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Initiation of differentiation of pluripotential embryonal carcinoma cells by retinoic acid exposure depends on cell density.

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UNIVERSITÉ D'OTTAWA
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Abstract.

Embryonal carcinoma (EC) cells are the stem cells of teratocarcinomas, and can differentiate in vivo and in vitro into a variety of cell types. EC cells are analogous to stem cells of the inner cell mass of the developing embryo, and some EC cell lines can contribute to normal tissues in chimeric animals. EC cells provide an excellent in vitro model for the study of development and differentiation.

Reports in the literature have suggested that P19 EC cells might be induced to differentiate with short exposures to retinoic acid (RA), and that they are only responsive to induction by RA in the G1 phase of the cell cycle (Gubler and Sherman, 1985; Mummery et al., 1987). We have found that a 4 hour exposure to RA induced the differentiation of P19 EC cells in both the G1 and S phases, or in exponentially growing cells. However, the density of the cells at the time of RA exposure was critical. Higher concentrations of RA were required for a 4 hr exposure to induce the differentiation of cells at higher density. Cells at higher density were more refractory to the induction of differentiation than cells at lower density. This density-dependence did not appear to be mediated by secreted or cell surface-associated factors, or by cell to cell interactions. Dose response curves at both cell densities indicated that a threshold of $4x10^9$ molecules of RA per cell was required to induce the differentiation of P19 EC cells with a 4 hr exposure. The fact that this threshold is expressed per cell suggests that the cells may be actively competing for the available RA during a 4 hr exposure, or for a secondary effector molecule.

When P19 EC cells are aggregated in the presence of 0.3 uM RA, each aggregate differentiates into neurons, glia, and fibroblast-like cells. We have induced P19 EC cells with a 4 hr RA exposure and analysed the differentiated progeny of single induced cells. We provide evidence that single induced cells differentiated into colonies consisting of fibroblasts only, neurons and fibroblasts, fibroblasts and glia, and all three cell types. Thus, induced P19 cells represent precursor cells with multiple developmental potential. The numbers of each differentiated cell type were variable from colony to colony, suggesting that P19 cells do not follow a strict cell lineage as they differentiate.

Résumé.

Les cellules embryonnaires carcinomateuses (EC) sont des cellules souches de tératocarcinomes qui peuvent être différentiées in vivo et in vitro en une variété de types cellulaires. Les cellules EC sont analogues aux cellules souches de la masse cellulaire interne de l'embryon en développement; elles peuvent également contribuer des tissus normaux dans des animaux chimériques. Les cellules EC constituent un excellent modèle in vitro pour l'étude du développement et de la différentiation.

Il a été rapporte dans la littérature que des cellules EC peuvent être induites par de brèves expositions à RA et qu'elles répondent à l'induction par RA dans la phase G1 du cycle cellulaire (Gubler et Sherman, 1985; Mummery et al, 1987). Nous avons démontré qu'une exposition de 4 heures à RA induit la differéntiation des cellules EC P19 durant la phase G1 ou S du cycle cellulaire ainsi que chez les cellules en phase de croissance exponentielle. Toutefois, la densité cellulaire au moment de l'exposition à RA est critique. De plus fortes concentrations de RA induisent la différentiation de cultures à haute densité avec une exposition de 4 heures. Cette dépendance sur la densité ne semble pas médiée par des facteurs associés à la surface cellulaire ou sécrétés ou par des interactions cellule-cellule. Des courbes dose-réponse aux deux densités cellulaires ont défini un seuil limite de $4x10^9$ molécules de RA par cellule, requis pour induire la différentiation de cellules EC P19 avec une exposition de 4 heures. Ceci suggère que les cellules compétitionnent activement pour s'approprier le RA disponible ou une autre molécule secondaire effectrice pendant l'exposition de 4 heures.

Lorsque des cellules EC P19 sont aggrégées en présence de RA à une concentration de 0.3 uM, chaque aggrégat se différentie en neurones, cellules gliales et cellules d'apparence fibroblastique. Nous avons induit des cellules EC P19 avec une exposition de 4 heures a RA et analysé la descendance différentiée de cellules induites isolées. Notre évidence expérimentale suggère qu'une cellule induite isolée se différentie en les trois types cellulaires déjà décrits et qu'elle est vraiment pluripotente. Egalement, nous suggérons que les cellules induites ne génèrent pas les mêmes quantités et types de cellules différentiées, qu'elles n'apparaîssent pas suivre un profil de différentiation strict.

Table of Contents.

Acknowledgements	i.
Abstract	ii.
Résumé	iv.
Table of Contents	vi.
List of Tables	vii.
List of Figures	viii.
List of Abbreviations	ix.

Chapter 1. Introduction.	1.
1.1. An experimental model.	1.
1.2. RA as a morphogen	3.
1.3. How does RA mediate its effects?	4.
1.4. Thesis project.	6.
Chapter 2. Experimental methods.	8.
2.1. Chemicals and Reagents.	8.
2.2. Cell culture.	8.
2.3. Cell synchronization.	9.
2.4. Chromosome preparations.	9.
2.5. Propidium iodide staining.	10.
2.6. Growth in semisolid medium and plating efficiency.	10.
2.7. Indirect immunofluorescence.	11.
Chapter 3. Results and Discussion.	13.
3.1. Differentiation of P19 EC cells induced by 4 hr exposure to RA.	13.
3.2. Cell cycle analysis.	22.
3.3. Effect of cell density at time of RA exposure.	29.
3.4. Threshold dose of RA required to induce differentiation with a 4 hr exposure.	38.
3.5. Analysis of the differentiation of cells induced by 4 hr RA exposure	48.
3.6. Discussion.	59.
Chapter 4. Conclusions.	65.
Appendix.	68.
Methods.	78.
Literature cited.	79.

List of Tables.

Table 2.1. Primary antibodies. Page 12.
Table A1. X-gal staining of pgk-lac Z transfected cell lines. Page 71.
Table A2. Percent of colonies stained changes with time. Page 73.

List of Figures.

Figure		Page
3.1.	Typical morphology of colonies derived from single P19 cells.	15.
3.2.	Indirect immunofluorescence detection of EC- and differentiation-specific antigens.	19-21.
3.3.	Progression of P19 cells through the cell cycle after synchronization by selective detachment of mitotic cells.	25.
3.4.	Response of synchronized and non-synchronized P19 EC cells to RA treatment.	28.
3.5.	Response of G1 and S phase P19 cells to various concentrations of RA.	31.
3.6.	Effect of proximity of neighboring cells on the induction of differentiation by a 4 hr RA exposure.	34.
3.7.	Effect of cell to cell contact on induction of differentiation by a 4 hr RA exposure.	37.
3.8.	Morphology of colonies before and after RA exposure.	40.
3.9.	RA dose response of P19 EC cells at various cell densities.	43.
3.10.	Response of P19 cells to 4 hr RA exposure in conditioned medium.	46.
3.11.	Removal of biologically active RA from medium by high density cultures of P19 cells.	50.
3.12.	Kinetics of the appearance of $B4F8^+$ cells in RA-treated cultures.	53.
3.13.	Distribution of $HNK\text{-}1^+$ cells in colonies derived from single RA-treated cells.	58.

List of Abbreviations.

α-MEM	alpha minimal essential medium
5-AC	5-azacytidine
B-gal	beta-galactosidase
cm	centimetre
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside
cRABP	cytosolic retinoic acid binding protein
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
EC	embryonal carcinoma
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
GFAP	glial fibrillary acidic protein
g	gram(s)
hr	hour(s)
mRNA	messenger ribonucleic acid
ug	microgram
um	micrometre
uM	micromolar
mL	millilitre
mm	millimetre
mM	millimolar
min	minute(s)
M	molar
nm	nanometre
nM	nanomolar
neo	neomycin phosphotransferase
PBS	phosphate-buffered saline
pgk-1	phosphoglycerate kinase-1
PE	plating efficiency
RA	retinoic acid
RAR	retinoic acid receptor
rpm	revolutions per minute
SSEA-1	stage-specific embryonic antigen-1
ZPA	zone of polarizing activity

For Justine.

1. Introduction.

Developmental biology is the study of the processes by which a single cell, the fertilized egg or zygote, becomes a mature organism. The zygote is totipotential, since every cell in the adult is derived from this single cell. As a multi-cellular organism develops, cells become restricted in their potential, and can only generate certain types of cells in the adult. These more restricted cells are thereby committed to certain developmental pathways or lineages. Most of these pathways end in the differentiation of immature precursor cells into cells with mature phenotypes and functions. Differentiation involves changes in gene and protein expression, cell morphology, cell cycle kinetics and other parameters.

1.1. An experimental model.

One approach to study cellular differentiation is the use of an experimental model, such as embryonal carcinoma (EC) cells (Graham, 1977; Hogan, 1977; Martin, 1980). EC cells are the stem cells of teratocarcinomas. These are tumors of embryonic origin which arise spontaneously in humans and mice, and can be induced in a variety of laboratory mouse strains. These tumors are composed of EC cells and a variety of differentiated cell types which are derived from the EC cells. Therefore EC cells are pluripotential (Kleinsmith and Pierce, 1964), and possibly even totipotential, since EC cells can contribute to virtually every tissue in chimeric mice (Papaioannou et al, 1975). Most EC cell lines differentiate in vitro, although not into the wide variety of cell types found in vivo. The differentiation of EC cells, and the associated changes in gene and protein expression, cell

morphology, cell cycle kinetics, and other parameters can be studied in vitro. Therefore EC cells provide an excellent model to study mammalian development.

The P19 line of EC cells was isolated from a teratocarcinoma derived by grafting a 7.5 day old mouse embryo onto the kidney capsule of a mouse. P19 EC cells have a euploid male karyotype (McBurney and Rogers, 1982), and can contribute to a variety of normal tissues in chimeric mice (Rossant and McBurney, 1982). In vitro, P19 EC cells can be induced to differentiate by aggregating the cells for 4 days in the presence of 1 nM to 0.1 uM retinoic acid (RA) or 1% dimethylsulfoxide (DMSO). Either of these treatments result in the formation of mesodermal cell types such as skeletal and cardiac muscle (Edwards and McBurney, 1983; McBurney et al, 1982). The gene and protein expression, morphological, and other changes accompanying this differentiation have been extensively studied (Rudnicki, 1988). When aggregates of P19 EC cells are exposed to 0.3 to 1.0 uM RA for 4 days, the differentiation follows a different pathway. Neuroectodermal cell types such as neurons and glia, and a fibroblastic cell type (Jones-Villeneuve et al, 1982) are formed. The changes associated with this differentiation pathway, and the cell types generated, have also been extensively characterized (McBurney et al, 1988). Thus the RA-induced differentiation of P19 EC cells provides an experimental model for the study of the development of the mammalian nervous system.

1.2. RA as a morphogen.

RA has a variety of effects on many EC and other cell lines, and on many tissues and organs (Sporn and Roberts, 1983). Specifically in EC cells, RA causes changes in growth kinetics and cellular morphology (Rayner and Graham, 1982; Mummery et al, 1984), and protein synthetic profiles (Schindler and Sherman, 1984). RA also causes an increase in the level of mRNAs for keratins 13 and 19 in epidermal cells (Eckert and Green, 1984), and collagen IV and laminin in F9 EC cells (Wang and Gudas, 1983). Recently, RA has been shown to directly activate transcription in F9 EC cells of a gene with unknown function, called Era-1 (LaRosa and Gudas, 1988). Although the mechanism for this increase is unknown, RA may activate transcription of these (and other) genes by binding to chromatin. Retinol, a biosynthetic precursor of RA, has been found to bind chromatin via a lipoprotein (Ferrari et al, 1988), although the same has not yet been shown for RA.

RA also has effects on embryo development: In the developing limb bud of the chick embryo, grafting cells from a posterior region, called the zone of polarizing activity (ZPA), to an anterior location causes a peculiar pattern of digit duplication (Tickle et al, 1975; Tickle, 1981). Implants of RA-soaked paper or beads in the anterior location cause an identical pattern of digit duplication (Tickle et al, 1982; Tickle et al, 1985). Furthermore, a concentration gradient of RA exists in the limb bud, with the highest concentration in the ZPA region (Thaller and Eichele, 1987). Anterior implants of RA-soaked beads result in a concentration gradient in the opposite orientation to this endogenous gradient (Eichele and Thaller, 1987).

These results suggest that RA is a natural morphogen in the chick limb bud (Slack, 1987). The effects of RA on "positional memory" in other situations, for example in regenerating anuran blastema cells (Kim and Stocum, 1986), and the identification of RA binding proteins (see below) in the cells of the developing mouse limb bud (Kwarta et al, 1985), suggest that RA may be widely employed as a signal molecule.

1.3. How does RA mediate its effects?

In vivo, RA is bound to several serum proteins, such as albumin, and a serum retinoid binding protein (Chytil and Ong, 1984). In tissue culture, retinoids are rapidly transferred through plasma membranes (Rando and Bangeter, 1982). Many cells which respond to RA, including EC cells (Jetten and Jetten, 1979), contain a cytosolic retinoic acid-binding protein (cRABP). Although a response to RA sometimes correlates with the presence of cRABP (Jetten et al, 1979; Plet et al, 1986), this is not always the case (Douer and Koeffler, 1982; Mukherjee et al, 1983). cRABP was found to rapidly translocate RA to the nucleus (Jetten and Jetten, 1979; Takase et al, 1986; Daly and Redfern, 1987). The RA remained in the nucleus, but the cRABP did not remain associated with the nucleus (Takase et al, 1986). In F9 EC cells, the RA was found associated with a nuclear RA binding component, which was distinct from cRABP, and was bound tightly to DNA (Daly and Redfern, 1987). Recently, two human genes have been cloned which encode nuclear RA receptor (RAR) proteins (Petkovich et al, 1987; Giguere et al, 1987; Brand et al, 1988). The RARs are highly homologous to steroid receptors, which bind specific ligands and activate transcription of steroid-responsive genes. These results suggest a pathway for the effects of RA: the cRABP transfers

RA from the cytoplasm to the nucleus, where RA binds to the RAR and affects transcription of genes, resulting in, for example, differentiation. An alternative fate for RA in cells is enzymatic breakdown. In hamster intestine and liver cells in tissue culture (Roberts et al, 1979) and several EC cell lines (Gubler and Sherman, 1985; Sherman et al, 1985), ^{3}H-labelled RA was rapidly metabolised to more polar compounds which were then secreted into the medium. Whether cRABP plays a role in this metabolism remains unclear, as does the possibility that one or more of these RA metabolites actually mediates the response of cells to RA.

The rapid metabolism of RA by EC cells suggested that a brief exposure to RA might be sufficient to induce their differentiation (Gubler and Sherman, 1985). Indeed, a 2 to 4 hr exposure of P19 EC cells to RA was found to induce their subsequent differentiation (Mummery et al, 1987). This effect was reported to be restricted to the G1 phase of the cell cycle. Cells in the S phase or exponentially growing were reported not to be induced to differentiate by a 4 hr RA exposure. The conclusion was that the commitment to differentiation induced by RA was G1 phase-specific. The commitment of EC cells to differentiation may be reversible (Sherman, 1986). By inducing cells with a short exposure to RA, one may be able to determine under which conditions the commitment is reversible or irreversible.

One might also be able to determine if the differentiation follows a programme or cell lineage. A cell lineage is used to describe the divisions of a precursor cell as it differentiates. In the development of the nematode worm Caenorhabditis elegans, the entire cell lineage from zygote to adult has been mapped, and is essentially invariant. The cell lineage has been studied

by the direct microscopic observation of developing C. elegans embryos (Sulston and Horvitz, 1977), the ablation of specific cells (Sulston and White, 1980), and the analysis of various developmental mutants (Sulston and Horvitz, 1981). From these studies, we know that the C. elegans cells are "programmed" to follow this lineage, with little influence from neighboring cells or the environment.

In the rat retina (Turner and Cepko, 1987), the RT4 rat tumor cell line (Droms and Sueoka, 1987), the rat optic nerve (Temple and Raff, 1986), and the neuroepithelium of the day 10 mouse embryo (Bartlett et al, 1988), cell lineages have also been studied. These studies all revealed the presence of bipotential precursor cells, which differentiated into both neurons and glial cells. However, the precursor cells did not seem to follow a strict lineage as they differentiated. A lineage analysis of differentiating EC cells has not yet been reported. Such an analysis might yield interesting information relevant to the development of the mammalian nervous system.

1.4. Thesis project.

The objectives of this study were to characterise further the initiation of differentiation of P19 EC cells by short RA exposure, and to exploit this initiation to study further the neuronal differentiation of P19 EC cells.

The initial objective was to confirm that a 4 hr RA exposure induced the differentiation of P19 EC cells, and that the initiation of differentiation of P19 cells by a 4 hr RA exposure was G1 phase-specific. The results in section 3.1 show that a 4 hr exposure to 1.0 uM RA was sufficient to induce the differentiation of P19 cells. The results in section 3.2 show that this induction of differentiation was not restricted to G1 phase cells.

Subsequently, experiments were designed to discover why these results were different from those in the literature. The results in section 3.3 show that the density of the cells at the time of RA exposure affected the induction of differentiation. This effect was not mediated by cell to cell interactions. In section 3.4, the results show that a dose of greater than $4x10^{9}$ molecules of RA per cell was required to efficiently induce the differentiation of P19 cells. Although a simple explanation for the dose per cell observation is that cells compete for RA from the medium, the cells did not appear to deplete the medium of RA during a 4 hr exposure.

Finally, in section 3.5, the initiation of differentiation of P19 cells by a 4 hr RA exposure was utilised to examine the questions of potentiality and cell lineage. The evidence indicated that single P19 cells have multiple developmental potential, but that their differentiation does not follow a programmed cell lineage.

2. Methods.

2.1. Chemicals and Reagents.

Except where noted, all chemicals and reagents were purchased from Fisher Scientific, Ottawa, Ontario, or Sigma Chemical Co., St. Louis, Mo.

2.2. Cell culture.

Cells were grown on tissue culture grade plastic in alpha minimal essential medium (α-MEM, GIBCO Laboratories, Grand Island N.Y.) supplemented with 2.5% fetal calf serum and 7.5% calf serum (Bokneck Industries, Rexdale, Ont.). This supplemented medium is refered to as "medium" throughout this thesis, and only where noted was there a change (e.g. serum free medium). Incubation was at 37°C in a 5% CO_2 atmosphere. To subculture, cells were rinsed with phosphate-buffered saline (PBS) and incubated at 37°C for 5 to 10 min in 0.025% trypsin (w/v), 1 mM ethylene diamine tetraacetate in PBS. Cells were pipetted vigorously to disperse into a single cell suspension. Cells were counted in a Coulter Counter model ZF with Channelizer (Coulter Electronics, Hialeah, Fla.). The antibiotics Fungizone (GIBCO) and gentamycin were used to combat fungal and bacterial contamination only when necessary. Cell lines tested negative for mycoplasma. A stock solution of RA (Eastman Kodak, Rochester, N.Y.) was prepared at 10^{-2} M in DMSO, and diluted to 10^{-4} M in medium immediately before use. Low density cultures were supplemented with 1 mM 2-mercaptoethanol to improve cell survival.

2.3. Cell synchronization.

Cells were plated at a density of 10^5 cells/mL 18 to 24 hours before synchronization in 25 or 150 cm^2 tissue culture flasks, depending on the number of cells required for the experiment. The medium was replaced with 2 or 10 mL of pre-warmed medium, and the loosely attached cells were removed by gently tapping the flask on the bench. The tapping was repeated three times at one half hr intervals, and the cells discarded to improve the degree of synchrony. After the third pre-shake, pre-warmed medium containing 0.06 ug/mL colcemid (Calbiochem, La Jolla, Ca.) was added and the cells incubated for 3 hours to improve the yield of mitotic cells. The flask was shaken again and the mitotic cells were collected for use.

2.4. Chromosome preparations.

Synchronized or trypsinized cells were suspended in 1 mL of PBS, and 10 mL of 0.56% (w/v) KCl was added. The cells were incubated at room temperature for 7 to 8 min, and centrifuged at 800 rpm for 5 min. The cells were fixed in 10 mL of 3:1 methanol:glacial acetic acid for 10 min at room temperature. The cells were centrifuged, washed in fixative, and recentrifuged. The cells were resuspended in fixative at 10^5 cells/ml, and dropped onto ethanol-cleaned slides. The fixative was evaporated under a 100 Watt light bulb, the slides were stained with 2% Giemsa (in water) for 3 to 5 min, and rinsed with distilled water.

2.5. Propidium iodide staining.

Cells growing on tissue culture dishes were rinsed once with serum free medium, and treated with 5 mL of propidium iodide (20 ug/mL) in PBS with 0.1% Triton X-100 for 10 to 15 min at $4^{o}C$. The cells were dispersed by pipetting, centrifuged at 800 rpm for 4 min at $4^{o}C$, and resuspended in 0.5 mL of PBS. Cells were fixed in 70% ethanol for 30 min on ice, centrifuged, resuspended in 1 mL of PBS and 7 units of bovine pancreatic ribonuclease (Boehringer Mannheim, Dorval, Que.) was added. The cells were incubated at $37^{o}C$ for 30 min, centrifuged, resuspended in PBS containing propidium iodide, and placed on ice for an additional 10 to 15 min. The cells were again centrifuged, resuspended in PBS and analysed using a fluorescence-activated cell sorter (FACS) by Mr. Martin White. A Becton-Dickinson FACS 440 with CellSort 4 software was used for the analysis.

2.6. Growth in semisolid medium and plating efficiency (PE).

12 g of methylcellulose was dissolved in 250 mL of boiling water and shaken until cool. 250 mL of cold 2x α-MEM was added to make a 2.4% w/v suspension. This methylcellulose stock was diluted to 0.8% with α-MEM, and 10^3 to 10^4 cells were added. The medium was supplemented with 7.5% calf serum, 2.5% fetal calf serum and 1 mM 2-mercaptoethanol. Cells suspended in 6 to 8 mL of methylcellulose medium were plated in 60 mm bacterial grade petri dishes. The cells were incubated 4 to 7 days, the methylcellulose was diluted with medium, and the colonies were transferred to tissue culture

dishes. To determine PE, the colonies were washed 2x with PBS, and stained for 10 min with methylene blue (2% in 95% ethanol). The stain was removed, the colonies were washed with water and counted.

2.7. Indirect immunofluorescence.

Cells growing on gelatin-coated (0.1% in water) coverslips were fixed in ethanol at -20^{o}C for 20 min, air dried, and rehydrated in PBS for 15 min. Incubation with appropriate dilutions of primary and secondary antibodies were for 45 min in a humidity chamber, each followed by three five min washes in PBS. The coverslips were mounted on glass slides with mounting medium, sealed with nail polish, and viewed under a Zeiss Photomicroscope 2 (Carl Zeiss, Inc., New York) equipped with epifluorescence optics. Table 2.1 lists the primary antibodies used, and their specificities, sources and references. For the antibodies AEC 3A1-9 and HNK-1, cells were fixed after incubation with primary and secondary antibodies. Secondary antibodies were fluorescein isothiocyanate (FITC)- or rhodamine-conjugated goat anti-mouse IgG (TAGO Inc., Burlingame, Ca.), FITC-conjugated rabbit anti-rat IgG, or FITC-conjugated swine anti-rabbit IgG (DAKOPATTS Inc., Denmark).

Table 2.1. Primary antibodies.

Antibody	Specificity	Source	Reference
AEC 3A1-9	Undifferentiated EC stem cells (SSEA-1)	Mouse monoclonal	Harris et al, 1984. Solter and Knowles, 1978.
B4F8	striated and smooth muscle α-actin	Mouse monoclonal	Lessard, 1988. Rudnicki, 1988.
Anti-GFAP	Glial fibrillary acidic protein (astrocytes, glia)	Mouse monoclonal	Debus et al, 1983. Altmannsberger et al, 1982.
HNK-1	natural killer cells, neural crest derivatives, P19-derived neurons	Mouse monoclonal	Abo and Balch, 1981. Abo et al, 1982. McBurney et al, 1988.
NF-1	Glial fibrillary acidic protein (astrocytes, glia)	Rabbit polyclonal (gift of V. Kalnins)	V. Kalnins, pers. comm.
TROMA-1	55 kD cytokeratin of endodermal epithelia, EC-derived endoderm	Rat monoclonal	Kemler et al, 1981. Boller and Kemler, 1983.

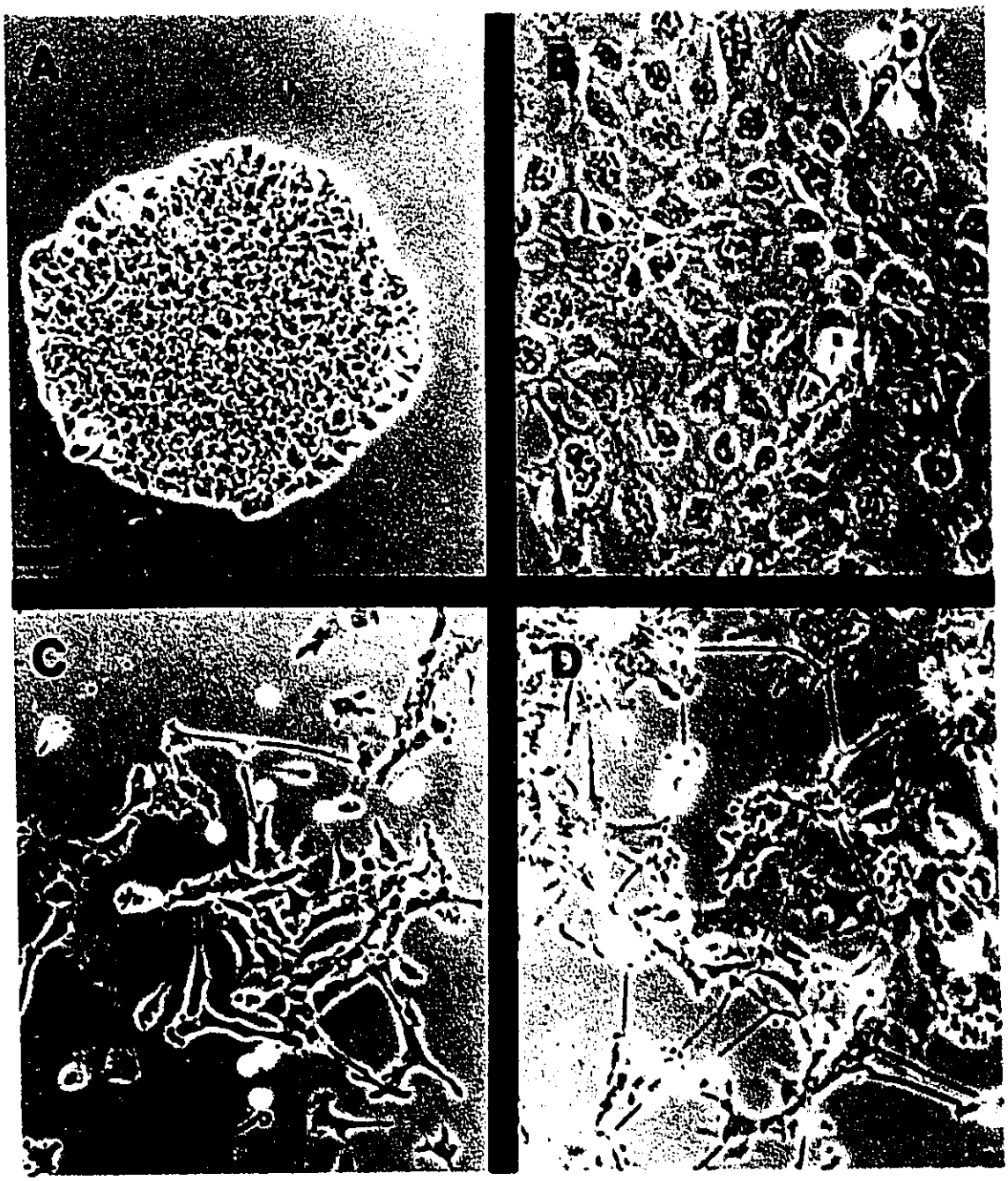
3. Results.

3.1. Differentiation of P19 EC cells induced by 4 hr exposure to RA.

We first confirmed that a brief RA exposure induced the differentiation of P19 cells. Cells were plated at $5x10^3$ cells/60 mm tissue culture dish, and treated with 1.0 uM RA for 4 hr. Following RA exposure, the cells were washed, and the medium was replaced with drug-free medium. The cells were incubated for 4 days, and observed by phase-contrast under the inverted microscope. Untreated cells developed into tightly packed EC cell colonies after 4 days (figure 3.1A). On day 4, colonies consisting of differentiated cells were readily distinguished from EC cell colonies in RA-treated cultures. A variety of morphologies were observed, two of which are shown in figure 3.1B and 3.1C. By day 7, neurons with extensive processes were observed in some colonies (figure 3.1D). In 12 samples in 6 independent experiments, 91% (values ranged from 74 to 100%) of the colonies in RA-treated samples were differentiated on day 4 after RA exposure. In the untreated samples, 5% (range 1 to 13%) of the colonies were differentiated, indicating the level of spontaneous differentiation.

The percentage of cells plated which formed colonies (PE) was 54% (range 46 to 60%) in untreated and 36% (range 32 to 41%) in RA-treated samples. Decreased PE on a tissue culture surface is an early event in the differentiation of P19 cells, and has been used as an indicator of commitment to differentiation (Campione-Piccardo et al, 1985a). Exposure of cell aggregates to 0.1 uM RA for 1 day reduced the PE to 5% of that of untreated controls. In our experiments, a 4 hr exposure to 1.0 uM RA resulted in 33%

Figure 3.1. Typical morphology of colonies derived from single P19 cells. Cells were plated at 5×10^3 cells/60 mm tissue culture dish, and were untreated (A), or exposed to 1.0 uM RA for 4 hr (B-D). The cells were washed, and the medium was replaced with drug-free medium. The cells were incubated, and photographed on day 4 (A-C) or day 7 (D). Bar=50 um.


A
B
C
D

reduction in PE. Although significant, this loss of PE following a 4 hr RA exposure is not large enough to account for the observed increase in percentage differentiation.

The loss of ability to form colonies in suspension culture in semisolid medium (i.e. loss of anchorage independent growth) is also associated with the differentiation of many EC cell lines (Jetten et al,1979; Strickland et al, 1980). We tested cells treated with RA for 4 hr to determine if they were anchorage independent. Cells were plated at $1x10^4$ cells/100 mm tissue culture dish and exposed to 1.0 uM RA for 4 hr. Following RA exposure, the cells were dispersed and plated in semisolid methylcellulose medium as described in methods. The cells were incubated for 4 days, by which time the cells had formed small colonies in suspension. The methylcellulose was diluted with medium, and the colonies were transferred to tissue culture dishes. The colonies attached to the substrate and spread out, and were scored by morphology as EC or differentiated. The colonies were stained with methylene blue and counted. 97% (average of 3 experiments, the values ranged from 95 to 98%) of the colonies were differentiated. Cells treated with 1.0 uM RA for 4 hr formed colonies in suspension with an efficiency 27% (range 19 to 39%) of that of untreated control cells. This result suggests that the loss of anchorage independent growth may precede the loss of PE following commitment to differentiation. Both in suspension and on a solid substrate, virtually all colonies formed following RA exposure were differentiated.

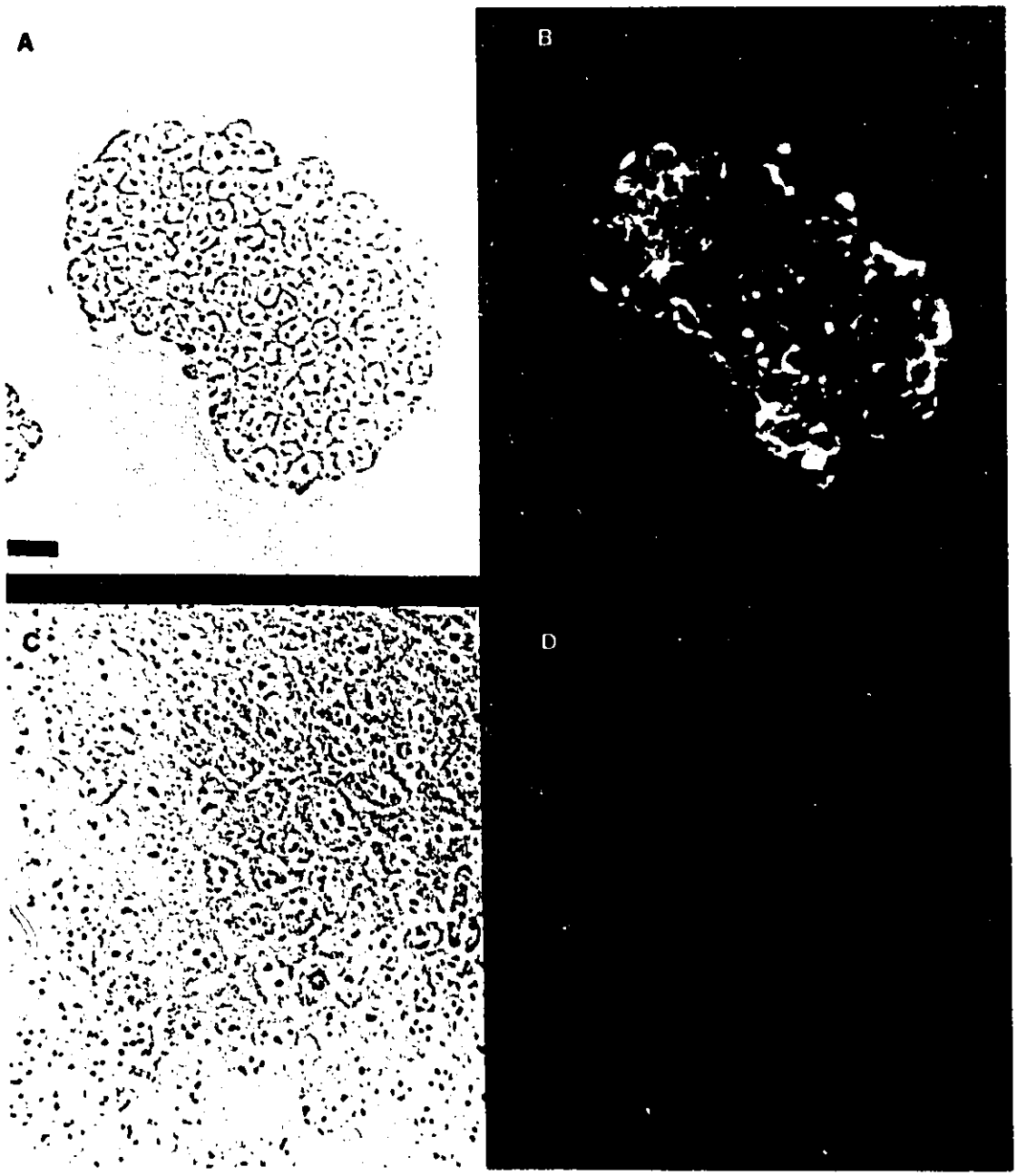
To identify the differentiated cell types present in RA-treated cultures, we performed indirect immunofluorescence experiments as described in methods. The antibodies used and their specificities are listed in Table

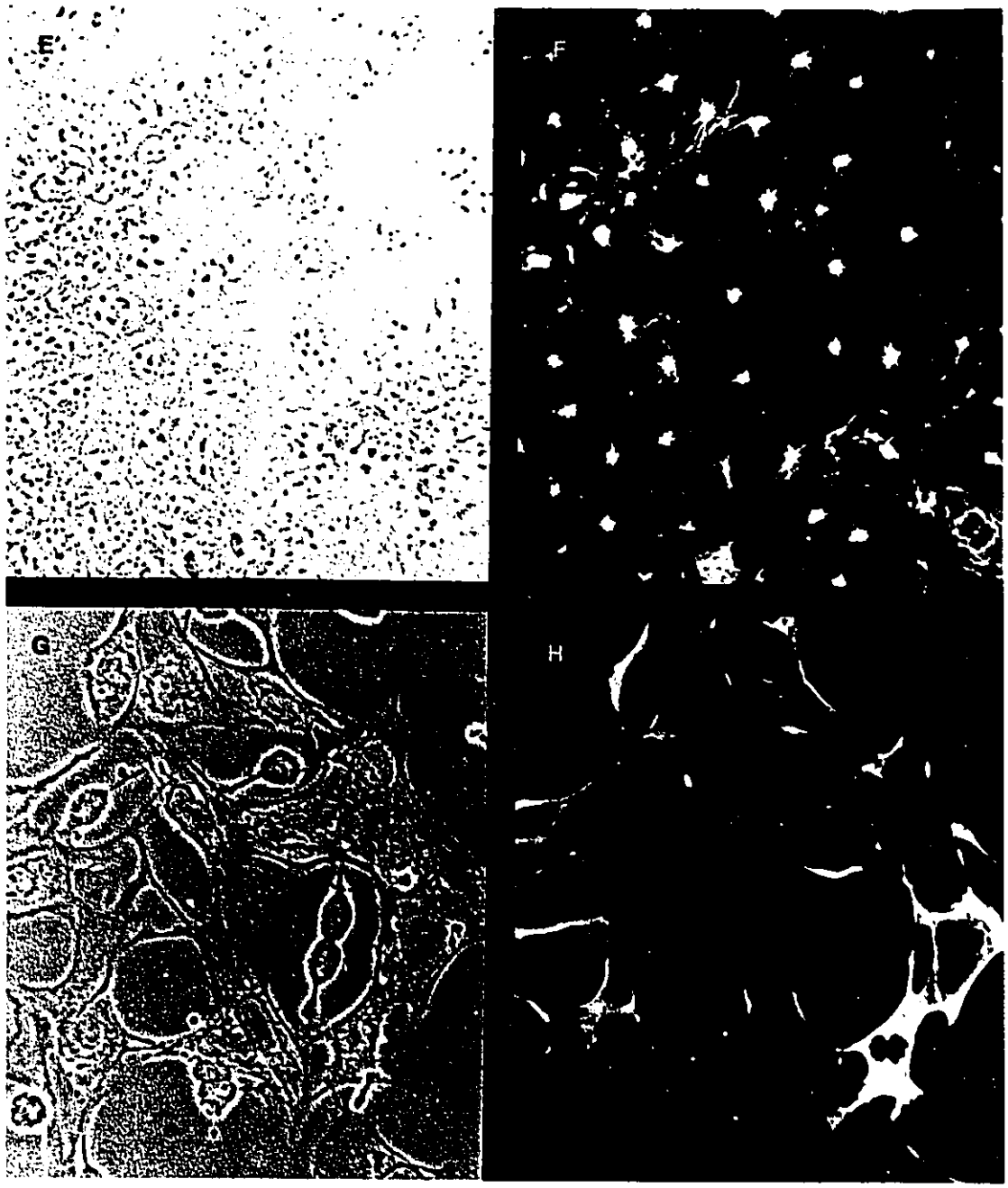
2.1. Cells were plated at $5x10^3$ cells/60 mm tissue culture dish, and treated with 1.0 uM RA for 4 hr. Following RA exposure, the cells were dispersed and plated onto gelatin-coated coverslips. The cells were incubated, and processed for indirect immunofluorescence on days 4 to 9. Quantitative data obtained by analysis of immunofluorescent staining is presented in section 3.5. Corresponding phase and fluorescence photomicrographs of representative fields of cells are shown beside one another in figure 3.2. EC cell colonies in untreated controls reacted with the stem cell-specific antibody AEC 3A1-9 (figure 3.2B, day 4) and were non-reactive with the other antibodies used (not shown).

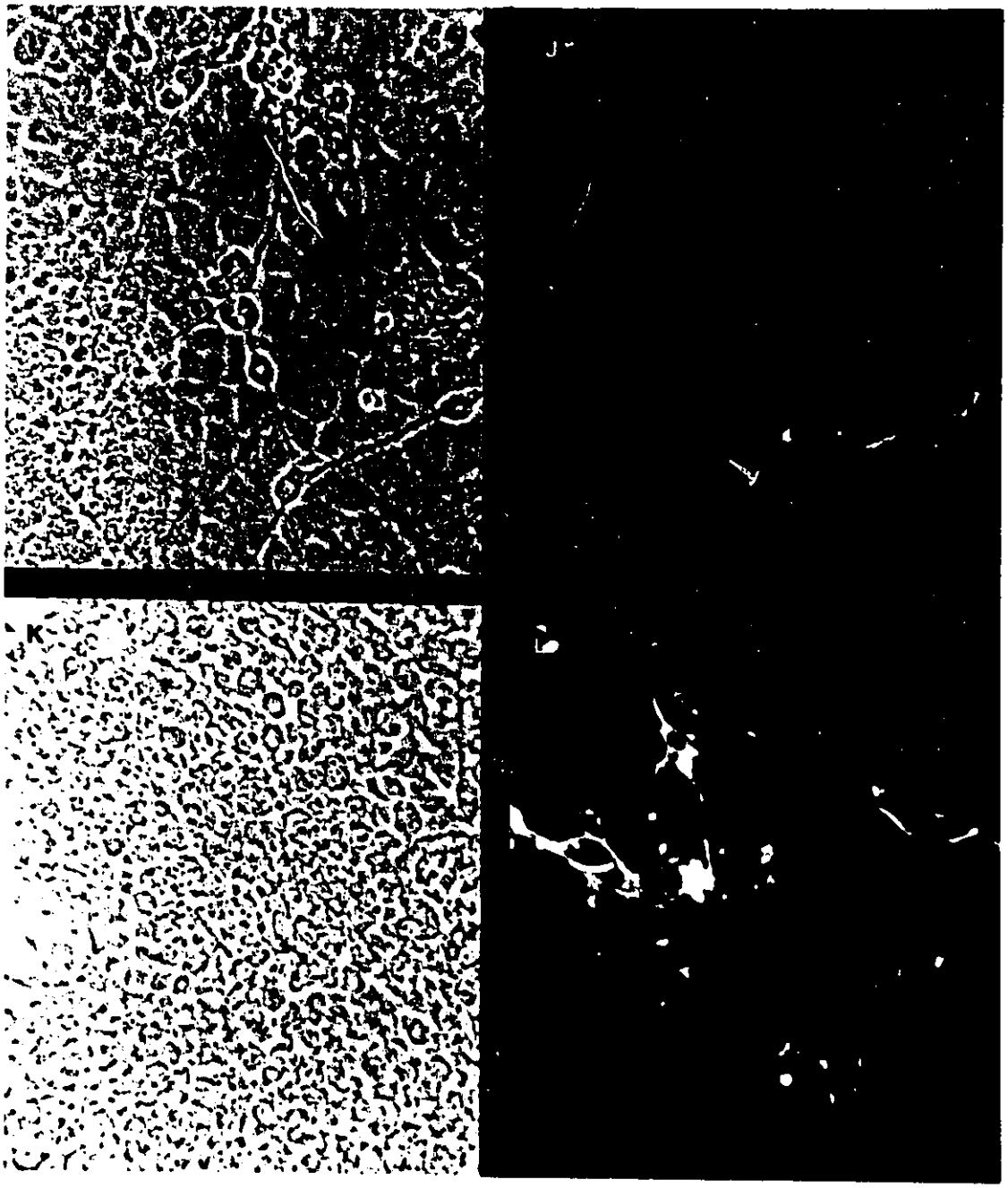
Differentiated cells in RA-treated samples were no longer reactive with the antibody AEC 3A1-9 by day 5 (figure 3.2D), but expressed differentiation-specific antigens. In most colonies, some or all of the cells were reactive with the antibody B4F8 (figure 3.2G and 3.2H, day 4). Although the stress fibers are not readily distinguished in this photograph, this antibody reacts exclusively with the abundant actin cables in these cells (Rudnicki, 1988). B4F8 recognizes muscle-specific α-actins and does not recognize β- or γ-cytoplasmic actins (Lessard, 1988).

Every cell in some differentiated colonies reacted with the antibody TROMA-1 (figure 3.2F, day 7). However, differentiated colonies in untreated controls also reacted with this antibody (not shown). This suggests that these TROMA-1^+ colonies may be derived from spontaneously differentiated cells. Neurons and their processes were reactive with the antibody HNK-1 (figure 3.2J, day 7). Some of the colonies contained cells which reacted with the anti-GFAP antibody (figure 3.2I, day 9). Thus RA-treated cells differentiated into cells expressing antigens characteristic of muscle, epithelial/endoderm, neuronal, and glial cells.

Figure 3.2. Indirect immunofluorescence detection of EC- and differentiation-specific antigens. Cells were plated at $5x10^3$ cells/60 mm tissue culture dish, and treated with 1.0 uM RA for 4 hr. The cells were dispersed and replated onto gelatin-coated coverslips. The coverslips were processed for indirect immunofluorescence on days 4 to 9 after RA exposure. Corresponding phase and immunofluorescence photographs are shown beside one another. Untreated cells (A,B) express the antigen SSEA-1 (B), while cells exposed to 1.0 uM RA for 4 hr (C-L) no longer express SSEA-1 on day 5 (D). RA-treated cells express the lineage-specific antigens recognized by the antibodies TROMA-1 (day 7, F), B4F8 (day 4, H), HNK-1 (day 7, J), and anti-GFAP (day 9, L). Bar=20 um.


A
B
C
D


E
F
G
H


J
K

These results confirmed that a 4 hr RA exposure induced the differentiation of P19 EC cells. The criteria we have used to define differentiation are colony morphology on day 4, anchorage dependence, and expression of lineage-specific antigens. In most of the experiments reported here, the tissue culture dishes were coded (to eliminate bias in scoring), and the criterion of colony morphology on day 4 was used to define differentiation.

3.2. Cell cycle analysis.

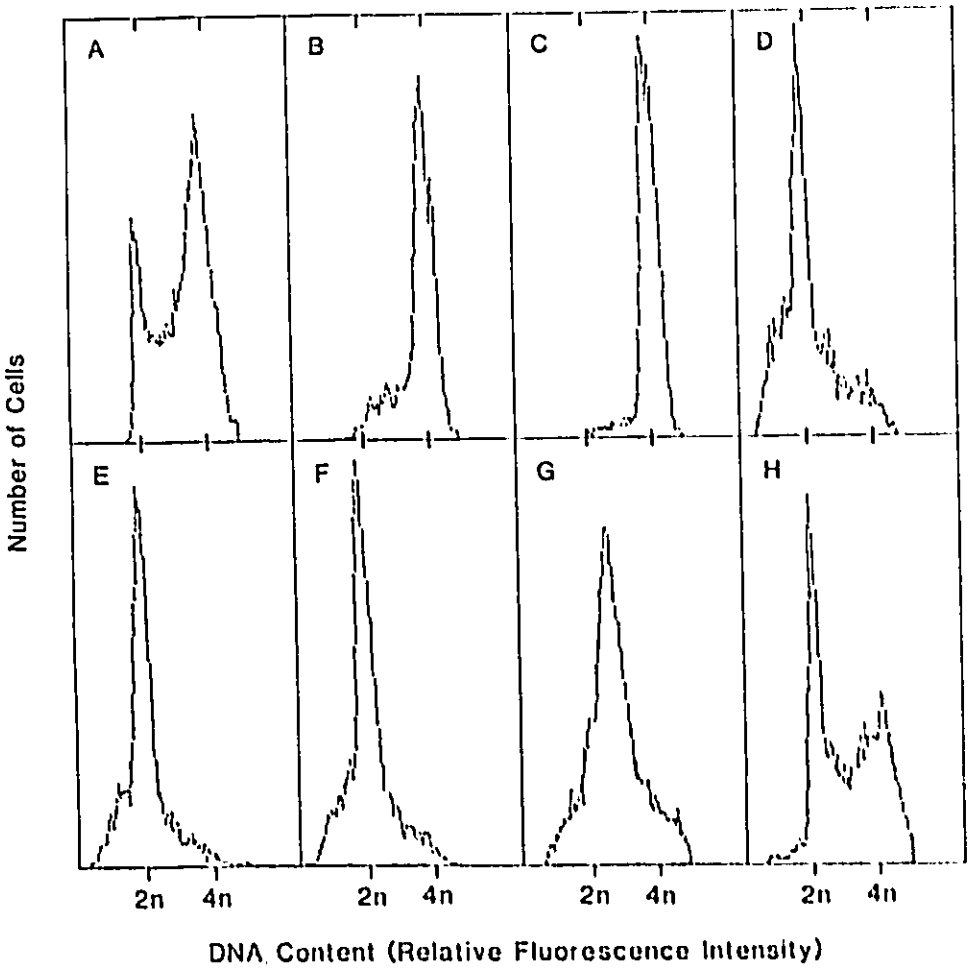
We next wanted to confirm that the commitment of P19 EC cells to differentiation induced by RA was G1 phase-specific, as was reported in the literature (Mummery et al, 1987). To synchronize cells in mitosis, we used the procedure of selective detachment of mitotic cells. Cells round up and become less adherent to the substrate as they proceed through mitosis, and can be removed from a tissue culture surface by gentle shaking. Initially, metaphase preparations of synchronized cells had very low mitotic indeces. Between 16 and 33% metaphases in a synchronized population was all that could be achieved. We found that discarding the cells removed with three 'pre-shakes' and the addition of colcemid for 3 hr increased the yield of mitotic cells. The standard synchronization procedure (as described in methods) yielded a mitotic index of 90% (average of 12 trials, the values ranged from 83 to 97% metaphases).

Although the effects of colcemid on microtubules seem to be rapidly reversed (Osborn and Weber, 1976), we wished to determine the timing of the G1 and S phases following our synchronization procedure. Cells were synchronized, and stained with propidium iodide (as described in methods) at

various times after incubation was resumed. The stained cells were analysed on a FACS for fluorescence intensity (indicating DNA content). 10^4 cells were analysed for each time, and the results are shown in figure 3.3. Greater than 92% of the cells exhibited a fluorescence intensity indicating a 4n DNA content immediately following synchronization (figure 3.3B). Between one half (figure 3.3C) and 1 hr (figure 3.3D) after synchronization, the fluorescence intensity of the majority of cells shifted to the 2n value. Thus by 1 hr after synchronization, most cells had completed mitosis and entered the G1 phase.

The majority of cells had the same fluorescence intensity at 2 hr (figure 3.3D) and 3 hr following synchronization (figure 3.3E). Thus the cells remained in the G1 phase up to at least 3 hr following synchronization. By 5 hr after synchronization, the fluorescence intensity of the majority of cells had shifted upwards (figure 3.3G). The cells entered the S phase and began to synthesize DNA within 5 hr after synchronization. P19 cell cycle data reported in the literature (Mummery et al, 1987) showed the G1 phase beginning one half hr after mitosis, and continuing for 3 hr. The S phase began 3 hr after mitosis, and continued for 9 hr. The G2/M phase began 2 hr before mitosis. Thus following our synchronization procedure, the cells completed mitosis somewhat more slowly, and entered the S phase later than previously demonstrated. However, times t=0 to 4 hr after synchronization overlaps the G1 phase, and t=6 to 10 hr correspond to the first part of the S phase of the cell cycle following our procedure for selective detachment of mitotic cells.

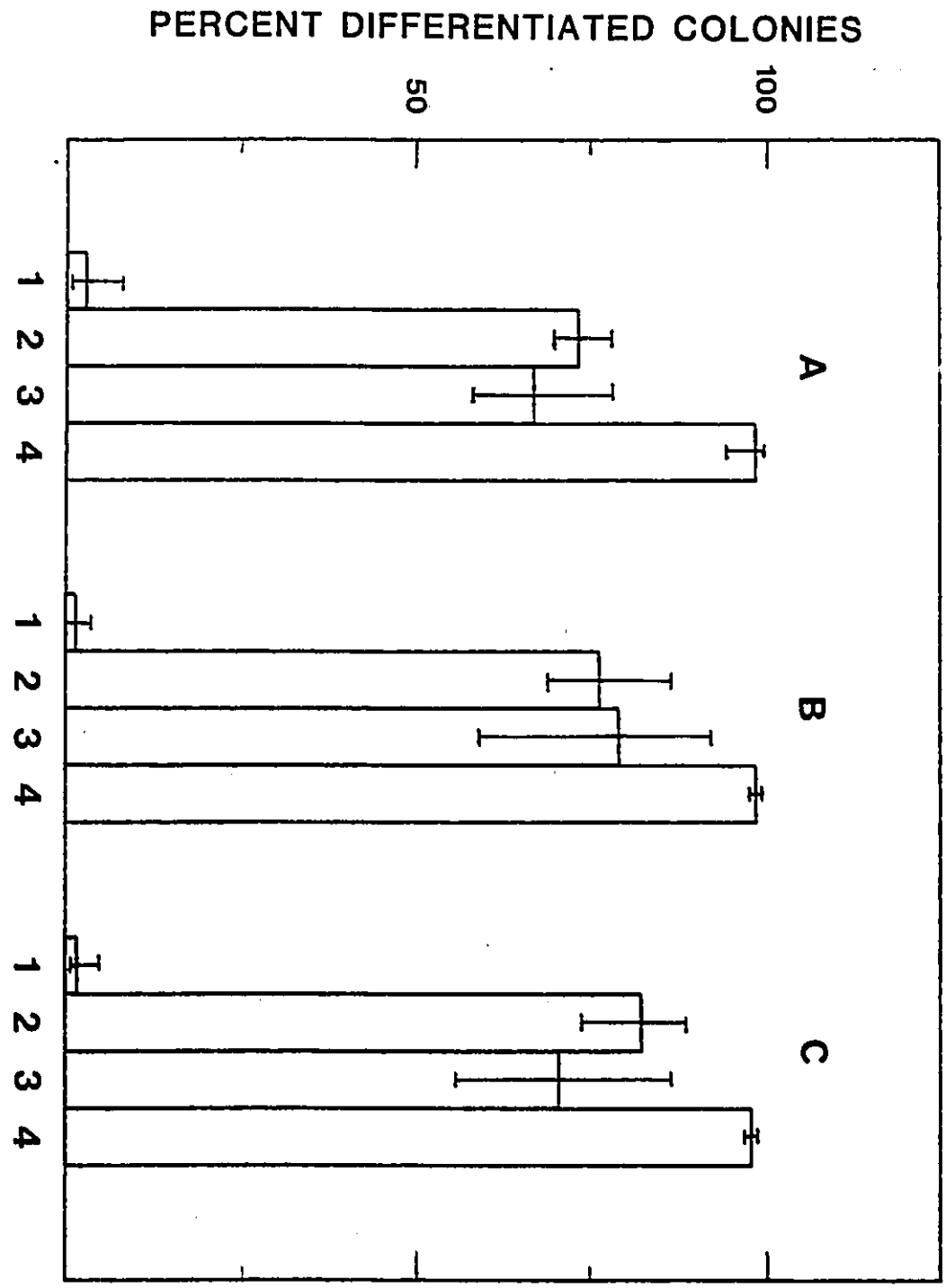
Figure 3.3. Progression of P19 cells through the cell cycle after synchronization by selective detachment of mitotic cells. P19 cells were synchronized as described in methods and plated onto tissue culture dishes to attach and divide. DNA content was determined by FACS analysis of propidium iodide fluorescence of 10^4 cells at times t=0 (B), and 1/2 hr (C), 1 hr (D), 2 hr (E), 3 hr (F), 5 hr (G), and 19 hr (H) after synchronization. Panel A shows an exponential population, from which the presumed 2n and 4n DNA content values were determined.


A
B
C
D
E
F
G
H
Number of Cells
2n
4n
2n
4n
2n
4n
2n
4n
DNA Content (Relative Fluorescence Intensity)

To determine if G1 phase cells were exclusively RA-responsive, cells were treated with RA for 4 hr in the G1 or S phase. In 16 preliminary experiments, we failed to observe a consistent difference in the RA response of G1 and S phase cells. The data shown in figure 3.4 are the results of 2 independent experiments (5 replicates of each condition). Cells were synchronized in mitosis, counted, and plated at $1x10^3$ cells/60 mm tissue culture dish. The cells were treated with 1.0 uM RA from times t=0 to 4 hr (G1 phase), or t=6 to 10 hr (S phase). Untreated cells and cultures continuously exposed to RA were also included. Cells which remained in the tissue culture flask after selective detachment of mitotic cells, and exponentially growing cells were counted, and plated at $1x10^3$ cells/60 mm tissue culture dish. These cells were exposed to 1.0 uM RA for the same time periods as the synchronized cells. Following RA exposure, the cells were washed and the medium was replaced with drug-free medium. The samples were coded, the cells were incubated, and colony morphology was scored on day 4. Cells in both the G1 and S phases were induced to differentiate with similar efficiencies by a 4 hr exposure to RA (figure 3.4A, bars 2 and 3). The colcemid-treated, non-synchronized cells (figure 3.4B), and the exponentially growing cells (figure 3.4C) were induced to differentiate with the same efficiency as the synchronized cells.

To further ensure that G1 and S phase cells were equally RA-responsive, cells were treated with various RA concentrations for 4 hr in the G1 or S phase. The RA exposures in this experiment were in serum free medium to eliminate the complication of RA binding to serum components (Chytil and Ong, 1984). Cells were synchronized in mitosis, counted, and plated at $3x10^3$ cells/60 mm tissue culture dish. Upon completion of mitosis, the dishes

Figure 3.4. Response of synchronized and non-synchronized P19 EC cells to RA treatment. Cells synchronized in mitosis (A), remaining in the flask after selective detachment of mitotic cells (B), or exponentially growing (C), were plated at $1x10^3$ cells per 60 mm tissue culture dish at t=0. 1.0 uM RA was omitted (1), or was added from t=0-4 hr (2), t=6-10 hr (3), or continuously (4). The cells were washed, and the medium was replaced with drug-free medium. The samples were coded, the cells were incubated, and colony morphology was scored on day 4. The mean and range of the data from 5 samples in 2 independent experiments are shown.

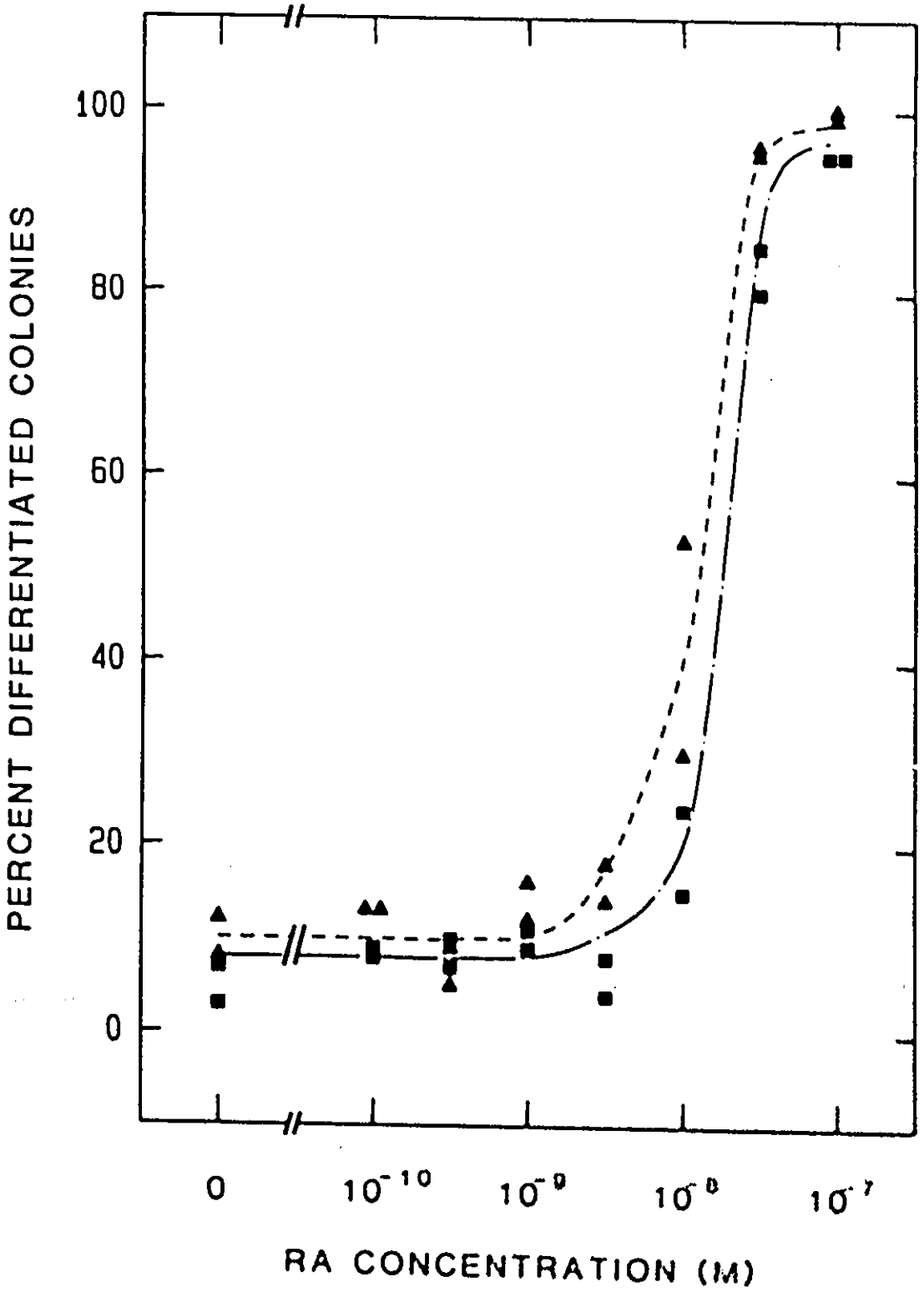

PERCENT DIFFERENTIATED COLONIES
50
100
1
2
3
4
A
1
2
3
4
B
1
2
3
4
C

should therefore contain $6x10^3$ cells. The cells were treated in serum free medium with 0.1 nM to 0.1 uM RA from times t-0 to 4 hr (G1 phase), or t-6 to 10 hr (S phase). Following RA exposure, the cells were washed and the medium was replaced with drug-free medium. The samples were coded, the cells were incubated, and colony morphology was scored on day 4. The data shown in figure 3.5 are the results of 2 samples in one experiment. The RA dose response curves were drawn by eye, and show that G1 and S phase cells were induced to differentiate to essentially the same extent following a 4 hr exposure to various doses of RA. Since our experiments indicated that the induction of differentiation by a 4 hr exposure to RA was not restricted to G1 phase cells, we used exponentially growing cells in the remainder of these experiments.

3.3. Effect of cell density at time of RA exposure.

During the course of our experiments, we noticed that the density of cells had a profound effect on the response to a 4 hr RA exposure. Cells at high density were less efficiently induced to differentiate than cells at low density. In one experiment, cells were plated at $5x10^5$ cells/60 mm tissue culture dish, and exposed to 1.0 uM RA for 4 hr. The cells were then dispersed and replated at $1x10^3$ cells/60 mm tissue culture dish. The cells were incubated for 4 days, and colony morphology was scored. Only 9% (average of 3 samples, the values ranged from 7 to 17%) of the colonies were differentiated. In untreated samples at this density, 2% (range 0 to 4%) of the colonies were differentiated. As shown above (section 3.1 and figure 3.4), a 4 hr exposure to 1.0 uM RA efficiently induced the differentiation of cells at low density.

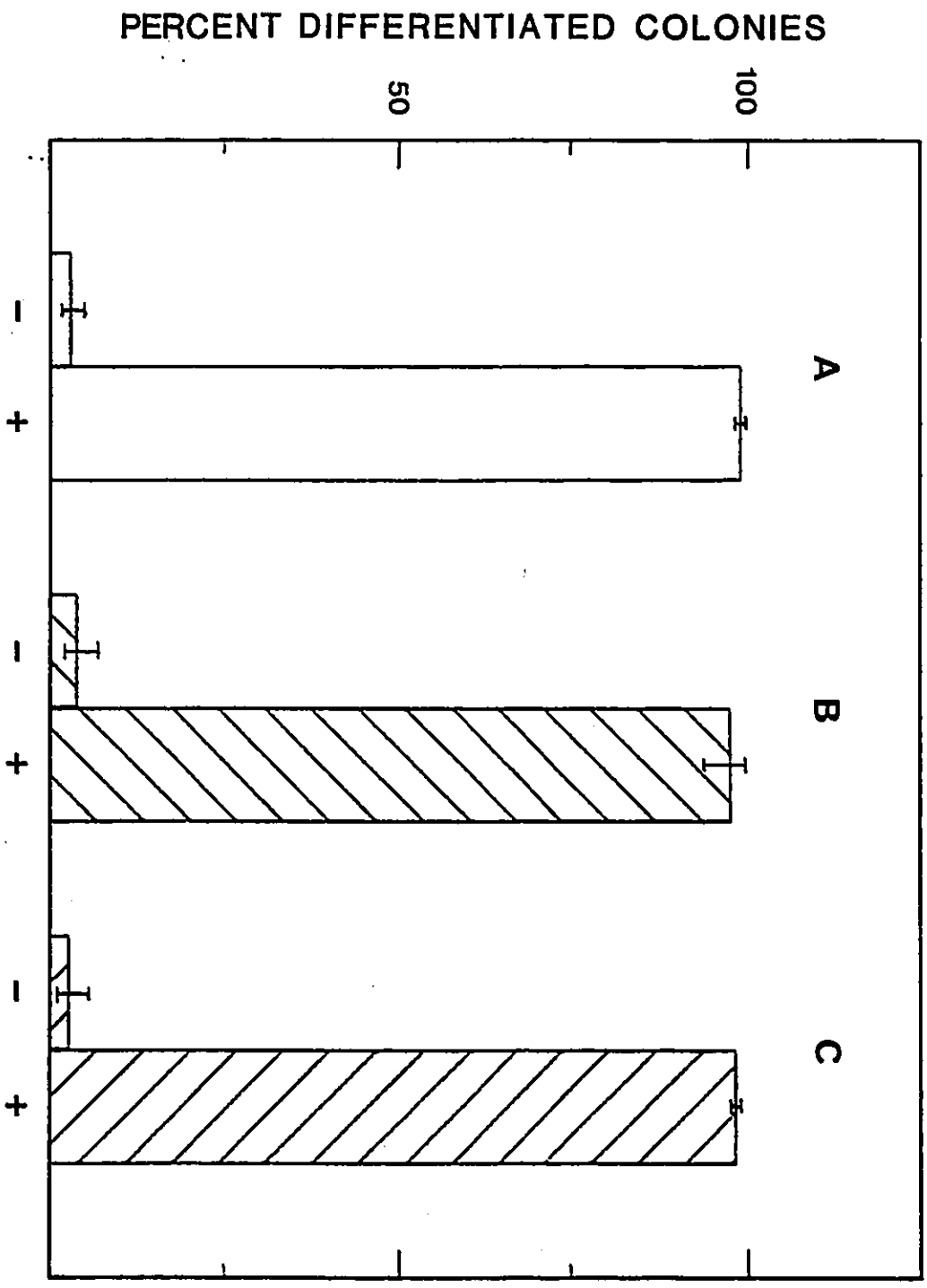
Figure 3.5. Response of G1 and S phase P19 cells to various concentrations of RA. P19 EC cells were synchronized by selective detachment of mitotic cells. $3x10^3$ cells were plated into 60 mm tissue culture dishes, and treated for 4 hr with various doses of RA in serum-free medium. RA treatment was from t=0 to 4 hr (▲, G1 phase) or from t=6 to 10 hr (■,S phase) after synchronization. The cells were washed, and the medium was replaced with drug-free medium. The samples were coded, the cells were incubated, and colony morphology was scored on day 4. Each symbol represents one sample, curves drawn by eye.


PERCENT DIFFERENTIATED COLONIES
100
80
60
40
20
0
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
RA CONCENTRATION (M)

We investigated the possibility that the RA insensitivity of high density cultures was the result of cell to cell interactions. To create conditions of high cell density and increased cell to cell contacts without changing the total cell number or amount of RA, we performed three experiments. The first was to vary the 'local' cell density (cells/cm^2) by plating the same numbers of cells in various volumes of medium. $5x10^3$ cells were plated in 20 uL, 100 uL, or 4.5 mL of serum free medium and allowed to attach to a tissue culture surface. The diameters that these amounts of medium occupied were measured as 0.6 cm, 1.2 cm, and 5.2 cm, respectively. The cells therefore occupied areas of 0.28, 1.22. and 21.24 cm^2, respectively. Medium or serum was added as required, so that each tissue culture dish contained 5 mL of serum containing medium. The cells were then exposed to 1.0 uM RA for 4 hr. Following RA exposure, the cells were dispersed and replated into new tissue culture dishes. Cells were incubated, and colony morphology was scored on day 4. Figure 3.6 shows the average result of 3 independent experiments. Over a 100-fold range in the density of $5x10^3$ cells, the cells were induced to differentiate with almost identical efficiencies by a 4 hr exposure to 1.0 uM RA.

In the second experiment to create conditions of increased cell to cell contact, cells were plated at low density and allowed to grow into small colonies. After incubation for 1 to 4 days, cells formed small colonies with extensive cell to cell contacts (see below). We treated these colonies with RA for 4 hr and then dispersed the cells into new tissue culture dishes. This

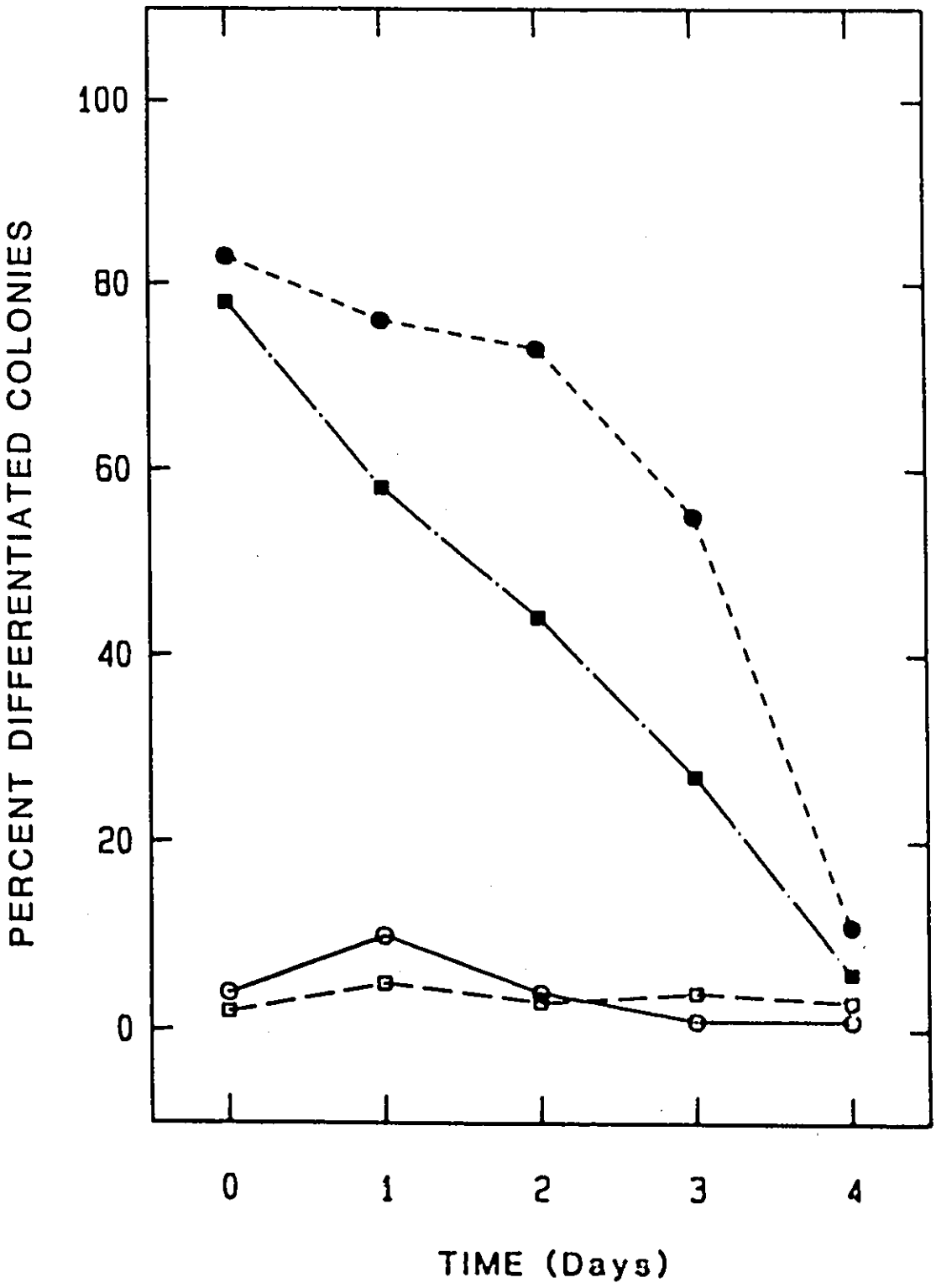
Figure 3.6. Effect of proximity of neighboring cells on the induction of differentiation by a 4 hr RA exposure. $5x10^3$ cells were plated in 4.5 ml ($2.4x10^2$ cells/cm^2, A), 100 ul ($4.1x10^3$ cells/cm^2, B), or 20 ul ($1.8x10^4$ cells/cm^2, C) of serum free medium, and allowed to attach to a tissue culture surface. Cells were treated in 5 mL of serum containing medium with (+) or without (-) 1.0 uM RA for 4 hr, dispersed to new tissue culture dishes, and colony morphology was scored on day 4. The mean and range of the data from 3 independent experiments are shown.


PERCENT DIFFERENTIATED COLONIES
50
100
-
+
A
-
+
B
-
+
C

allowed us to score colonies which developed from single cells which had been exposed to RA while in contact with many neighboring cells. Cells were plated on day 0 at $1x10^3$ cells/dish in a series of 60 mm tissue culture dishes. At daily intervals, the cells in one set of dishes were treated with 0.3 uM RA for 4 hr, and then dispersed into new dishes. In another set, the cells were dispersed into new tissue culture dishes and then exposed to 0.3 uM RA for 4 hr. Following RA exposure, the cells were washed and the medium was replaced with drug-free medium. Cells in a third and fourth set of dishes were dispersed at the same times, but not treated with RA. Cultures were incubated, and colony morphology was scored 4 days after RA exposure. The result of one experiment is shown in figure 3.7.

The percentage of cells which were induced to differentiate decreased with time. 80% of the cells treated on day 0 were induced to differentiate, whereas only 10% of the cells treated on day 4 were induced to differentiate by a 4 hr exposure to 0.3 uM RA. The RA response of these cells presumably decreased due to cell proliferation and increasing cell density. At every time tested, the percentage of cells which were induced to differentiate was greater in the samples dispersed after RA exposure. There was no evidence for increased RA resistance of cells exposed to RA while in contact with neighboring cells in these colonies. This experiment was repeated 3 times, and we failed to observe a consistent difference in the RA response of cells dispersed before and after RA exposure.

Figure 3.7. Effect of cell to cell contact on induction of differentiation by a 4 hr RA exposure. Cells were plated at $1x10^3$ cells/60 mm tissue culture dish on day 0, treated with 0.3 uM RA for 4 hours (filled symbols), or without RA (open symbols), at the times indicated. Cells were dispersed into new tissue culture dishes prior to (squares) or after (circles) RA exposure. The cells were washed, and the medium was replaced with drug-free medium. The cells were incubated, and colony morphology was scored 4 days after the RA exposure in each case.

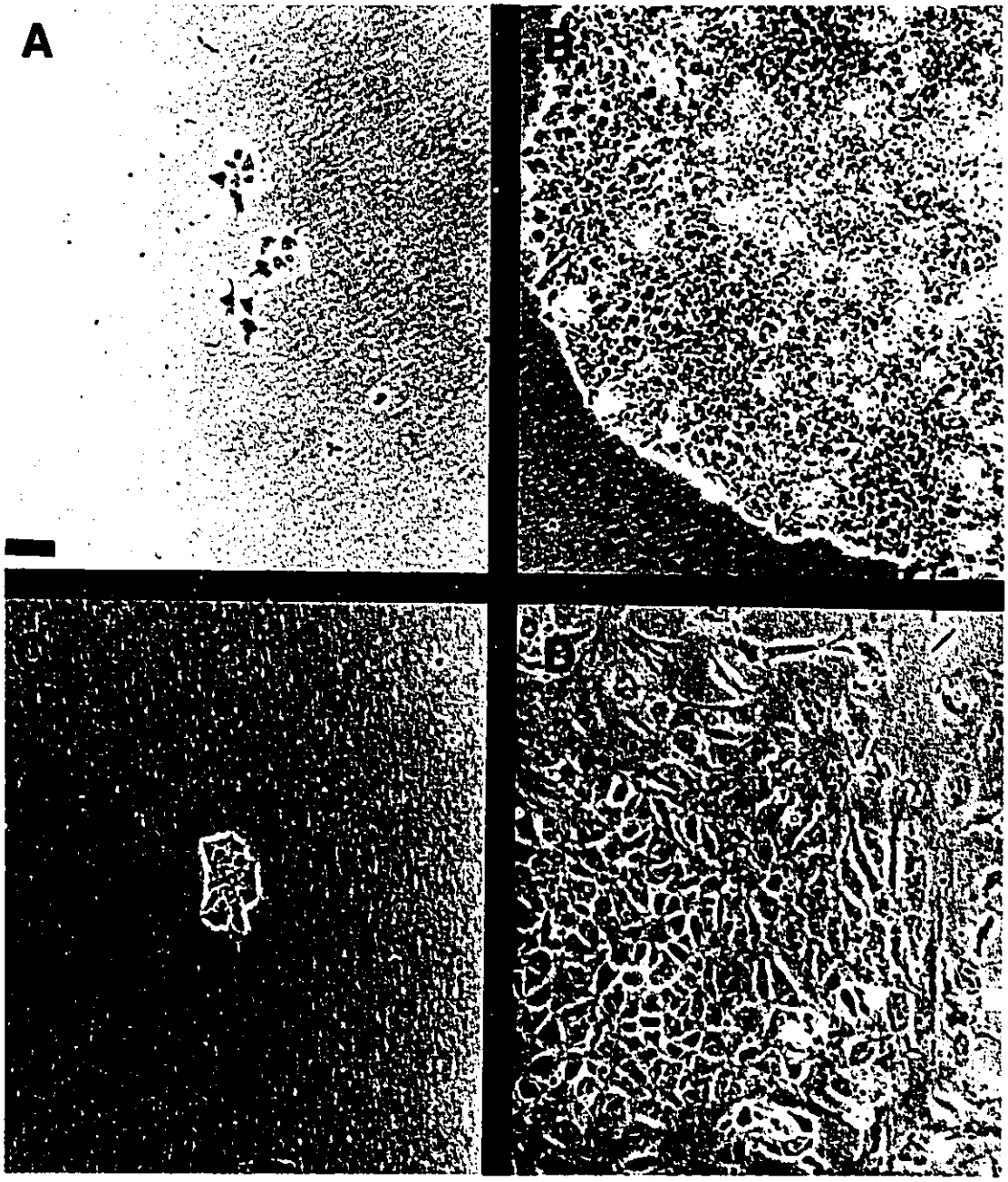

PERCENT DIFFERENTIATED COLONIES
100
80
60
40
20
0
0
1
2
3
4
TIME (Days)

The third experiment to create conditions of increased cell to cell contact is shown in the photomicrographs of figure 3.8. Cells were plated at $5x10^2$ cells/60 mm tissue culture dish and incubated for 2 days. Figures 3.8A and 3.8C show two colonies photographed on day 2. The colonies were marked on the underside of the tissue culture dishes so that they could be located again and rephotographed. 1.0 uM RA was added to the medium in the tissue culture dish which contained the colony in figure 3.8C, and the cells were incubated for 4 hr. Following RA exposure, the cells were washed and the medium was replaced with drug-free medium. The colony in figure 3.8A was in an untreated control dish. The cells were incubated for an additional 4 days after RA exposure, and these colonies were rephotographed on day 6. The untreated colony developed further into a large undifferentiated colony (figure 3.8B). The RA-treated colony developed into a colony containing only cells with differentiated morphology (figure 3.8D). The cells in the colony shown in figure 3.8C were induced to differentiate by a 4 hr exposure to 1.0 uM RA while in contact with many neighboring cells. These three experiments provided evidence that the RA insensitivity of high density cultures was not the result of cell to cell interactions.

3.4. Threshold dose of RA required to induce differentiation with a 4 hr exposure.

To examine more closely the relationship between cell density and RA response, we treated cells at three densities with various RA concentrations. Cells were plated in serum free medium at $5x10^3$, $5x10^4$, and $5x10^5$ cells/dish in a series of 60 mm tissue culture dishes. The cells were treated with 0.1 nM to 10 uM RA for 4 hr. Following RA exposure, the cells

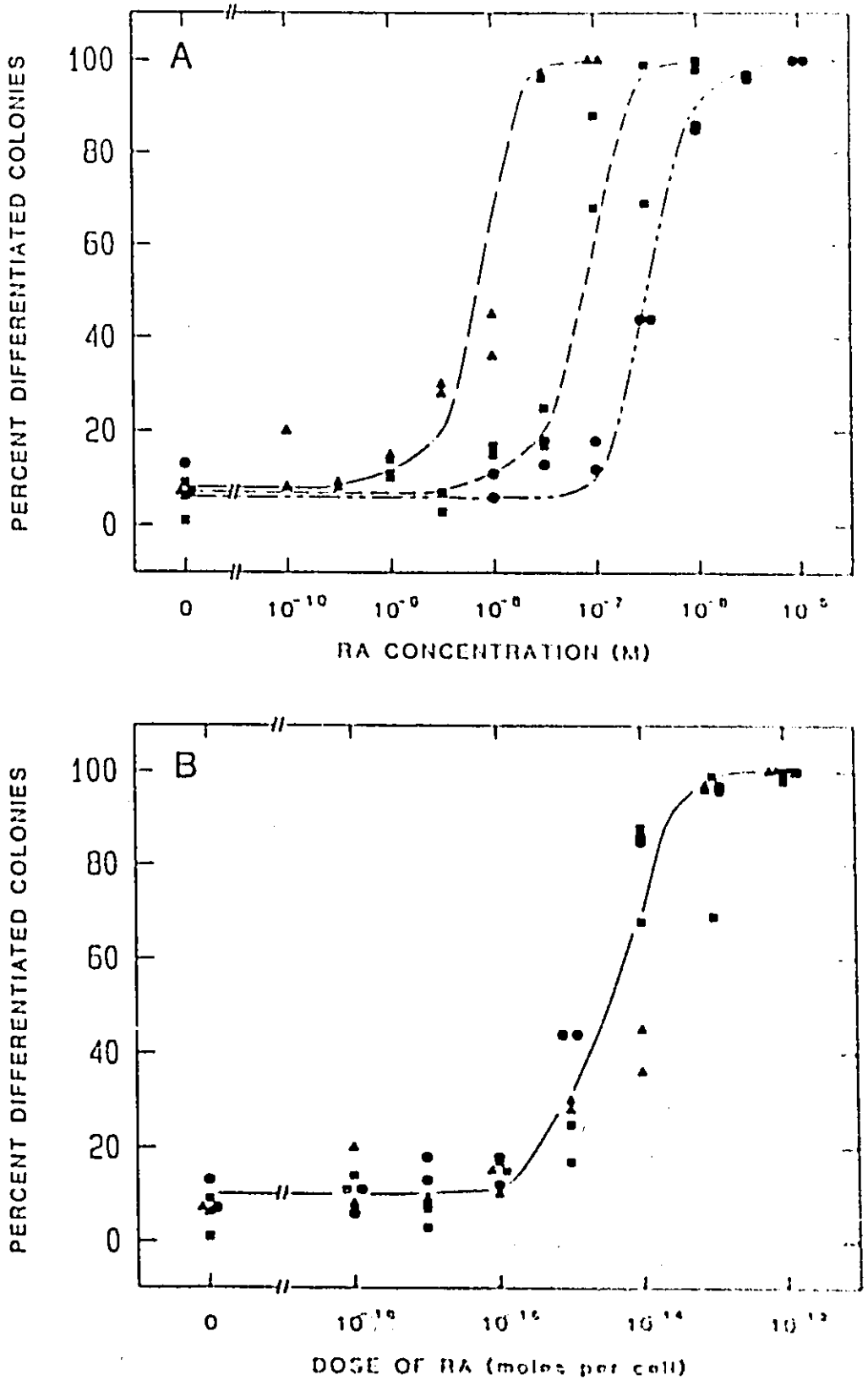
Figure 3.8. Morphology of colonies before and after RA exposure. Cells were plated at $5x10^2$ cells/60 mm tissue culture dish on day 0 and colonies were photographed on day 2 (A,C). The colony in A was in an untreated control dish. The colony in C was in a dish treated with 1.0 uM RA for 4 hr on day 2. The cells were washed, and the medium was replaced with drug-free medium. The cells were incubated for an additional 4 days, and the colonies rephotographed on day 6 (B,D). Bar=50 um.


A
B
B

were dispersed and replated at $1x10^3$ cells/60 mm tissue culture dish in drug-free medium. The samples were coded, the cells were incubated, and colony morphology was scored on day 4. The results of one experiment are shown in figure 3.9. At all three cell densities, the cells exposed to lower doses of RA were not induced to differentiate. Cells which were exposed to higher doses of RA were induced to differentiate. However, higher RA concentrations were required to induce differentiation of cells at higher density. The dose response curves (drawn by eye) were separated along the abscissa by approximately 10-fold increments in RA concentration. As the cell density increased 10-fold, the dose of RA required for a 4 hr exposure to induce differentiation also increased approximately 10-fold. This experiment was repeated 3 times with similar results.

The RA concentrations were converted to doses of RA in units of moles per cell. These values were used to plot the same data in figure 3.9B. A smooth curve was drawn (by eye) to pass through the means of the six datapoints for each dose of RA per cell. The inflection point of this curve is at approximately $6x10^{-15}$ moles/cell, or $4x10^9$ molecules of RA per cell. A dose of $4x10^9$ molecules of RA per cell induced the differentiation of 50% of the cells. This value represents a threshold dose of RA which is required to induce the differentiation of P19 cells with a 4 hr exposure. The RA dose response of P19 cells is profoundly affected by cell density. The critical parameter of cell density is total cell number, rather than cell to cell interactions.

Figure 3.9. RA dose response of P19 EC cells at various cell densities. Cells were plated in serum free medium at $5x10^3$ (▲), $5x10^4$ (■), or $5x10^5$ (●) cells/60 mm tissue culture dish. The cells were exposed to various RA concentrations for 4 hr. Following RA exposure, the cells were dispersed and replated into new tissue culture dishes at $1x10^3$ cells/dish. The dishes were coded, the cells were incubated, and colony morphology was scored on day 4. In Panel A the data show the response of P19 cells at the 3 cell densities to various RA concentrations. In Panel B the same data show the response of P19 cells to various doses of RA per cell. Curves drawn by eye.

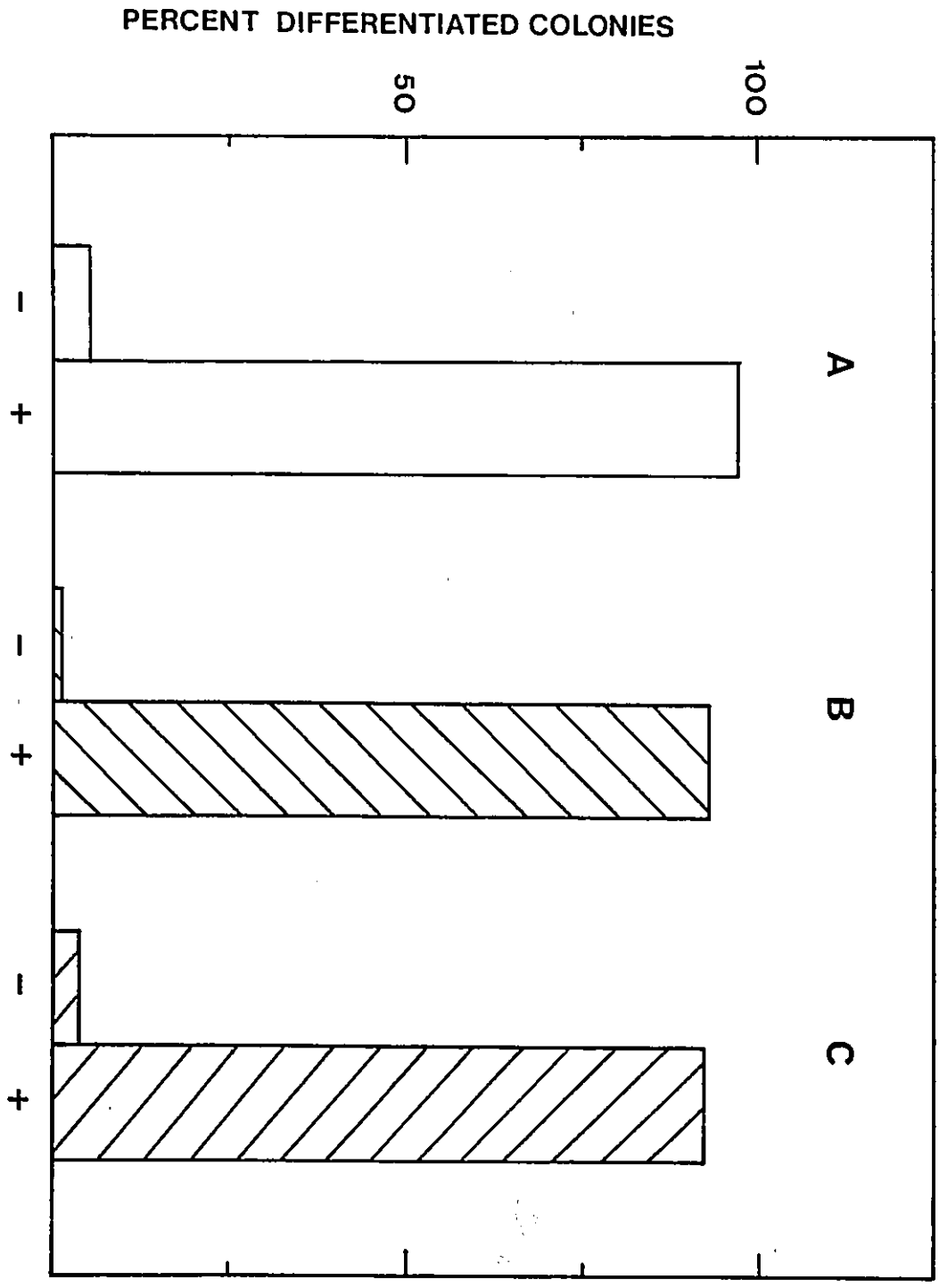

A
PERCENT DIFFERENTIATED COLONIES
100
80
60
40
20
0
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
RA CONCENTRATION (M)
B
PERCENT DIFFERENTIATED COLONIES
100
80
60
40
20
0
0
10^{-15}
10^{-15}
10^{-14}
10^{-13}
DOSE OF RA (moles per cell)

Many EC cell lines are known to synthesize growth factors (Rizzino et al, 1983; Heath and Isacke, 1984). We therefore investigated the possibility that high density cultures of P19 EC cells secreted a factor which stimulated proliferation of EC cells and inhibited RA-induced differentiation. Cells were plated at $5x10^5$ cells/dish in a series of 60 mm tissue culture dishes. One set was untreated, and one set was treated with 1.0 uM RA for 4 hr. The cells were washed, the medium was replaced with drug-free medium, and the cells were incubated for 2 days. The medium was removed, placed into new tissue culture dishes, and then tested for differentiation-inhibiting activity.

$5x10^3$ cells/60 mm tissue culture dish were plated in these media. One sample was untreated, and one was treated with 1.0 uM RA for 4 hr. The cells were washed and the medium was replaced with drug-free medium. The cells were incubated for 4 days and colony morphology was scored on day 4. The result of one experiment is shown in figure 3.10. The RA-treated cells in each type of medium were induced to differentiate by a 4 hr exposure to 1.0 uM RA to the same extent as the control cells in fresh medium (figure 3.10). We were unable to detect any activity secreted by high density cultures of P19 EC cells which inhibited the induction of differentiation. A 4 hr exposure to 1.0 uM RA did not appear to induce high density cultures to secrete such an activity.

The fact that the threshold of the dose response curves is expressed in units of RA per cell suggests that the cells may be 'competing' for the available RA during a 4 hr exposure. To examine the kinetics by which RA is removed from the medium by P19 cells, we treated high density cultures with

Figure 3.10. Response of P19 cells to 4 hr RA exposure in conditioned medium. $5x10^3$ cells/60 mm tissue culture dish were plated with (+) or without (-) 1.0 uM RA for 4 hr in fresh medium (A), or medium in which $5x10^5$ cells/60 mm tissue culture dish were grown for 2 days untreated (B), or after they were exposed to 1.0 uM RA for 4 hr (C, see text). The cells were washed, and the medium was replaced with drug-free medium. The cells were incubated, and colony morphology was scored on day 4.


PERCENT DIFFERENTIATED COLONIES
50
100
-
+
A
-
+
B
-
+
C

RA for various times. The medium was then tested for any remaining differentiation-inducing activity (which we assume is RA). Cells were plated at $5x10^5$ cells/60 mm tissue culture dish and treated with 1.0 uM RA for 4 hr, 1 day, or 2 days. The medium was removed and placed into new 60 mm tissue culture dishes. $5x10^3$ cells were plated in each of these media and incubated for 4 hr. The cells were washed, and the medium was replaced with fresh medium. In one series of dishes, the cells were incubated continuously in the medium being tested. The samples were coded, the cells were incubated, and colony morphology was scored on day 4. The results shown in figure 3.11 are from one experiment only.

The medium in which high density cells were incubated with 1.0 uM RA for 4 hr efficiently induced the differentiation of a low density culture with a 4 hr exposure (figure 3.11A). The medium in which high density cells were incubated with 1.0 uM RA for 1 day did not induce the differentiation of low density cultures with a 4 hr exposure, but did induce differentiation with a continuous exposure (figure 3.11B). The medium in which high density cells were incubated with 1.0 uM RA for 2 days did not induce the differentiation of low density cultures even with continuous exposure (figure 3.11C). Medium incubated for 2 days with 1.0 uM RA (without cells) efficiently induced differentiation with a 4 hr exposure (figure 3.11D). Thus although a 4 hr exposure to 1.0 uM RA did not induce the differentiation of high density cells, the medium was not depleted of RA during this exposure. By day 1, the high density cells had depleted some, but not all, of the RA from the medium. By day 2, when the high density cells were induced to differentiate (not shown; see Jones-Villeneuve et al, 1982), no biologically active RA was

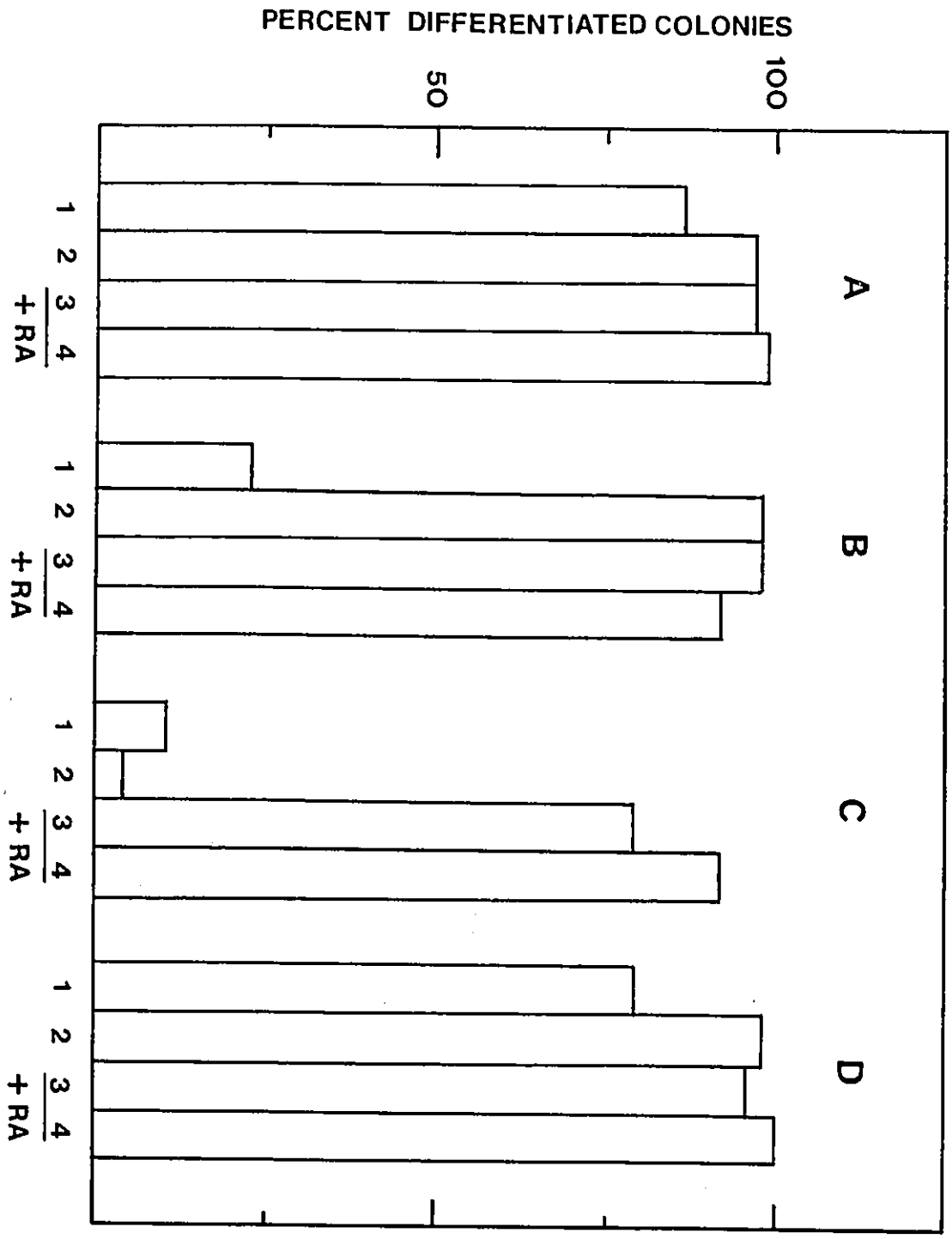
detectable in the medium. Since the cells did not deplete the medium of RA in 4 hr, it seems that they were not competing for the available RA during the 4 hr exposure.

We also tested these media for an activity (factor) which inhibited the induction of differentiation (see figure 3.10). 1.0 uM RA was added to one set of the media being tested, and $5x10^3$ cells were plated in these media for 4 hr. The cells were washed, and the medium was replaced with drug-free medium. The samples were coded, the cells were incubated, and colony morphology was scored on day 4. All of the cultures treated with RA were efficiently induced to differentiate by a 4 hr exposure (figure 3.11, +RA lanes). As in figure 3.10, there was no evidence for an activity (factor) in any of these media which inhibited the induction of differentiation by a 4 hr exposure to RA.

3.5. Analysis of the differentiation of cells induced by 4 hr RA exposure.

We have shown that a 4 hr exposure to RA induced the differentiation of P19 EC cells (section 3.1), provided the dose of RA per cell was above a threshold (section 3.4). We next studied the differentiation of single P19 cells after this induction. We wanted to determine if single induced cells have multiple developmental potentials, and to determine if there is a programmed cell lineage in their differentiation.

Figure 3.11. Removal of biologically active RA from medium by high density cultures of P19 cells. P19 cells at $5x10^3$ cells/60 mm tissue culture dish were exposed for 4 hr (1,3) or continuously (2,4) to medium in which $5x10^5$ cells/60 mm tissue culture dish had been grown in the presence of 1.0 uM RA for 4 hr (A), 1 day (B), or 2 days (C). See text for detailed explanation. Group D is medium which was incubated with 1.0 uM RA for 2 days without cells. An additional 1.0 uM RA was added to one sample of each media (+ RA) at the beginning of the exposure of the test ($5x10^3$) cells. After the 4 hr RA exposure, the cells were washed, and the medium was replaced with drug-free medium. The cells were incubated, and colony morphology was scored on day 4.

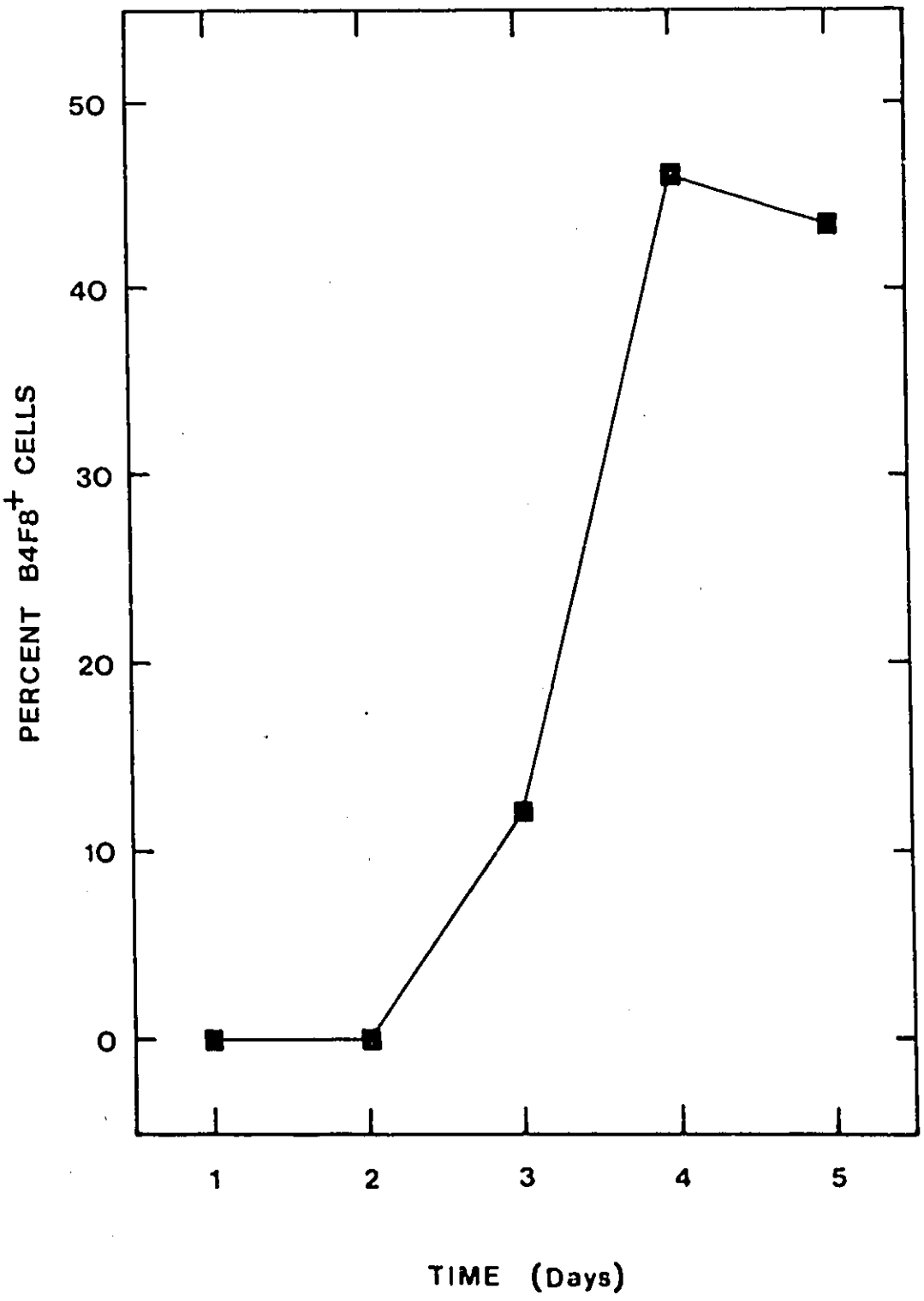

PERCENT DIFFERENTIATED COLONIES
50
100
A
B
C
D
1
2
3
4
+RA

One of the differentiated cell types which appeared following a 4 hr RA exposure (see section 3.1) expressed the muscle-specific actin isoform recognized by the antibody B4F8 (Jessard, 1988; Rudnicki, 1988). We analysed the kinetics of the appearance of $B4F8^+$ cells by indirect immunofluorescence. Cells were plated at $5x10^4$ cells/60 mm tissue culture dish and treated with 10 uM RA for 4 hr. Following RA exposure, the cells were dispersed and replated onto gelatin-coated coverslips. At daily intervals, coverslips were fixed and processed for indirect immunofluorescence as described in methods, using the antibody B4F8. The percentage of $B4F8^+$ cells in one experiment is plotted vs. time in figure 3.12. $B4F8^+$ cells first appeared on day 3, and increased to a maximum of approximately 45% by day 4.

Some of the differentiated colonies on the day 5 coverslips were scored individually to determine the number of $B4F8^+$ and $B4F8^-$ cells in each colony. 20 colonies were scored, although we cannot be certain that every colony arose from a single cell. We found that every differentiated colony contained at least one $B4F8^+$ cell. In fact, 2 of these colonies (10%) were composed entirely of $B4F8^+$ cells; however, the majority of the colonies (90%) contained both $B4F8^+$ and $B4F8^-$ cells.

This experiment provided preliminary evidence that some induced cells differentiated into more than one cell type. To gather more evidence for the multiple developmental potential of single induced cells, we wanted to plate single cell suspensions of RA-treated cells into semisolid medium. In this way we could be sure that the colonies we scored were derived from single cells. For this experiment, we used the EC cell line P19(ras), which was derived from P19 by transfecting the activated Ha-ras oncogene (Bell et al,

Figure 3.12. Kinetics of the appearance of B4F8^{+} cells in RA-treated cultures. 5x10^{4} cells/60 mm tissue culture dish were treated with 10 uM RA for 4 hr, and replated onto gelatin-coated coverslips. At the times indicated, the coverslips were processed for indirect immunofluorescence with the antibody B4F8, and the cells were scored by fluorescence. 100, 175, and 350 cells were scored for days 1-3, 4, and 5, respectively.


50
40
30
20
10
0
PERCENT B4F8+ CELLS
1
2
3
4
5
TIME (Days)

1986; Rudnicki, 1988). P19 cells are anchorage dependent in the continuous presence of RA, while P19(ras) cells cultured in semisolid medium have equal PE in the presence and absence of RA. P19(ras) cells aggregated in the presence of RA differentiate like P19 cells into neurons, glia, and smooth muscle cell types (Bell et al, 1986; Rudnicki, 1988). We first compared the RA dose response of P19(ras) and P19 cells. Cells of both lines were plated at $5x10^3$ cells/60 mm tissue culture dish and exposed to various RA concentrations in serum free medium. Both cell lines were induced to differentiate to the same extent following a 4 hr exposure to various doses of RA (not shown). We have assumed that P19(ras) cells also show the same density-dependent response as P19 cells to a 4 hr RA exposure.

We then plated single cell suspensions of RA-treated P19(ras) cells into semisolid medium, and analysed the resulting differentiated colonies. In one experiment, P19(ras) cells were plated at $5x10^4$ cells/60 mm tissue culture dish and exposed to 10 uM RA for 4 hr. Following RA exposure, the cells were dispersed and plated into semisolid medium. Immediately after plating, the cultures were examined under the inverted microscope, to determine the proportions of single cells and groups of two or more cells. 95% of the cells plated were single cells, and only 5% were in groups of two or more. These cultures were incubated for 4 days, by which time the cells had formed small colonies in suspension. The methylcellulose was diluted with medium, and the colonies were transferred to tissue culture dishes, or onto gelatin-coated coverslips (see below). The morphology of the colonies in the tissue culture dishes was examined on day 7. The differentiated colonies were scored for the presence or absence of neurons (with extended processes), as shown in figure 3.1D. Of the 390 differentiated colonies scored, 36% had

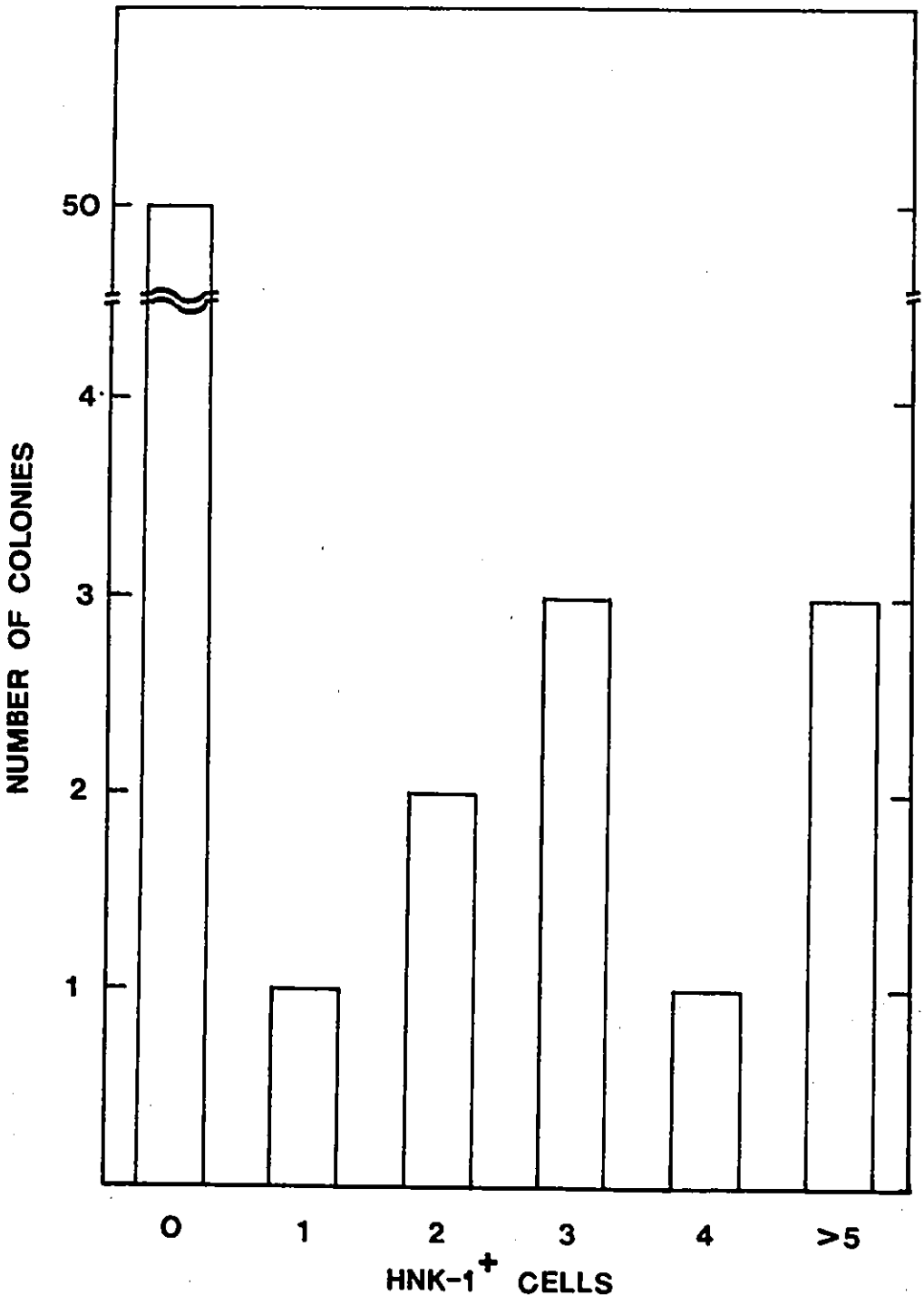
morphologically indentifiable neuronal and non-neuronal cells. Single P19(ras) cells induced by a 4 hr RA exposure differentiated into more than one morphologically identifiable cell type.

To distinguish between glial and smooth muscle cells in these colonies, some of the coverslips were processed for indirect immunofluorescence on day 9. We used the antibodies NF-1 to recognize glia, and B4F8 to detect smooth muscle cells. The secondary antibodies were FITC-conjugated anti-rabbit, and rhodamine-conjugated anti-mouse, so that the glial cells fluoresced green and the smooth muscle cells red. Where possible, the colonies were scored for the presence of neurons with visible processes. 16 differentiated colonies were scored in which we could determine by morphology whether or not neurons were present. 8 of these colonies were $B4F8^{+}$, $NF\text{-}1^{-}$, $neuron^{-}$, 6 colonies were $B4F8^{+}$, $NF\text{-}1^{-}$, $neuron^{+}$, and 2 of the colonies were $B4F8^{+}$, $NF\text{-}1^{+}$, $neuron^{+}$. Although based on a small sample size, these results showed that single RA-induced cells differentiated into two and sometimes three cell types.

To determine if the induced cells followed a programmed cell lineage as they differentiated, we counted the number of neurons in some of these colonies. The neurons are post-mitotic, and therefore the number of neurons in a colony might give an indication of the cell lineage leading to the neurons. If a programmed cell lineage were being followed, one might expect all colonies to have the same number of neurons. And, one might expect the number of neurons in each colony to be $2n$ or 2^{n}, similar to production of terminally differentiated cells in the skeletal myogenesis in the chick (Quinn et al, 1987).

Some of the coverslips were processed for indirect immunofluorescence on day 7, using the antibody HNK-1. The number of HNK-1^+ cells (neurons) counted in each of the 60 colonies scored is shown in the histogram of figure 3.13. Most of the colonies had no neurons. Only 10 (17%) of the colonies contained HNK-1^+ cells. This is an apparent discrepancy with the scoring on the basis of morphology, where 36% were scored as containing neurons. This is due to the fact that the colonies scored for fluorescence were not scored first for differentiated morphology. That is, some of the colonies with 0 neurons were undifferentiated. In the colonies containing neurons, there is no clear pattern with respect to the number of HNK-1^+ cells in each colony. Some of the colonies contained only 1 neuron, and the maximum number scored was 14 HNK-1^+ cells. The colonies did not necessarily contain 2n or 2^n neurons. From this experiment, there was no indication that the induced cells followed a programmed cell lineage as they differentiated.

Figure 3.13. Distribution of HNK-1^{+} cells in colonies derived from single RA-treated cells. Cells were plated at 5x10^{4} cells/60 mm tissue culture dish and treated with 10 uM RA for 4 hr. Following RA exposure, the cells were dispersed, and replated into semisolid medium. Cells were incubated for 4 days until colonies had grown. Colonies were transferred to gelatin-coated coverslips, processed for indirect immunofluorescence on day 7 using the antibody HNK-1, and cells were scored by fluorescence.


NUMBER OF COLONIES
50
4
3
2
1
0
1
2
3
4
>5
HNK-1+ CELLS

3.6. Discussion.

Commitment of L6E9 rat myoblasts to differentiation was found to be a stochastic event which occurred exclusively in the G1 phase (Nadal-Ginard, 1978). In mouse MM14 myoblasts, G1 phase-specific commitment to differentiation occurred in the absence of fibroblast growth factor (Clegg et al, 1987). Commitment of P19 EC cells to differentiation induced by RA was also reported to be G1 phase-specific (Mummery et al, 1987). The observation was that G1 phase cells exposed to RA for 4 hr and then incubated in drug-free medium for 3 days were anchorage dependent (i.e. differentiated). On the other hand, S phase and exponentially growing cells exposed to RA for 4 hr were anchorage independent on day 3. However, our results show that G1 and S phase cells exposed to various RA concentrations for 4 hr were induced to differentiate with equal efficiencies. Furthermore, exponentially growing cells were also efficiently induced to differentiate by a 4 hr RA exposure.

Our results are in direct contrast to those in the literature (Mummery et al, 1987). There are differences in the experimental approaches, such as type of medium, serum concentrations, and the assay used to assess differentiation. However, the most significant difference is in the density of the cells at the time of RA exposure. Our results in figure 3.9 show that changes in cell density profoundly affect the induction of differentiation of P19 cells. In the experiments published in the literature (Mummery et al, 1987), the cells were exposed to 1.0 uM RA at high density ($2x10^4$ cells/cm^2). Our experiments have shown that at this cell density and RA concentration in serum-containing medium, the total number of cells is critical. The difference in cell density during the RA exposure is the likely explanation

for the contrasting results. The conclusions of Mummery et al were incorrect due to the high density of the cells during the 4 hr RA exposure.

We have found that cells at higher densities are more refractory to the induction of differentiation by brief RA exposure. This density-dependent resistance to the initiation of differentiation by RA did not appear to be mediated by secreted or cell surface-associated factors, or by cell to cell interactions. We have determined that a dose of greater than $4x10^9$ molecules of RA per cell is required to initiate the differentiation of P19 cells with a 4 hr exposure. Since RA induces differentiation of P19 cells in a cell-autonomous fashion (Campione-Piccardo et al, 1985b), and biochemical processes normally depend on total substrate or reagent concentration, the fact that the threshold is expressed as RA per cell is highly unusual. The most likely explanation for the dose to be expressed as RA per cell is that the cells deplete the medium of RA during the 4 hr exposure. Figure 3.11 appears to rule this out, as even a 24 hr exposure of high density cells does not deplete the medium of RA. This experiment is, however, complicated by the presence of serum in the medium. We cannot be sure how much of the RA is bound reversibly to the serum albumin and other proteins.

To resolve the issue of the number of RA molecules the cells take in during a 4 hr exposure, two types of experiments are needed. The first are dose response experiments, similar to those in figure 3.9. By using the same RA-containing medium on sequential plates of cells, one could determine how quickly the cells are removing RA from the medium and reducing the RA concentration to below the threshold dose per cell. Secondly, one could add ^{3}H-labeled RA to P19 cells for 4 hr and analyse of the distribution of label

in the cells and the medium. This would allow a direct calculation of the number of molecules of RA the cells take in during a 4 hr exposure. Some experiments have been reported in which ^{3}H-labeled RA was added to EC cells (Gubler and Sherman, 1985). These experiments showed that less than 5% of the label was associated with F9 EC cells, even after a 24 hr incubation. After only a 5 hr exposure, most of the added label was found as RA metabolites in the medium. The cells depleted the medium of RA after a 24 hr exposure. Even with this information we cannot be certain whether or not P19 EC cells internalize $4x10^{9}$ molecules of RA per cell during the 4 hr exposure. This is simply the amount of RA which must be present (in serum-free medium) for a 4 hr exposure to induce the differentiation of 50% of the cells.

Provided RA is the actual inducer, and not one of its metabolites, the presence of RA inside the cell initiates differentiation. Whether or not the cells are competing for RA from the medium, and in spite of the fact that most of the RA is metabolised to inactive compounds, the cell must have a mechanism to respond to an above-threshold level of RA. The intracellular fate of the RA may provide an explanation for the threshold, and a mechanism for the cell to 'decide' if the dose of RA is high enough to induce differentiation. The most likely explanation is that RA is bound by the cRABP (Chytil and Ong, 1984), and translocated to the nucleus and the RAR (Daly and Redfern, 1987; Brand et al, 1988). The RAR then activates transcription of certain genes resulting in the differentiation of the cell. A given RAR molecule may have a specific probability of activating one or more genes at the beginning of a cascade of genes required for differentiation. Or, perhaps a certain minimum number of RAR molecules must

be bound to RA and the DNA encoding the RA-responsive gene to activate transcription. This would provide a response to RA depending upon the intracellular concentration of RA.

This model can also incorporate length of RA exposure as a critical variable. 1.0 uM RA (in serum containing medium) is below the threshold for a 4 hr exposure to induce $5x10^5$ cells/60 mm tissue culture dish to differentiate. Yet a 48 hr exposure to 0.3 uM RA induces $5x10^5$ cells/60 mm tissue culture dish to differentiate (Jones-Villeneuve et al, 1982). With a short exposure, the number of RA molecules which bind to cRABP and are translocated to the RAR in the nucleus is limiting. With a longer exposure, the cRABP is able to translocate several molecules of RA to the nuclear RAR, thereby overcoming the threshold necessary to induce the differentiation of the cells. The paucity of cRABP molecules and the presence of RAR in P19 lend some support to this model. However, it will be difficult to prove the involvement of cRABP and RAR in the RA-induced differentiation of P19 cells.

We have used a 4 hr RA exposure to induce cells and analysed the subsequent differentiation of P19 cells. When aggregated in the presence of 0.3 uM RA, P19 cells differentiate into cells of the neuroectodermal lineage (neurons, glia, and fibroblast-like cells; Jones-Villeneuve et al, 1982). Each aggregate of cells treated with 0.3 uM RA produces these three cell types. Like the RT4 cell line (Droms and Sueoka, 1987) and the neuroepithelial cells of the 10 day old mouse embryo (Bartlett et al, 1988), P19 cells have neuronal and glial potential. We have provided evidence that individual RA-induced P19 cells have multiple developmental potential. And, as seen in the frog (Wetts and Fraser, 1988) and rat (Turner and Cepko, 1987)

retina, the differentiation of P19 cells did not seem to follow a programmed cell lineage. To provide further evidence for these two statements concerning the differentiation of induced P19 cells, we would like to induce cells with a 4 hr RA exposure, and analyse the differentiated progeny from cell aggregates, using a lineage marker to follow the fate of single induced cells (see Appendix).

EC cells are used here as an experimental model for the development of the mammalian embryo. We would like to suggest that these results concerning the differentiation of P19 cells also apply to the cells of the inner cell mass of the developing embryo. That is, that precursor cells with multiple developmental potential exist, and that their differentiation does not necessarily follow a programmed cell lineage. Although some cells may be part of a programmed cell lineage, we suggest that those giving rise to the neuroectodermal cell types (as P19 EC cells do) do not have a strict cell lineage. The differentiation of these cells types may be affected profoundly by neighboring cells and the environment.

The concept of a per cell threshold dose for a response to a brief exposure to an inducer is relevant to developmental biology only if the endogenous inducer is spatially or temporally restricted. Here we have reported a per cell threshold response of P19 cells to RA as an inducer of differentiation. Much evidence has accumulated that RA is a natural morphogen, at least in the chick limb bud (Slack, 1987). Although we are unaware of evidence of temporal regulation, it is clear that gradients of RA exist in the limb bud (Thaller and Eichele, 1987). The cells in the limb bud respond to RA, differentiating into various cell types, such as cartilage,

mesenchyme, and bone. The cells form various organizations of these cells, i.e. different digits, depending on the concentration of RA (Tickle et al, 1985).

P19 cells also differentiate into various cell types, depending on the concentration of RA. Aggregates of P19 cells exposed to 1.0 nM to 0.1 uM RA for 4 days differentiate into mesodermal derivatives, including cardiac and skeletal muscle (Edwards and McBurney, 1983). Aggregates of P19 cells exposed to 0.3 uM RA differentiate along the neuroectodermal cell lineage, producing neurons, astrocytes, and fibroblast-like cells (Jones-Villeneuve et al, 1982). Furthermore, we report here that P19 cells respond to a 4 hr RA exposure, depending on the dose of RA per cell. If the dose of RA is below $4x10^9$ molecules per cell, the cells will not be induced to differentiate but will proliferate. If the dose is above $4x10^9$ molecules per cell, the cells are induced to differentiate into neuroectodermal cell types, but not following a strict cell lineage. These results are important to our understanding of the RA-induced differentiation of P19 EC cells, of mammalian developmental biology, and of RA as a morphogen.

4. Conclusions.

There are several conclusions from this work on the initiation of differentiation of P19 EC cells:

1. A 4 hr exposure to RA can induce the differentiation of P19 EC cells.

2. The RA-induced differentiation of P19 EC cells is not restricted to cells in the G1 phase.

3. The initiation of differentiation is dependent on the cell density during the 4 hr exposure.

4. A threshold dose of 4×10^9 molecules of RA per cell is required for a 4 hr exposure to induce the differentiation of P19 EC cells.

5. Single P19 cells induced by a 4 hr RA exposure differentiate into more than one cell type.

6. We have been unable to find any evidence for the involvement of a cell lineage in the differentiation of P19 EC cells.

Some further experiments become obvious to supplement this work and help support these conclusions. Repeating the dose response experiments, treating two or three sets of plates with the same media sequentially would determine

whether the cells are competing for the available RA during the 4 hr exposure. To determine if cells are taking in $4x10^9$ molecules of RA during the 4 hr exposure, ^3H-labelled RA could be added to P19 cells. The distribution of radioactive label in the medium and the cells after 4 hr would allow a calculation of the number of molecules of RA cells take in during a 4 hr exposure. This approach could also determine if RA is translocated to the nucleus by cRABP or metabolised by P19 cells.

An ongoing experiment in the laboratory is attempting to determine if the RAR is involved in the RA-induced differentiation of P19 EC cells. Chimeric genes have been made which encode proteins which will bind RA and activate transcription of normally glucocorticoid-responsive genes rather than RA-responsive genes. If such chimeric proteins were introduced into P19 EC cells, one would expect a shift in the RA dose response curve since these receptors would compete with the endogenous RAR for RA molecules. This experiment would show if the intra-cellular concentration of RA is limiting and if the RAR is involved in the initiation of differentiation of P19 EC cells by RA.

We have shown that individual P19 EC cells differentiate into more than one mature cell type. We would also like to know if a monopotential precursor for neurons or glia exists. It would be very interesting if one could clone out such monopotential precursors from differentiating cultures. One could induce P19 EC cells to differentiate with a 4 hr RA exposure, and after one (or several) cell divisions, pick single cells to clone. Of course, these cells would be committed to terminal differentiation, but perhaps the addition of certain growth factors would allow for the continued

self-renewal of such precursor cells.

Finally, for further evidence that there is no reproducible cell lineage in the differentiation of P19 EC cells, a prospective lineage analysis would be ideal. With an appropriate lineage marker, one could culture a single, induced, marked cell within a cell aggregate of unmarked cells. From studies of such mixed aggregates, one could ensure that the differentiation of P19 EC cells does not follow a programmed cell lineage. Furthermore, these experiments could determine effects of the environment and neighboring cells on the differentiation, by varying the size of the aggregate, and changing the proportion of induced and non-induced cells within the aggregate.

Appendix.

The experiments discussed here were undertaken in order to carry out a prospective lineage analysis of differentiating P19 EC cells. We planned to introduce a genetic marker (see below) into P19 EC cells to derive cell lines which could be distinguished from the parental P19 EC cells. We wanted to characterize the growth and differentiation of these cell lines, to ensure that the introduction of the marker gene had no adverse effects. We then intended to induce the differentiation of these cells with a 4 hr RA exposure, and place single cells into aggregates of induced P19 EC cells. Analysis of the numbers and types of differentiated cells labelled with the lineage marker would determine if a programmed cell lineage were involved in the differentiation of P19 EC cells.

We chose to use a genetic marker for a variety of reasons. A micro-injected lineage tracer, such as FITC-labeled tubulin, would probably be diluted by the numerous cell divisions we wanted to allow. Also, the process of micro-injection itself may have effects on the cells or their differentiation, whereas a genetic marker allows for characterization of the growth and differentiation of labelled cell lines. The genetic marker we chose was the Escherichia coli lac Z gene, encoding the enzyme B-galactosidase. B-gal can be detected in situ with a histochemical staining procedure (X-gal stain), by indirect immunofluorescence using anti-B-gal antibodies, or quantitated with a spectrophotometric assay. To promote transcription of this gene in P19 EC cells, we used one of two mammalian

gene promoters or a viral promoter. One plasmid construct containing the lac Z gene under the transcriptional control of the murine heat shock protein (hsp) 70 promoter (pCH126A2) was obtained from Dr. R. Kothary of the Mount Sinai Research Institute, Toronto, Ontario. This promoter is inducible, and only directs transcription of genes following cellular stress. Therefore the gene product (B-gal) would only be expressed after stressing the cells, for example by heat shock. The promoter region of the murine phosphoglycerate kinase-1 (pgk-1) gene and the SV40 promoter were chosen as constitutive promoters. Constructs with the lac Z gene under the transcriptional control of each of these promoters were made by Mr. Chaker Adra. Within P19 EC cells, these plasmid constructs could serve as genetic lineage markers, since only cells containing a plasmid would express B-gal.

Calcium phosphate mediated DNA transfection was used to introduce these plasmids into P19 EC cells. Each plasmid was co-transfected with pSV2neo, which confers resistance to the antibiotic drug G418. Cells which incorporated pSV2neo into the genome were selected in the continuous presence of 400ug/mL G418, and expanded into cell lines. These cell lines were tested for co-transformation (incorporation of both plasmids) by X-gal staining, following heat shock for those transfected with the hsp-lac Z plasmid. Many of the cell lines isolated were co-transformed, but we did not observe staining in every cell. Some of the cell lines containing the pgk-lac Z plasmid were chosen for further analysis, since they had high percentages of cells stained with X-gal. All of these cell lines were derived from one transfection, so the possibility exists that they are clonally related (see below).

One explanation as to why all of the cells did not stain with X-gal is that these were not clonal cell lines. They may have originated from more than one cell, which did not all contain the B-gal plasmid. We therefore sub-cloned these cell lines, to ensure that we had cell lines derived from a single cell. Cells were plated at 10^3 cells/60 mm dish and incubated 4 to 6 days to allow single cells to form colonies. The dishes were stained with X-gal, and the colonies were scored as having all cells stained, all cells not stained, or some cells stained and some not. Results from several cell lines are presented in Table A1. With all of the cell lines which were tested in this experiment, at least some of the colonies consisted entirely of stained cells. This indicated that sub-cloning might produce cell lines in which every cell expressed B-gal.

Before staining some of these dishes, one half of the cells from some of the colonies were removed with a drawn pipette, and replated into separate tissue culture dishes. The remainder of the colonies were then stained with X-gal. The cells from a number of colonies in which all of the cells were stained were then expanded into cell lines. When these cell lines were then stained with X-gal, all of the cells did not stain. The clonal cell lines generated were heterogeneous in the expression of the lineage marker. However, a prospective lineage analysis does not absolutely require the lineage marker to be present in every descendant of the labelled precursor. The results must be analysed carefully though, since unlabelled cells are not necessarily unrelated, they may simply fail to express the lineage marker.

Table A1. X-gal staining of pgk-lac Z transfected cell lines.

Cell line	Percent cells stained (est.)	Sub-cloning: All stained #	(%)	All unstained #	(%)	Some stained #	(%)
B9	N.D.[a]	98	(36.7)	76	(28.5)	93	(34.8)
		70	(29.5)	62	(26.2)	105	(44.3)
D1	75	58	(45.3)	25	(19.5)	45	(35.2)
		228	(78.1)	15	(5.1)	49	(16.8)
C5	50-75	166	(68.6)	9	(3.7)	67	(27.7)
		110	(47.4)	30	(12.9)	92	(39.7)
C9-15	50-70	226	(64.8)	50	(14.3)	73	(20.9)
B9-24	30-50	76	(27.7)	149	(54.4)	49	(17.9)
		53	(17.7)	211	(70.3)	36	(12.0)
B9-33	25	35	(15.5)	140	(61.9)	51	(22.6)
B9-24-3	75-90	242	(83.2)	36	(12.4)	13	(4.5)
		201	(73.9)	51	(18.8)	20	(7.4)
		83	(69.7)	15	(12.6)	21	(17.6)

a - not determined.

We next induced the differentiation of these cells, to determine if the introduction of the lineage marker had any effects, and if the differentiated cells expressed the lineage marker. Cells were aggregated for 4 days in the presence of 0.3 uM RA, and the aggregates were plated into tissue culture dishes. The cultures were indistinguishable from similar cultures of the parental P19 cells, and contained the same morphologically identifiable differentiated cell types. The differentiated cultures were then stained with X-gal. The proportion of cells stained was much lower than in the undifferentiated cells. Morphologically identifiable neurons were seldom stained with X-gal. Most of the cells which had spread out from the aggregates were not stained. Thus most of the differentiated cells derived from these transfected cell lines did not express B-gal.

To determine when the differentiating cells stopped expressing B-gal, we stained cells at various times after the initiation of differentiation. Cells were plated at $5x10^3$ cells/60 mm tissue culture dish, and left untreated, or treated with 1.0 uM RA for 4 hr. The cells were washed, refed drug-free medium, and stained with X-gal on days 4 to 8 after RA treatment. Colonies were scored for the number of cells stained (all, none, or some). The results are shown in Table A2. Firstly, this table shows the heterogeneity in the EC cells. For example, cell lines D1, C5, and C9-15 all had decreasing percentages of 'all stained' colonies over time. The percentages of colonies not stained stayed the same, and the percentage of mixed colonies increased. This suggests that some of the cells in colonies which were all stained at earlier days had lost the expression of B-gal. Secondly, table A2 shows that the percentage of colonies stained in the + RA conditions are significantly lower in all cases by Day 5. Generally, the

Table A2. Percent of colonies stained changes with time.

Clone	Day[a]	-RA			+RA		
		All[b]	None	Some	All	None	Some
D1	5	64	6	30	16	54	30
	6	52	7	41	7	48	45
	7	22	9	69	15	55	30
	8	1	10	89	2	70	28
C5	5	65	9	26	13	55	32
	6	23	12	65	3	68	29
	7	12	12	76	2	60	38
	8	3	12	85		N.D.	
C9-15	5	50	30	20	26	35	39
	6	25	32	43	11	39	51
	7	20	31	49	14	42	44
	8	0	45	55	5	38	57
B9-24-3	4	72	16	12	3	64	33
	5	60	24	16	11	54	35
	6	56	11	33	19	58	23
	7	62	13	25	13	64	23

a - days after plating and RA exposure.
b - see table A1 for explanation of column headings.
Approximately one hundred colonies were scored in each case, except for B9-24-3 + RA, where only 50 colonies were scored.

reduction is accompanied by a corresponding increase in the percentage of unstained colonies. We noted that many of the colonies which were scored as all stained were morphologically undifferentiated. For example, when scoring day 7 plates from cell line D1, we noted that all of the stained cells were in colonies with characteristic EC morphology. Thus by day 4 or 5, when the differentiated cells are identifiable by morphology, they no longer expressed B-gal.

The loss of expression of B-gal upon differentiation of these cell lines may be due to any of a number of reasons (see below). We wanted to determine if these cells also lost expression of the other transfected gene (neoR) upon differentiation. Cells were aggregated in the presence of 0.3 uM RA and 400 ug/mL G418 for 4 days. The aggregates were plated in tissue culture dishes with G418 and without RA. None of the cells in these experiments survived (they were G418 sensitive), suggesting that the cells no longer expressed the neo product upon differentiation.

To provide further evidence for the loss of expression of these genes, differentiated cell lines were derived. Cells were aggregated in 0.3 uM RA for 4 days, and plated in tissue culture dishes in the continuous presence of RA. After several passages, a differentiated cell line was derived. When stained with X-gal, no staining was observed. When cultured in the presence of G418, the cells did not survive. This differentiated cell line no longer expressed either the neo or the lac Z gene. When these transfected cell lines differentiated, they did not express the lineage marker. Therefore these cell lines were not useful for a prospective lineage analysis.

The reason for the heterogeneous expression of B-gal in these cell lines remains unclear. This could be a general phenomenon of transfected or exogenous DNA sequences. The explanation could be that chromosome or chromatin structure or accessibility changes within cells as the cell lines are expanded. There could be DNA sequences in the plasmids (vector, bacterial, coding, or 3' end sequences) which introduce some variability in, for example, accessibility to cellular transcription machinery. The stability of the mRNA or protein may be variable from cell to cell. The heterogeneity could be simply a staining artefact, although this is unlikely for a number of reasons. We have used a variety of fixative procedures for both X-gal and immunofluorescence staining, and various conditions for X-gal staining, and we have always observed this heterogeneity. Others have used the same X-gal staining procedure and have observed staining in every cell of cell lines infected with a B-gal coding retrovirus.

The reason for the loss of B-gal expression in the differentiated cells is also unclear. The explanation for this could be a decrease in mRNA or protein stability in the differentiated cells. There could be decreased transcription in differentiated cells due to the site of integration of the plasmid. The proportions of active and inactive chromatin change as EC cells differentiate, and the plasmid may have integrated into a site which becomes inactive upon differentiation. The plasmid may have integrated near an EC-specific enhancer (or differentiation-specific negative enhancer). Since a number of cell lines show the same phenomenon, these possibilities are less likely. However, the possibility exists that these cell lines are all derived from the same integration event (see below). Alternatively, transcription or translation may be decreased due to competition for cellular

factors, due to an increase in production of differentiation-specific mRNAs or proteins. A final possibility is the phenomenon of X-inactivation, which occurs in female EC cells upon differentiation. Since pgk-1 is an X-linked gene, the promoter region may have critical sites for X-inactivation. A tandem array of multiple copies of this plasmid may have created a 'mini X chromosome', which inactivates upon differentiation.

To determine the reason for the heterogeneous expression of B-gal in the EC cell lines, and the loss of expression in differentiated cells, a number of experiments are needed. Firstly, a Southern blot, probed to detect lac Z sequences will determine if the B-gal plasmid is integrated, and if there are separate integration sites in these cell lines. Also, a Southern analysis could determine if integrated copies of the plasmid are lost during sub-cloning or exponential growth of sub-clones, or during differentiation.

Secondly, Northern blot analysis of differentiating B-gal cell lines will determine if the mRNA level decreases during differentiation. If the mRNA level does not decrease, then the decrease in staining observed is likely due to decreased translation of mRNA or stability of protein. However, persisting EC cells in differentiating cultures might mask a small decrease in mRNA levels in the population. If the mRNA level was found to decrease, this could be due to decreased transcription or mRNA stability. One could measure the half-life (i.e. stability) of the mRNA to distinguish these two possibilities.

If these cell lines are in fact derived from the same integration event, then the explanations which involve the integration site become more

realistic. If the cell lines were found to be independent integrations, then a decrease in transcription of this transfected gene might reflect a general decrease in transcription upon differentiation. This may indicate that a gene requires a differentiation-specific enhancer element to be transcribed in differentiating cells.

Finally, to test the X-inactivation possibility, one could treat the differentiated cell line with 5-azacytidine. XX EC cell lines have been shown to re-activate an inactive X chromosome upon 5-AC treatment. If the decrease in B-gal production in these cell lines is explained by X-inactivation, then treating with 5-AC may reactivate this gene as well.

To continue with the prospective lineage analysis, a different lineage marker is required. The marker must be expressed in differentiated cells, and preferably in every cell of a cell line, EC and differentiated. Some sequence changes in the plasmids may be required, or a different gene promoter might be used. For example, a neuron-specific promoter such as that of a neurofilament gene to direct transcription of B-gal in neurons may be useful to examine neuronal cell lineages. Alternatively, using a retrovirus to introduce B-gal into P19 cells may generated a suitable cell line. Although the cell lines generated so far were not useful for a prospective lineage analysis, they may generate some interesting results with respect to EC- and differentiation-specific transcription and translation of genes.

Methods.

Plasmid were constructed and prepared according to the methods described (Maniatis et al, 1982). Transfection was done according to established methods (Graham and van der Eb, 1973). For X-gal staining, cells were fixed for 5 min at 4^{o}C in 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). The cells were rinsed twice with cold PBS and stained with X-gal stain (0.2 mL of X-gal (2% in dimethyl formamide), 4.5 mM potassium ferricyanide, 4.5 mM potassium ferrocyanide in 10 mL of phosphate buffer).

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