DMSO.

	% muscle myosin positive cells (day 9)	% EC antigen positive cells (day 6)
OL1 untreated	2.8	89.6
OL1 1% DMSO	9.4	17.8
D3 untreated	0	99.2
D3 1% DMSO	0	96.4

products, three hybrid cell lines were established (M.W. McBurney and E.M.V. Jones-Villeneuve, Depts. of Biology and Medicine, University of Ottawa, unpublished data). Two of these, HY-1 and HY-2, were derived from fusions between FAC6-5 and C1A1 cells. The other, C65D3-2, was the fusion product of the two variant cell lines D3 and FAC6-5. The chromosome number for each of these hybrids was quite variable, but was always higher than the chromosome number in either of the parental cell lines. HY-1 and HY-2 had chromosome numbers between 69 and 80, with a few cells with more than 100 chromosomes. C65D3-2 had a modal chromosome number of 83, and each cell contained the long marker chromosome found in D3.

HY-1 and HY-2 developed muscle when treated with DMSO (Table 3) and their response was intermediate between the responses of the two parental cell lines. They showed a very low level of response to butyrate or 6-TG (Table 2). HY-1 did not differentiate into cardiac muscle at low doses of RA, but did form neurons and skeletal muscle at RA concentrations above  $10^{-6}$  M.

C65D3-2, like the D3 parental line, showed no differentiation in response to DMSO (Fig. 3), and developed neurons at high concentrations of RA, but no muscle at lower ones.

The variation in sensitivity to the inductive effects of DMSO amongst these mutant and hybrid cell lines was not connected with variation in their sensitivity to the cytotoxic

Figure 4

Aggregates of D3 cells produce neurons in response to high retinoic acid concentrations. Aggregates of D3 cells were exposed to various concentrations of RA, and scored for the presence of neurons (open circles) and undifferentiated EC cells (closed circles) as in the usual protocol. This experiment was repeated three times, and the figure shows the results of one representative experiment.

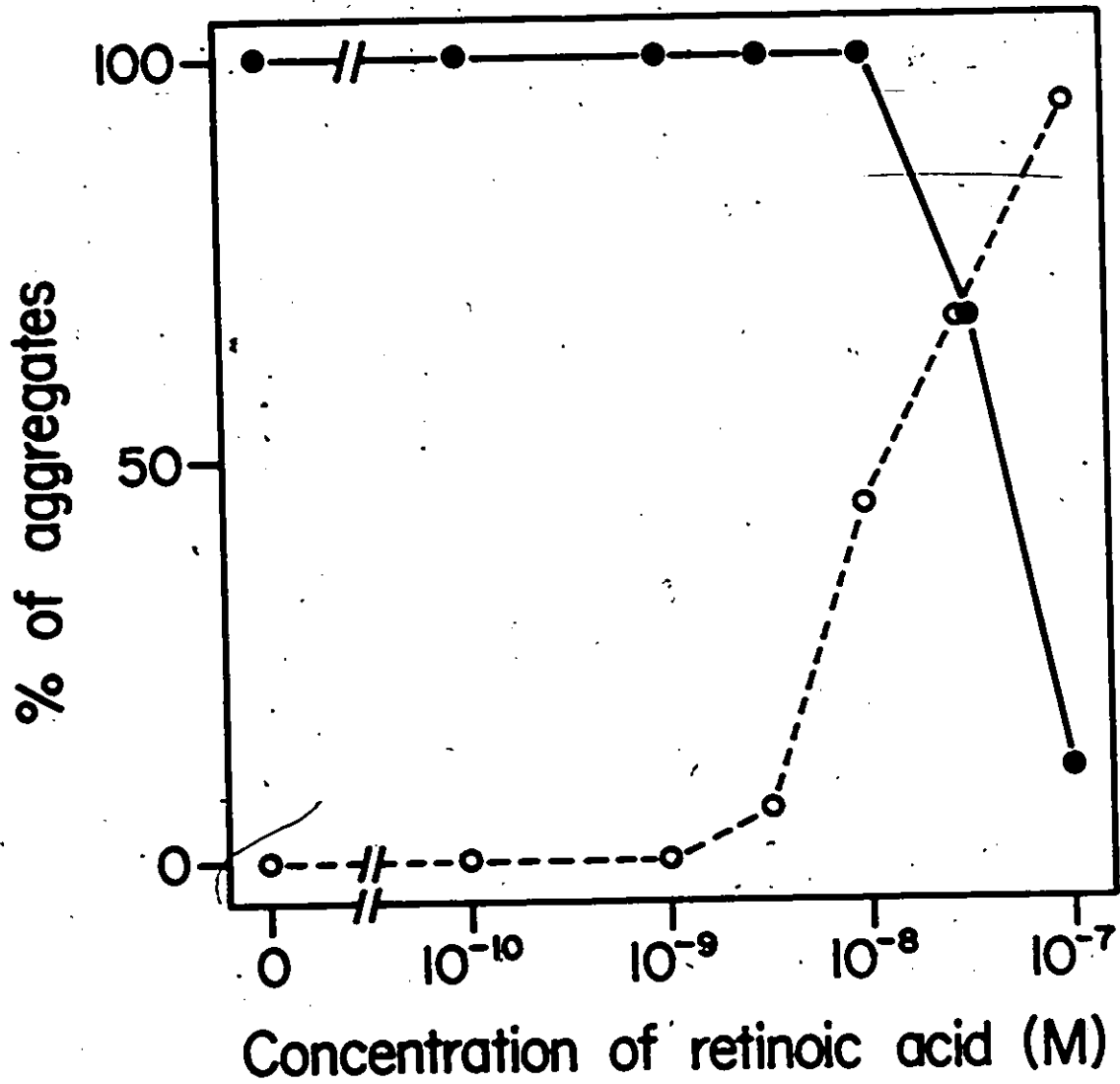


TABLE 2

Response of mutant and hybrid cell lines  
to butyrate and 6-thioguanine.

		% of aggregates			
		E.C.	beating muscle	skeletal muscle	neurons
D3	butyrate 1mM	100	0	0	0
	6-TG 60mM	100	0	0	0
RAC6-5	butyrate 1mM	100	0	0	0
HY-1	butyrate .5mM	80	10	15	0
	1mM	100	31.3	6.3	0
HY-2	butyrate .5mM	100	0	10	0
	1mM	85	5	20	0
	2mM	80	5	35	5

**NEXT**

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**FICHE**

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**SUIVANTE**

TABLE 3

Response of hybrid cell lines to DMSO.

	% DMSO	% of aggregates with EC cells	% of aggregates with beating muscle
HY-1 *	0	96.1	2.9
	0.5	92	6.6
	1	75	23
HY-2 *	0	93.2	4.5
	0.5	78.9	22.2
	1	57.6	38.8
C65D3-2	0	100	0
	0.5	100	0
	1	100	0

\* average values for 3 experiments

effects of the drug at higher concentrations. DMSO did not affect the plating efficiency of any of these cell lines at the concentrations used in differentiation experiments.

#### DISCUSSION

DMSO non-responsive variants in ME1 cells are most often non-responsive to other inducers as well, though exceptions exist in which cells respond to one inducer but not to another (Nasi et al, 1978, Rovera and Surrey, 1978, Harrison et al, 1978, Harrison, 1977). Rovera and Surrey (1978) have argued that this sort of mutant, responsive to one treatment but refractory to another, shows that the two treatments act on normal cells by different mechanisms. The mutation in the D3 cell line affects differentiation in response to DMSO, purvrate, 6-TG, or low doses of RA. D3 could, however, form neurons efficiently in response to high RA concentrations. Thus, by the argument above, the parental C1A1 cells may respond via different cellular mechanisms to the different concentrations of RA.

The extent of both neural and muscle differentiation was variable among the cell lines that were responsive to RA and DMSO. For example, P19 differentiated less extensively into muscle than C1A1 cells when exposed to DMSO. P19 was responsive to doses of RA lower than those required for neural development, but differentiated mostly into fibroblast-like cells (Jones-Villeneuve et al, 1982) whereas C1A1 formed both

cardiac and skeletal muscle under these conditions. This may be a further example of the effects of HPRT deficiency in O1A1 cells, already discussed in chapter 2. Higher levels of endogenous purines in these cells, compared with (HPRT<sup>+</sup>) P19 cells, may render them more likely to differentiate into muscle, both in untreated aggregates and in response to inducers.

The responses of O1A1 to high doses of RA and to DMSO are probably mediated by different cellular mechanisms. The effects of the drugs are not additive (Chapter 3, Fig. 4) and procaine inhibits the myogenic response to DMSO, but not the neural differentiation in response to RA. HY-1 and HY-2, hybrids between FAC6-5 and O1A1, developed muscle in response to DMSO and neurons in response to high doses of RA, but both responses were intermediate between those of the two parental lines. Thus both parental genomes appeared to have some effect on the hybrid cell, a sort of co-dominant genetic expression.

C65D3-2, the hybrid between FAC6-5 and D3, had about 83 chromosomes, and the total expected if both parental genomes were present in full is about 120. This considerable chromosome loss makes the relative genetic importance of the two parental genomes in this hybrid much less clear. These cells did not differentiate in response to DMSO, like both parents, but did develop neurons in response to high doses of RA like the D3 parent. One obvious conclusion from the C65D3-2 data

is that the two mutations affecting DMSC response in RAC6-5 and D3 do not show complementation when present in the same hybrid cell. The other conclusion is that the mutation in RAC6-5 affecting its response to high doses of RA was recessive to the D3 genome. Because of the chromosome loss in this hybrid, however, these interpretations are only weakly supported.

## Chapter VI

### CONCLUSIONS

The P19 cell line and its derivatives appear to offer a culture system in which the differentiation of pluripotent cells can be induced, and directed, by cell aggregation and the addition of particular compounds to the medium. The addition of DMSO, butyrate, or 6-IG, results in a differentiation pattern in which muscle, both cardiac and skeletal, is the characteristic cell type. RA, in concentrations of  $10^{-7}$  M or more, induces a different pattern, characterized by neurons, and lower concentrations produce a muscle differentiation pattern similar to DMSO. This system is not ideal for the study of individual commitment pathways; other cell types besides muscle and neurons are produced, and the response is complicated by dosage effects and the requirement for aggregation.

The relationship between the responses of P19 or O1A1 cells to DMSO and RA is not clear. The effects of the drugs were not additive, and the myogenic response to DMSO, but not the neural differentiation response to RA, was inhibited by procaine, which suggests that different mechanisms are involved. However, there is an intriguing similarity in the spectrum of cell types observed after treatment of O1A1

cells with varying doses of DMSO, other MEL cell inducers, and RA, which suggests that there are some common aspects to the cellular response to these drugs. RA, DMSO, butyrate, and purines are also all effective in inducing differentiation of the human promyelocytic cell line HL-60 (Erejtman et al, 1980, Collins et al, 1980). The effect of RA on O1A1 cells is particularly of interest since it is a naturally occurring compound, and effective as an inducer at physiological concentrations, well below toxic levels. As discussed in Chapter 4, retinoids may be one class of morphogen in normal embryogenesis. The other drugs may act on O1A1 cells, albeit by different mechanisms, to produce the same intracellular conditions as RA, which allow commitment to particular cell types to take place. Thus further study of the relationship between the responses to RA and DMSO may reveal common factors which are important to the commitment process. The dose-dependant response, the cell types produced other than muscle and neurons, and the involvement of cellular interactions, which complicate this O1A1 system, may be encouraging indications of its relevance as a model for normal cell commitment in the embryo.

Even though our initial objective of simplifying the differentiation patterns of EC cells in culture has only been partially met, the response of O1A1 cells to DMSO and RA offers some unique opportunities to exploit the EC cell system. A pluripotent cell line can be induced to form predic-

table limited groups of embryonic cell types in culture. Virtually all the cells can be induced to differentiate over a relatively short period of time. Tissue-specific gene expression, the nature of the cellular interactions allowing the response to the drugs, and the common aspects of the responses to DMSO and low doses of RA, which lead to muscle differentiation, are all areas which could be explored using the C1A1 cell line.

## Appendix A

### CREATINE PHOSPHOKINASE IN O1A1 CULTURES

Creatine phosphokinase (CPK, E.C.2.7.3.2) is a dimeric enzyme that has been widely used as a marker for muscle differentiation. Neural tissue contains fairly high levels of the BB isoenzyme of CPK. In mammals, both adult skeletal and cardiac muscle contain predominantly the MM form of the enzyme, and cardiac muscle also has significant levels of the MB heterodimer (Adamsen, 1976).

During maturation of skeletal muscle in the rat there is a dramatic rise in the total CPK enzyme activity. This is accompanied by a decrease in the relative activity of the BB isoenzyme, a transient peak in the MB at about 17 days gestation, and a rise in the MM form that accounts for the overall increase in activity. In cardiac muscle, the same pattern of isoenzyme transition is observed, though the total activity is less, and the MB form persists in the adult (Zitter, 1974)

In myogenic cell cultures, either from embryonic skeletal muscle or myoblast cell lines, there is a similar rise in CPK activity after myoblast fusion (Shainberg et al, 1971, Turner et al, 1974), with a similar isoenzyme transition pattern (Perriard et al, 1978, Dym et al 1978). Undifferentiated

EC cells contain only the BE form of CK. Gearhart and Mintz (1975) cultured embryoid bodies from the peritoneal cavities of mice injected with EC cells. These embryoid bodies developed areas of recognizable skeletal muscle in culture, and showed an increase in CK activity, though this was all BE-CK. Adamson (1976) also observed only the BE form in cultures of embryoid bodies, but found a small amount of ME-CK in one differentiating culture of an EC cell line.

To determine if DMSO treated C1A1 aggregates show an increase in CK activity, a standard spectrophotometric assay (Shainberg, Yagil, and Yaffe, 1971) was used. As a preliminary check on this assay, replicate cultures of the L6 myoblast cell line (Yaffe, 1968) were harvested after various times in culture by scraping the cells into phosphate-buffered saline, and extracts were prepared by sonication. The specific CK activity in these cultures rose after myoblast fusion (Fig. 1), and the values obtained were similar to those reported by Yaffe (1968). The modified Lowry method of Hartree (1972) was used for protein determination. Similar results were also obtained using a commercially available CK assay kit (Sigma). DMSO treated C1A1 aggregates, harvested in the same way, had a high initial level of specific CK activity relative to the L6 myoblasts. The untreated C1A1 aggregates displayed a drop in the total CK activity to day 6 of the experiment, then recovery to the initial levels by day 12 (Fig. 2). Untreated control aggregates

showed a steady level of CPK activity in this experiment, comparable with the levels found by Adamsen (1976) for embryoid bodies that had not begun to differentiate in culture.

Parallel cultures of O1A1 aggregates were also harvested into Tris-HCl buffer for separation of CPK isoenzymes by DEAE-Sephadex column chromatography (Lough and Eischhoff, 1977, Lough, 1980). These samples were run on DEAE-Sephadex minicolumns, using stepwise elution buffers containing 50, 100, and 300 mM NaCl, and the fractions tested for CPK activity as above.

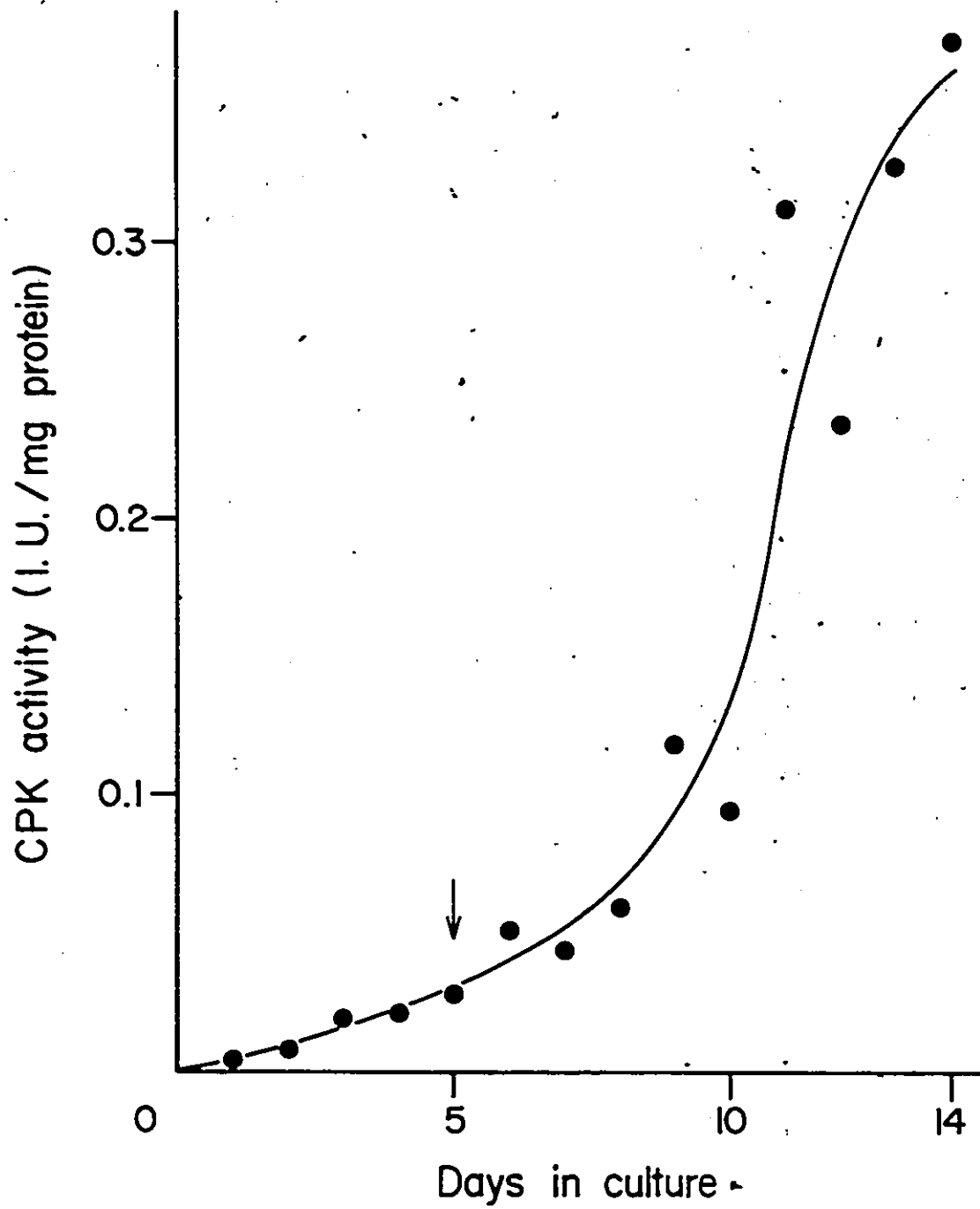
Preliminary tests using homogenates of adult mouse tissues showed that these elution buffers eluted mouse MM, ME, and BE-CPK isoenzymes in that sequence as the NaCl concentration was increased. Brain, thigh muscle and heart homogenates were tested by cellulose acetate strip electrophoresis (Turner et al, 1974), and showed the expected banding pattern: one anodal band (BE-CPK) in the brain sample, one cathodal band (MM-CPK) in the skeletal muscle sample, and two bands in the heart sample, one at the cathodal (MM) position and one at an intermediate position (ME). These tissue homogenates were run on the DEAE-Sephadex columns using the stepwise buffers mentioned above. All the recovered activity from the skeletal muscle homogenate came off the column at 50 mM NaCl, whereas no activity from the brain sample was recovered except at 300 mM NaCl. 100 mM NaCl eluted

CPK activity off the column only from the heart sample. Thus the activity eluted off the column at 50 mM NaCl is presumed to represent the MM form, 100 mM NaCl, the ME, and 300 mM NaCl, the BB form of CPK.

When the O1A1 extracts were tested on these columns, the results were as shown in Table 1. Untreated monolayer controls contained predominantly the BB form of CPK, and DMSO treated aggregates developed only very minor amounts of ME or MM-CPK after 11 or 13 days of culture. The rise in CPK activity in myoblast cultures after fusion is due to accumulation of the MM form. (Lough and Fischhoff, 1977). The appearance of some MM-CPK in 13-day O1A1 cultures may represent the beginning of this process, but cannot account for the rise in CPK activity between days 6 and 12. Thus the biochemical marker of M-form CPK is present in DMSO treated O1A1 cultures, but at very low levels, perhaps because of the limited time span of the experiment, or because of limitations on muscle maturation imposed by culture conditions, the DMSO treatment itself, or the presence of other cell types.

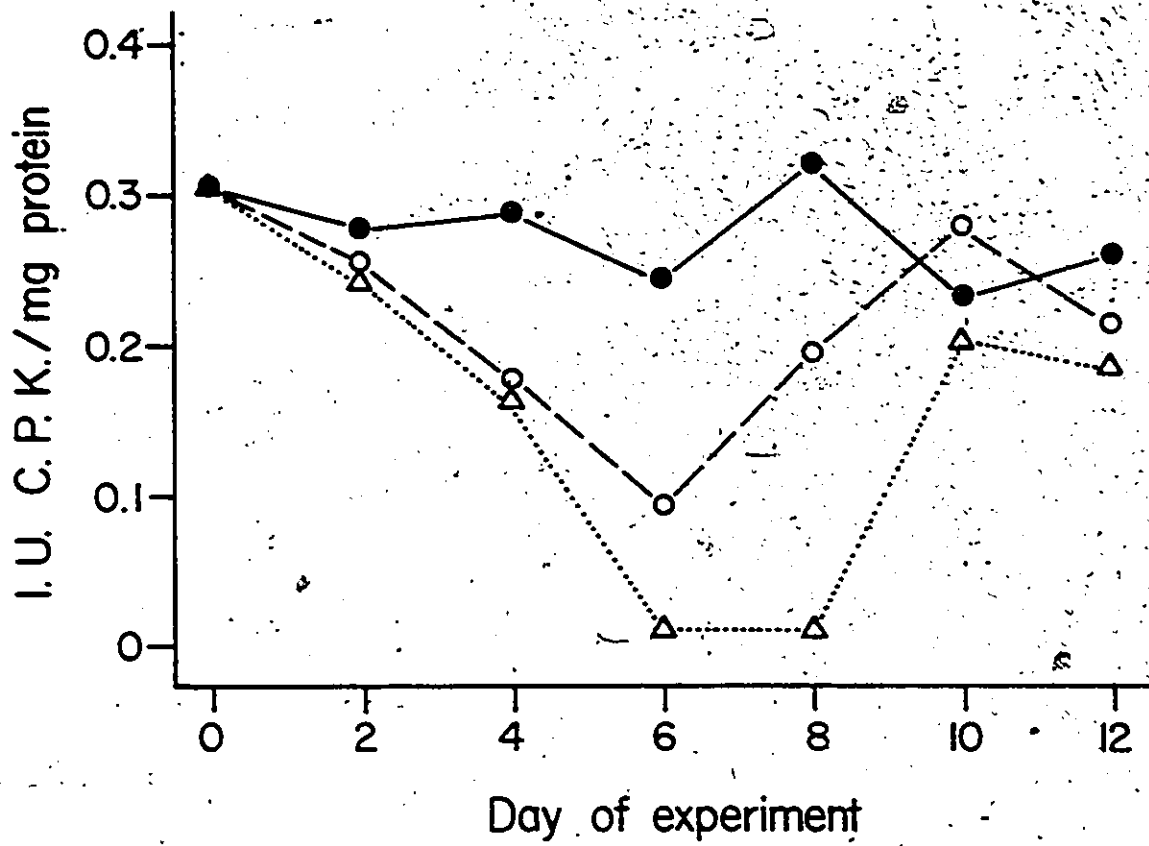
**Figure 1**

Specific CPK activity rises in cultures of L6 cells after myoblast fusion. Replicate cultures of L6 cells were harvested after various lengths of time in culture, and extracts were assayed for protein content, and CPK activity. The myoblasts began to fuse at day 5 (arrow).



**Figure 2**

Total specific CPK activity in O1A1 cultures. O1A1 aggregates that were untreated (closed circles), or treated with 0.5% (open circles) or 1% (triangles) DMSC according to the usual protocol were harvested at various times and extracts were assayed for protein content and CPK activity.



8

8

7

TABLE 1

Distribution of CPK isoenzymes in mouse tissue and OL1A1 cell extracts, as determined by ion exchange column chromatography.

% of recovered CPK activity

CPK isoenzyme	MM	MB	BB
NaCl (mM)	50	100	300
adult mouse tissue homogenates			
brain	0	0	100
skeletal muscle	100	0	0
heart	62.2	37.8	0
2.5:1 mixture of skeletal muscle:brain	64.7	0	35.3
OL1A1 cell extracts			
untreated monolayer 1	0	2.6	97.4
untreated monolayer 2	0	0	100
1% DMSO treated aggregates			
day 11	0	5	95
day 13	2.8	3.2	94

Appendix B

MCBURNEY ET AL, 1982

This appendix consists of a paper containing the first report of the response of O1A1 cells to DMSO.

## Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line

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Pluripotent murine embryonal carcinoma cells can differentiate in culture into many tissue types similar to those normally found in early embryos<sup>1</sup> and may be useful in investigating some developmental events<sup>1,2</sup>. Central to our understanding of embryonic development are explanations of cellular determination, that is, the commitment of early embryonic cells to form divergent cell types. Of relevance is recent work with the F9 line of embryonal carcinoma cells which suggests that certain extra-embryonic cell types are specifically formed following treatment of undifferentiated cells with drugs<sup>3,4</sup> and the manipulation of culture conditions<sup>5</sup>. We report here that the P19 line of embryonal carcinoma cells<sup>6</sup> may provide an analogous system in which drugs can be used to manipulate the formation of tissues which normally comprise the fetus. In the presence of dimethyl sulphoxide (DMSO) aggregates of P19 cells differentiate rapidly to form large amounts of cardiac and skeletal muscle but no neurones or glia. We have previously shown that in the presence of high concentrations of retinoic acid ( $>5 \times 10^{-7}$  M), aggregates of these same cells develop into neuronal and glial tissues but not muscle<sup>7</sup>. Thus, drugs can be used to generate two quite different spectra of embryonic tissue types from the same population of embryonal carcinoma cells.

P19 is a euploid (40:XY) embryonal carcinoma cell line derived from a teratocarcinoma induced in C3H/He strain mice<sup>6</sup>. For the experiments described below, we used P19S1801A1, a ouabain-resistant and 6-thioguanine-resistant subclone of P19 isolated without mutagenesis<sup>8</sup>. Suspensions of dispersed cells were plated onto bacterial-grade plastic surfaces to which cells do not adhere<sup>6</sup>. Cells adhere to each other to form small aggregates. These aggregates were cultured in suspension for 4–5 days in the presence or absence of DMSO. They were then plated into tissue culture-grade plastic dishes.

In the absence of drug, the plated aggregates contained undifferentiated embryonal carcinoma cells along with small numbers of extra-embryonic endodermal cells<sup>9</sup> (Fig. 1a). The presence of DMSO in the culture medium produced effects which became clear 1–2 days after plating, that is, 6–7 days after initiation of the experiment. In cultures exposed to 0.25% (v/v) DMSO, most plated aggregates contained embryonal carcinoma cells, rhythmically contracting muscle and fibroblast-like cells. At concentrations of 0.5, 0.75 and 1.0% DMSO, none of the plated aggregates contained embryonal carcinoma cells (identified by morphology), virtually all contained areas of rhythmically contracting muscle, and all contained cells with fibroblast-like morphology (Fig. 1c). By 10–12 days the amount of contracting muscle had increased (Fig. 1d, e). Also at this time many of the DMSO-treated aggregates developed areas of bipolar myoblasts which fused into myotubes (Fig. 1f). These myotubes were usually non-contractile but often developed spontaneous twitching activity by 14 days.

Electron microscopy of the cells in DMSO-treated cultures indicated that the rhythmically contracting cardiac muscle cells contained glycogen granules, large numbers of mitochondria, and numerous areas of thick and thin filaments which were not organized into mature myofibrils (Fig. 2a). The multinucleate skeletal muscle cells were similar in appearance (Fig. 2b). Thus both muscle types seemed to be immature. Many of the non-muscle cells had abundant rough endoplasmic reticulum and

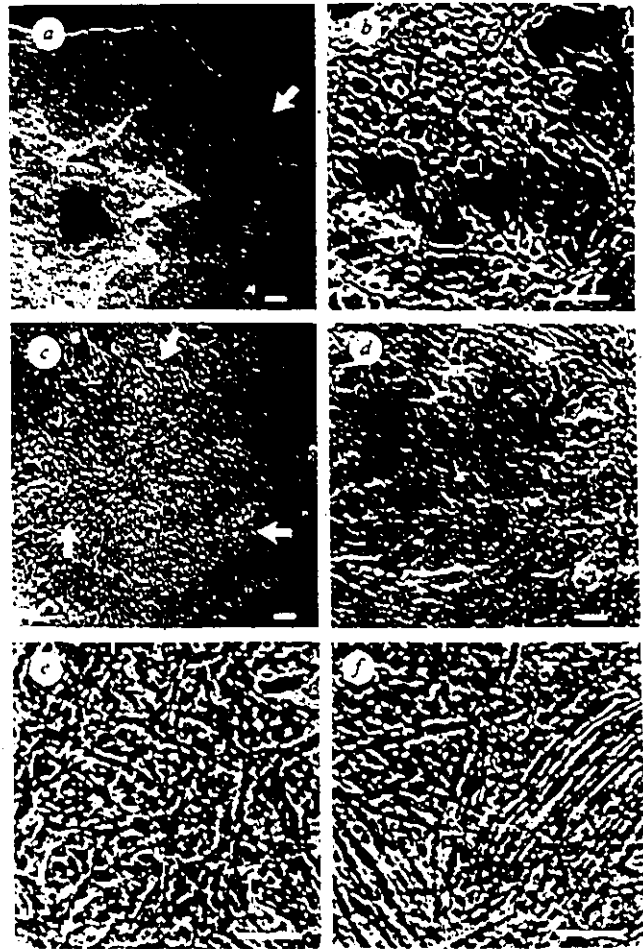


Fig. 1 Phase contrast photomicrographs of live teratocarcinoma cells. The conditions for cell culture<sup>6,7,8</sup> and aggregation<sup>8</sup> have been described previously. Cell aggregates were formed from a stock culture of P19S1801A1 cells and parallel cultures were carried in: a, normal medium ( $\alpha$ -medium plus 10% fetal bovine serum<sup>10</sup>); b, in medium containing  $5 \times 10^{-7}$  M retinoic acid; and c–f, in medium containing 0.5% (v/v) DMSO. Aggregates were cultured in suspension in bacterial-grade Petri dishes for 5 days before being plated on to tissue culture-grade plastic surfaces. Photographs were taken 2 days (a–c) or 9 days (d–f) later. a, Untreated aggregates contain embryonal carcinoma and a few extra-embryonic endoderm cells (arrow). b, Aggregates of cells which had been cultured in the presence of  $5 \times 10^{-7}$  M retinoic acid contain neurones and astrocyte glial cells<sup>7</sup>. c–f, Aggregates cultured continuously in the presence of 0.5% DMSO contain small areas of rhythmically contracting cardiac muscle (arrows in c) which become more extensive with time (d). At higher magnification are: e, areas of rhythmically contracting mononucleate cardiac muscle and, f, multinucleate skeletal muscle. Scale bars, 200  $\mu$ m.

some were surrounded by extracellular matrix which included collagen fibres (Fig. 2c).

The DMSO-treated aggregates of P19S1801A1 cells developed muscle but neither neurones nor glia. Treatment of the same cells with retinoic acid resulted in the development of neurones (Fig. 1b), glial cells, fibroblast-like cells, but no muscle. Cultures exposed to both retinoic acid ( $5 \times 10^{-7}$  M) and DMSO (0.5 or 1.0%) developed as if exposed only to retinoic acid, that is, neurones and glia but no muscle were formed.

Differentiated cultures contained more actin than did untreated cultures (Table 1). Much of the actin in DMSO-treated cultures was  $\alpha$ -actin, the type present only in skeletal and cardiac muscle cells<sup>9</sup>. Muscle-specific myosin was also detected in both cardiac and skeletal muscle by immunofluorescence using monoclonal antibodies directed against muscle myosin. About 15% of all cells were muscle myosin positive in these cultures by 8 days but none were detected in untreated or in retinoic acid-treated cultures.

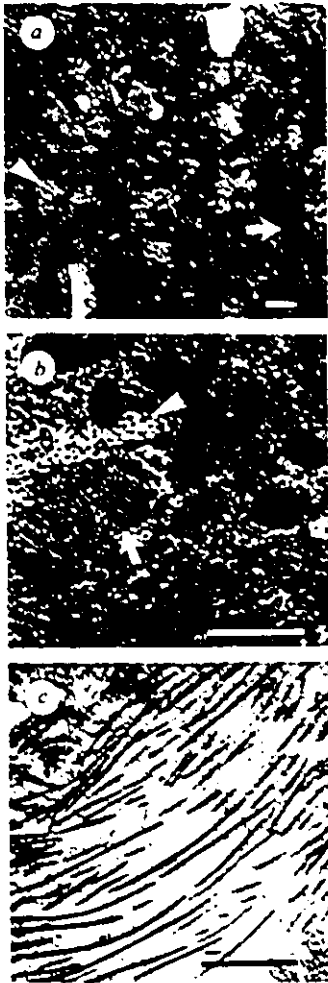


Fig. 2 Electron micrographs of some of the tissues formed in DMSO-treated cultures. a, A section through cardiac muscle shows bundles of thick and thin filaments in longitudinal section (arrows), glycogen granules (arrowhead) and numerous mitochondria. b, A section through a multinucleated myotube shows the thick and thin filaments in cross section (arrow) and glycogen (arrowhead). Many of the non-muscle cells in these cultures secreted collagen (c) which was often seen forming part of an intercellular matrix. Scale bars, 0.5  $\mu$ m.

When 10  $\mu$ M adrenaline was added to DMSO-treated cultures, the cardiac muscle responded by a 2–2.5-fold increase in contraction frequency and some previously quiescent areas of the culture were stimulated into rhythmic activity. Therefore  $\beta$ -adrenergic receptors were present. Such receptors are apparently acquired by cardiac muscle after the acquisition of spontaneous contractility<sup>10</sup>.

DMSO was not demonstrably cytotoxic to the P19S1801A1 cells at concentrations effective in differentiation experiments. Figure 3 shows that the efficiency of colony formation was unaffected by DMSO at concentrations up to 1.0%. Virtually all colonies formed in DMSO contained only embryonal carcinoma cells. In other experiments, monolayers of cells were cultured for 20 days in 1% DMSO without change in growth rate or morphology. At the end of this 20-day period, the DMSO-treated cells were aggregated in the presence or absence of DMSO (0.5%). Those aggregates formed in the absence of DMSO did not differentiate while those cultured in the drug formed muscle and fibroblasts in the usual way. Thus, it seems that the DMSO had no effect on the P19S1801A1 cells cultured as monolayers and that both the drug and cell aggregation are necessary for muscle differentiation. DMSO could be removed after 2–3 days but cardiac muscle still developed at 6–7 days as in the continuous presence of the drug.

The effects of DMSO described above were observed not

Table 1 Presence of  $\alpha$ -actin in DMSO-treated cultures

Treatment	Total actin*	$\alpha$ -actin*	% Muscle actin
Untreated, day 7	1.91 $\pm$ 0.07	0.16 $\pm$ 0.02	8.4
Retinoic acid, day 7	2.31 $\pm$ 0.03	0.30 $\pm$ 0.02	13.0
DMSO, day 7	2.62 $\pm$ 0.15	0.52 $\pm$ 0.01	19.8
DMSO, day 11	3.05 $\pm$ 0.14	0.68 $\pm$ 0.02	22.3

Aggregated cultures were prepared as described in Fig. 1 legend. Two days after plating (day 7) or 6 days after plating (day 11) the cultures were collected for analysis. The DMSO-treated day 7 culture contained rhythmically contracting but not multinucleate muscle. By day 11 both muscle types were present. Protein and peptide isolation was carried out as previously described<sup>24</sup> except for the use of trypsin instead of chymotrypsin for peptide generation. The actin contents were calculated by measuring the amounts of material which co-purified during electrophoresis at pH 6.5, 2.1 and 3.5 with tryptic peptides generated from muscle actin. Total actin values were calculated from the amounts of radioactive material co-migrating with the two chemically modified peptides CmCys-Asp-Ile-Asp-Ile-Arg and CmCys-Phe. All known actins contain peptides which should co-purify with these two.  $\alpha$ -Actin values were calculated from the amount of material co-purifying with an 18-residue CmCys-containing peptide generated from the N-terminal region of  $\alpha$ -actin. Actins from other tissues differ from  $\alpha$ -actin in this region<sup>9</sup> so should not co-purify with this peptide. Total actin is higher in differentiated than in undifferentiated cultures and there is more muscle-specific  $\alpha$ -actin in DMSO-treated cultures. The 8–13% of muscle actin present in untreated and in retinoic acid-treated cultures may represent a background of radioactive label derived from non- $\alpha$ -actin peptides which co-purify with the legitimate peptide.

\* mg actin per 100 mg total protein.

only on P19S1801A1 cells, but also on the parental P19 cells and on all of the subclones from this line which were tested. However, DMSO had no effect on the differentiation of the embryonal carcinoma cell lines F9<sup>11</sup>, OC15S1<sup>12</sup> and C86S1<sup>12</sup> whereas some clones of P10 cells<sup>13</sup> appear to form an excess of neurons in the presence of DMSO (G. D. Paterno and M.W.M., unpublished). Variation has also been observed in the response of different embryonal carcinoma lines to retinoic acid<sup>2,7</sup> and to aggregation in the absence of drugs<sup>1,2</sup>.

DMSO is an inducer of Friend cell differentiation<sup>14</sup> as are 6-thioguanine<sup>15</sup>, butyrate<sup>16</sup> and ouabain<sup>17</sup>. The effects reported above for DMSO have also been observed with non-toxic concentrations of 6-thioguanine and butyrate but not with ouabain. Another Friend cell inducer, hexamethylene bisacetamide (HMBA), has previously been shown to influence the differentiation of some other embryonal carcinoma cell lines<sup>18,19</sup> but we have not tested this compound with P19 cells.

Many papers have reported the formation of a limited range of cell types following spontaneous or induced differentiation of lines of teratocarcinomas and of embryonal carcinoma cells<sup>20–23</sup>, but it is not clear whether this is the result of differential selection or of the occurrence of a limited number of

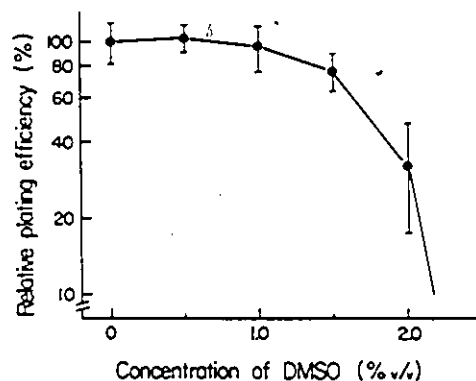


Fig. 3 The efficiency of colony formation of P19S1801A1 embryonal carcinoma cells in the presence of DMSO indicates the absence of toxicity at concentrations of less than 1% (v/v). About 200 cells were introduced into replicate 60-mm diameter dishes containing various concentrations of DMSO dissolved in  $\alpha$ -medium supplemented with 10% fetal bovine serum and  $10^{-4}$  M  $\beta$ -mercaptoethanol. Incubation was for 8 days at 37°C. All colonies consisted of cells with embryonal carcinoma morphology. Those colonies formed in 1.5% DMSO were smaller than control colonies, indicating that at these concentrations, the rate of cell proliferation was decreased.

determinative events. We think it is unlikely that differential selection can account for our observations because: (1) the cells did not differentiate into embryonic cell types in the absence of drugs, (2) the cell types formed in DMSO-treated cultures were substantially different from those formed by the same cells in parallel cultures exposed to retinoic acid, (3) neither drug appeared to be toxic, (4) all subclones responded to both drugs, (5) the drugs were effective even when cultures were exposed to them for 48 h at the beginning of an experiment, and (6) DMSO did not inhibit the formation of neurones in cultures exposed to both retinoic acid and DMSO. The simplest interpretation of these data seems to be that each drug acts by 'inducing' uncommitted embryonal carcinoma cells to differentiate along a limited number of developmental avenues. If the drugs act by bringing about intracellular changes which mimic the results of certain embryonic decisions, it may be possible to use the drugs to identify parts of the cellular decision-making apparatus.

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