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IN VITRO DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS:
THE ROLE OF AGGREGATION AND THE PRODUCTION OF AN ENDOGENOUS
INDUCER OF DIFFERENTIATION

by

Steven C. Smith

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

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To Ann

ABSTRACT

Embryonal carcinoma (EC) cells are the malignant, pluripotent stem cells of teratocarcinomas. P19 is a line of EC cells which are capable of differentiating in vitro into a broad spectrum of cell types. This occurs in response to a variety of inducing drugs, notably retinoic acid (RA) and dimethylsulphoxide (DMSO). This differentiation is, however, dependent upon the formation of aggregates of the cells, particularly during DMSO-induced differentiation.

In order to further explore this requirement for aggregation, the relationship between aggregate size and differentiation was studied. It was found that there was a threshold starting size which DMSO-treated aggregates had to exceed in order for muscle differentiation to ensue. In contrast, differentiation of extraembryonic endoderm was found to be inversely related to aggregate size. The proportion of endoderm was found to decrease with increasing aggregate size.

The internal structure of differentiating DMSO-treated aggregates was studied through the use of frozen sections and indirect immunofluorescence. Examination of these aggregate sections revealed that muscle formed just beneath the periphery of the aggregate, immediately below a sheath of extraembryonic endoderm which formed prior to the appearance of muscle. This spatial and temporal

relationship of endoderm and muscle differentiation led to a working hypothesis whereby the formation of an external sheath of extraembryonic endoderm was causally implicated in the differentiation of muscle beneath it.

One of the ways in which extraembryonic endoderm was thought to be capable of influencing muscle formation was through the production of a diffusible inducer of differentiation. Thus, a study of media conditioned by extraembryonic endoderm was initiated. When these media were placed on P19 cells growing in monolayer culture, the cells differentiated rapidly and extensively into a tissue morphologically resembling extraembryonic endoderm. This effect could not be attributed to residual RA, depletion of medium or serum, or to selection of a sub-population of P19 cells. Therefore, secretion of a differentiation-inducing factor by extraembryonic endoderm was suggested.

This factor acted rapidly on P19 cells, decreasing their growth rate and thereby their plating efficiency. In addition, DMSO enhanced the differentiation-inducing ability of the factor. The factor, in turn, enhanced the effect of RA. The effect of the conditioned media was dependent upon cell density, however, with less differentiation ensuing in crowded cultures.

Partial biochemical characterization of the factor revealed that it was not sensitive to exhaustive staphylococcal nuclease or trypsin digestion, or to boiling. As well, evidence is presented that suggests that the

factor may be associated with some serum component(s). By ultrafiltration, the factor had an apparent molecular weight of less than 10 000. Therefore, the factor did not appear to be any of the recently described differentiation-inducing factors, but, rather, a novel morphogen, defined as a diffusible, differentiation-inducing substance.

RESUME

Les cellules de carcinomes embryonnaires (CE) sont des cellules souches malignes à caractère pluripotentiel dont sont constitués les tératocarcinomes. P19 est une lignée cellulaire de CE capable de se différencier in vitro en plusieurs types cellulaires. Cette différenciation peut être provoquée de façon artificielle par certains agents inducteurs tels que l'acide rétinoïque (AR) et le sulfoxide diméthylé (DMSO) mais s'avère toutefois entièrement dépendante de l'agrégation des cellules. Cette observation est particulièrement remarquable lorsque la différenciation est induite par le DMSO. Dans le but d'élucider pourquoi la différenciation cellulaire chez P19 nécessite la formation d'agrégats, une étude fut effectuée pour établir un rapprochement entre le niveau de différenciation et la grosseur des agrégats formés.

Il fut alors observé que pour assurer la formation de cellules musculaires (à l'aide du DMSO), les agrégats, à l'origine, devaient excéder une grosseur critique et que la proportion d'endoderme extraembryonnaire variait de façon inverse avec la masse des agrégats.

L'architecture interne des agrégats traités avec DMSO a également été étudié à l'aide de sections congelées combinées à l'immunofluorescence indirecte. L'examen de ces sections d'agrégats a démontré que les cellules musculaires se forment juste en-dessous de la périphérie des agrégats,

x

immédiatement sous une gaine d'endoderme extraembryonnaire formée avant l'apparition des cellules musculaires. Cette relation temporelle et spatiale entre la formation d'endoderme et l'apparition du muscle a donné naissance à l'hypothèse suivante. La formation d'une gaine endodermique est impliquée dans la différenciation des cellules musculaires.

Il a été postulé que l'endoderme extraembryonnaire pourrait être capable d'induire la différenciation musculaire par l'entremise d'une substance diffusible. Ainsi, une étude des milieux conditionnés par l'endoderme extraembryonnaire a été initiée. Ces milieux placés sur les cellules P19 en monocouches ont induit la différenciation des cellules de façon rapide et prononcée en un tissu morphologiquement semblable à l'endoderme extraembryonnaire. Puisque cet effet ne peut être attribué ni à l'AR résiduel, ni à l'absence de facteurs sériques ou fournis par le milieu, ni à la sélection d'une sous-population prédéterminée à la formation d'endoderme, il fut alors proposé que l'endoderme extraembryonnaire soit responsable de l'élaboration d'un facteur pouvant induire la différenciation.

Ce facteur semblait agir rapidement au niveau des cellules P19 diminuant à la fois leur taux de croissance ainsi que leur efficacité de clonage. Or, bien sûr que le DMSO semblait potentialiser l'effet du facteur sur la différenciation, le facteur, pour sa part, pouvait

potentialiser l'effet de l'AR. Enfin les milieux conditionnés peuvent stimuler la différenciation en fonction de la densité cellulaire, quoique cet effet fut réduit chez des cultures très confluentes.

La caractérisation partielle du facteur nous a révélé que ce dernier était résistant à la digestion par la nucléase staphylococcale et la trypsine ainsi qu'à l'ébullition. De plus, ce facteur semble associé à une ou plusieurs composantes sériques et possède un poids moléculaire apparent de moins que 10 000 (obtenu par ultrafiltration). En somme, ce facteur ne semble pas s'apparenter à aucun des facteurs de différenciation récemment décrits mais serait plutôt un nouveau composé morphogène, diffusible et capable d'induire la différenciation.

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LIST OF ABBREVIATIONS

CM	Conditioned medium
cm	centimetre(s)
cm ²	square centimetre(s)
CS	Calf serum
DBA	<u>Dolichos biflorus</u> agglutinin
DMSO	Dimethylsulphoxide
EC	Embryonal carcinoma
ECDGF	Embryonal carcinoma-derived growth factor
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
GAG	Glycosaminoglycan
Kd	Kilodalton(s)
M	Molar
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
PBS	Phosphate buffered saline
RA	Retinoic Acid
SC	Serum-containing
SC-CM	Serum-containing conditioned medium
SEM	Standard error of the mean
SF	Serum-free
SF-CM	Serum-free conditioned medium
T-EDTA	Trypsin and EDTA in PBS
ug	microgram(s)
ul	microlitre(s)
um	micrometre(s)

CHAPTER I

INTRODUCTION

Since the beginnings of the study of living things, one of the central, unsolved questions of biology has been that of the development of higher animals. The poorly understood processes by which a single cell, formed through the fusion of two gametes, is transformed into a complex, multicellular organism composed of individual, irreversibly specialized tissues are still the subject of intense study today. The transformation of the apparently identical cells of the early embryo into specialized cells is termed "differentiation".

A variety of systems have been developed to study the processes involved in cellular differentiation, and the more complex processes of tissue formation and organogenesis.

1.1 Embryonic Systems:

The most obvious system for the study of the processes of development is the embryo itself. This was the system of the classical embryologists. Traditionally, the formation of different tissues and organ systems was followed through manipulations of the embryo - usually through extirpation, transplantation, or explantation. Alternatively, tracing experiments could follow the movement of cells through the embryo and the resulting differentiation of these cells.

The major and most obvious advantage of these systems is that they are normal developing systems. As well as cellular differentiation, higher levels of organization, such as tissue and organ formation, can be studied using embryos. From classical studies, the ideas of morphogenetic (inductive) fields developed (see Yamada, 1967; Saunders, 1982, for reviews). Eventually, these studies led to the idea that differentiation in some systems was mediated by chemical "morphogens" which either permitted, enabled, or actually instructed responding tissues to differentiate.

The direct study of embryos has several disadvantages, however. Foremost among these is the complexity of the system and the resultant difficulty of determining the exact factor(s) involved in the differentiation of individual tissues. In addition, the amount of material available is limited by the small size of the embryos, and their inaccessibility (particularly in mammalian systems). The use of explants tends to minimize the first drawback, but only at the cost of accentuating the second. The use of larger amphibian embryos provides a somewhat greater abundance and accessibility of working materials, but is still often greatly dependent upon the amorous whims of the organisms involved.

1.2 In Vitro Systems:

With the advent of tissue culture, studies on the commitment and subsequent differentiation of cells had the

advantage of a larger availability of material. The systems of choice were (and still are) often composed of cells already precommitted to a single or limited range of differentiated cell types. These included blood cell lineages (Preisler et al, 1975; Preisler and Lyman, 1975; Marks and Rifkind, 1978; Till and McCulloch, 1980; Harrison et al, 1982) , myoblast cell lines (Yaffe and Saxel, 1977; Senechal et al, 1983) , and neuroblastoma cell lines (Sueoka et al, 1982; Thiele et al, 1985) to name a few. When exposed to the appropriate conditions, these cells complete their developmental repertoire, and become terminally differentiated cells.

Although these systems have provided a wealth of information on the genetic and biochemical changes occurring during the later phases of differentiation, the fact that these cells are already committed to form a pre-determined spectrum of cell types precludes the study of the early stages of commitment. In addition, unless short term primary cultures are used, the cells in culture are generally abnormal, at least until they differentiate. As well, unlike embryonic systems, levels of development and organization higher than cellular differentiation cannot be studied.



1.3 Teratocarcinomas, Embryonal Carcinoma Cells, and Cancer:

Teratocarcinomas are malignant but relatively rare tumors of the gonads which arise in a variety of species, including humans (see Stevens, 1983 for review; Rossant and Papaioannou, 1984). These tumors are usually composed of a cortex of various differentiated tissues. The differentiated tissues are benign, but the tumors often contain a core of undifferentiated stem cells. Tumors without these stem cells, as a result, are benign and are termed teratomas.

The stem cells, known as embryonal carcinoma (EC) cells, are the malignant cells of the tumor and are developmentally pluripotent, giving rise to the variety of differentiated tissues which surround them. Teratocarcinomas arise spontaneously with high frequency in some strains of mice, or they may be induced by transplanting early embryos or their genital ridges to ectopic sites in syngeneic mice (usually under the kidney or testis capsule). These may be maintained as transplantable tumors in mice (either solid or ascites) or the EC cells at the centre of these tumors can be established in tissue culture as either ascites-like "embryoid bodies" (aggregates; see Amano *et al*, 1978; Uno, 1982) or as monolayers.

EC cells offer unique advantages to the study of early

developmental processes. Since they can be maintained in tissue culture and usually grow very quickly, virtually unlimited amounts of material are available. As well, their ability to differentiate into a wide variety of cell types allows the early processes of commitment to be studied. This is further enhanced by the use of inducing agents which can direct (or restrict?) the cells into a limited spectrum of tissue types (see Strickland and Mahdavi, 1978; Solter et al, 1979; Speers et al, 1979; Jones-Villeneuve et al, 1982; Edwards and McBurney, 1983). As well, this offers a direct chance to study the processes involved in induction, as the inducing agent and the responding tissue are effectively isolated from the complex environment of the embryo.

This system, like any other, is not perfect, however. It must always be remembered that levels of development higher than cytodifferentiation cannot be effectively studied using an EC cell system. The cells can be induced to differentiate, but the formation of organized tissues is rarely, if ever, seen.

In addition, it must be remembered that EC cells are abnormal, tumorigenic cells. This, however, must be qualified by the fact that this tumorigenicity disappears upon differentiation, when the cells become benign (see Martin, 1975; Vandenberg et al, 1975; Solter et al, 1979; Speers et al, 1979). As well, many lines of EC cells have been shown to have greatly reduced tumorigenic potential.

when exposed to an embryonic milieu. Indeed, many mouse lines have been shown to participate in the formation of chimaeric embryos when associated with mouse blastocysts (see Lehtonen, 1984; Rossant and Papaioannou, 1984, 1985). Therefore, although these cells are tumorigenic, their tumorigenicity can, at least sometimes, be regulated by some of the same things which presumably regulate early embryonic development.

Herein lies one of the relationships of the study of in vitro differentiation of EC cells to cancer research - the regulation of malignancy by differentiation. Cancer can be regarded as a developmental problem (Martin, 1975; Vandenberg et al, 1975; Speers et al, 1979; Solter et al, 1979; Sporn and Roberts, 1983). During normal development, the seemingly uncontrolled growth of a ball of cells is eventually controlled as differentiation ensues. This growth regulation normally continues in the adult organism, with some cells replicating at a reduced rate, so as to replace those lost, and some not at all. During tumorigenesis, some of this homeostasis is lost, and a tumor is formed. Indeed, many of the so-called oncogenes and their normal counterparts (proto-oncogenes), which are believed to be at least partially responsible for the transformation of normal cells into neoplastic ones, have recently been shown to be developmentally regulated (Muller et al, 1983; Muller and Wagner, 1984; Slamon and Cline, 1984; Thiele et al, 1985; Rodrigues et al, 1985; Lev et al,

1984, 1985; Bell et al, 1986).

EC cells provide an excellent system of study for this because they are tumorigenic. During differentiation, however, the growth of the cells is halted, and the differentiated cell types lose their tumorigenicity. Indeed, loss of colony-forming ability is used as one of the measures of differentiation (see Rosenstrauss and Levine, 1979; Campione-Piccardo et al, 1985). As well, at least one of the drugs commonly used to induce the differentiation of EC cells (retinoic acid) has been found useful for the prevention and treatment of neoplasia in some animal systems (see Sporn and Roberts, 1983).

1.4 The P19 EC Cell System:

P19 is a line of euploid, male EC cells isolated from C3H/He mice (McBurney and Rogers, 1982). In vitro, P19 can be induced to differentiate into a variety of tissues (Fig 1.1), depending upon the culture conditions (Fig. 1.2). As well, P19 cells may be formed into floating aggregates by the method of Martin and Evans (1975). This is briefly explained in Section 2.2.

When P19 is allowed to form aggregates in the presence of high doses (5×10^{-7} M) of retinoic acid (RA), and these aggregates are allowed to subsequently spread out on tissue culture surfaces, a variety of neuroectodermal derivatives are seen. These include neurons, glial cells, and fibroblast-like cells (Jones-Villeneuve, 1982;

Figure 1.1: Schematic representation of the differentiation patterns of P19 cells. In 10^{-7} M RA, P19 cells form neurons. In 10^{-8} M and 10^{-7} M RA these cells form skeletal and cardiac muscle, respectively. Treatment with 1% DMSO results in the formation of the striated muscle types only.

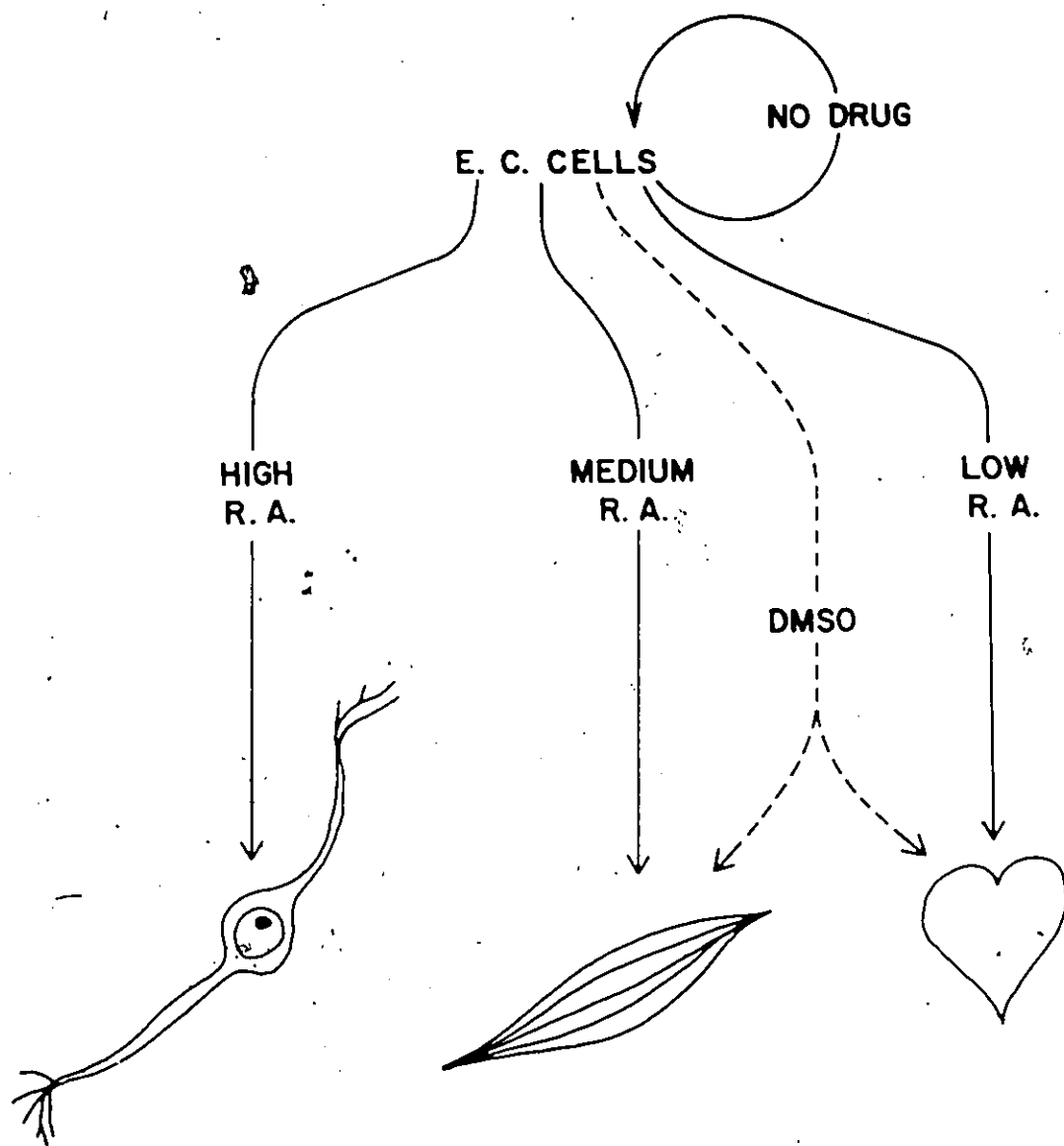
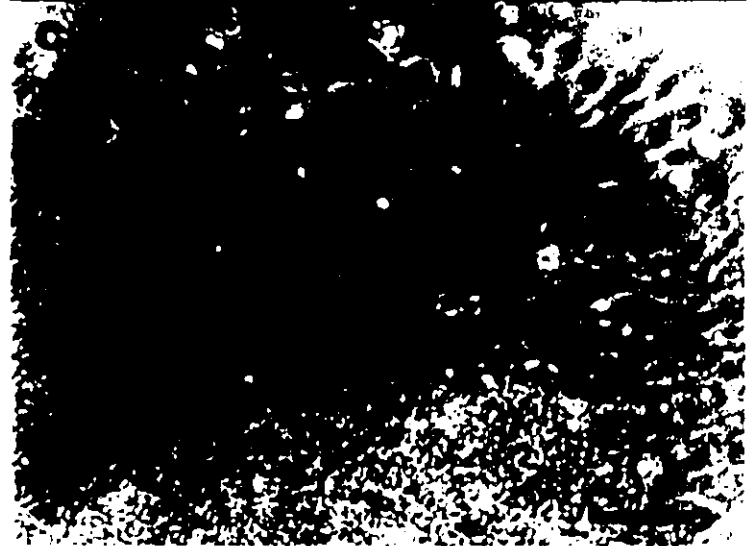
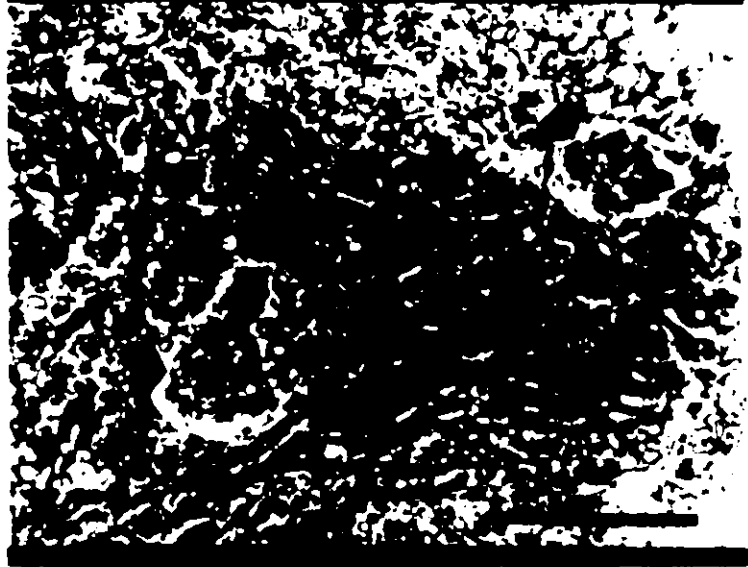
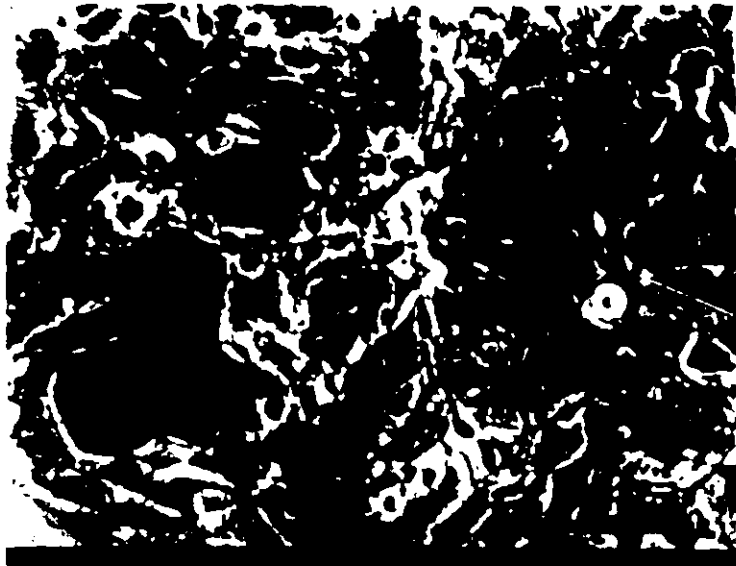


Figure 1.2: Photomicrographs of P19 cells following aggregation and treatment with (a) 5×10^{-7} M RA (b) and (c) 1% DMSO. Note the areas of beating cardiac muscle (b; arrows) and the large amounts of a tissue resembling extraembryonic endoderm (c). Bars = 100 μ m.



Jones-Villeneuve et al, 1982, 1983; McBurney et al, 1982; Edwards and McBurney, 1983). These cells were identified by immunofluorescent detection of neurofilament and glial fibrillary acidic proteins (GFAP), the increase in acetylcholine esterase, and by their morphology. The formation of neurons is dependent upon aggregation of the cells, however. In monolayers treated with RA, few neurons or glial cells are formed, although many fibroblast-like cells do appear (Jones-Villeneuve et al, 1982).

In lower doses of RA (10^{-8} M and 10^{-7} M), combined with aggregation, skeletal and cardiac myocytes (respectively) appear, along with a number of fibroblast-like cells and a rather large amount of extraembryonic endoderm (Fig. 1.2; Edwards and McBurney, 1983; Edwards, 1983). This differentiation into striated muscle cells also occurs when aggregates of these cells are treated with dimethylsulphoxide (DMSO) at concentrations ranging from 0.5% to 1.5% (McBurney et al, 1982; Edwards et al, 1983).

Muscle cells were identified through immunofluorescence using an antibody directed against muscle-specific myosin, as well as through visual identification of skeletal myoblasts and myotubes, and the spontaneous beating exhibited by patches of cardiac muscle.

Once again, aggregation of the cells is an absolute requirement for differentiation. In monolayer, no myocytes are ever seen and, indeed, the vast majority of the cells

remain undifferentiated (McBurney et al, 1982). Also worthy of note is the fact that, while RA treatment could proceed aggregation, DMSO treatment absolutely required simultaneous aggregation. Treatment of the cells with DMSO in monolayer, immediately prior to aggregation, yielded the same result as untreated aggregates.

The presence of an inducing drug is an absolute necessity. When P19 cells are aggregated in the absence of any inducing agent, no differentiation into either neurons or muscle is seen. A small amount of extraembryonic endoderm forms in some aggregates, but the majority of the cells remain undifferentiated (Fig. 1.2).

Since P19 cells can differentiate into derivatives of both ectoderm (neurons) and mesoderm (muscle), as well as extraembryonic endoderm, it can be stated that they are pluripotent, if not totipotent. As such, they are a viable in vitro model of the cytodifferentiation involved during the development of mammalian embryos. As with all EC cell systems, however, it must be remembered that beyond the molecular and cellular level, little can be deduced from this system. The formation of tissues and organogenesis are beyond the scope of in vitro EC cell systems. This is perhaps especially true of P19, as these cells often do not participate in normal development when injected into mouse blastocysts, and all chimaeric fetuses with a high P19-derived component are abnormal (Rossant and Papaioannou, 1985).

1.5 Thesis Project:

As mentioned in the previous section, the in vitro differentiation of P19 cells is dependent upon the aggregation of these cells. This is particularly true during DMSO-induced differentiation of the cells into cardiac muscle. Why aggregation is an absolute requirement is poorly understood. This project began as an attempt to elucidate the role of aggregation in DMSO-induced differentiation.

The role of aggregation was studied by examining one of the parameters of aggregation, and the internal architecture of developing DMSO-treated aggregates. As the project progressed, however, it became apparent that extraembryonic endoderm, which was formed in the same aggregates, may have been linked to the formation of muscle.

As a result, I began a study of the effect of medium conditioned by extraembryonic endoderm on P19 cells. Since an effect was discovered, the remainder of the thesis deals with an elucidation of the biological effects of endoderm-conditioned media on P19 cells and a partial biochemical characterization of the factor(s) involved.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell Culture Conditions:

The cell lines used in this study are listed in Table 2.1. All cells were maintained as monolayer cultures on Falcon plastic tissue culture surfaces (catalogue numbers 3002 and 3003, Becton, Dickenson and Co., Oxnard, CA, USA.). These dishes were coated with a 0.1% solution of gelatin and air dried for F9 cells. All cells were maintained in alpha minimal essential medium without nucleosides (cat. no. 330-2561, Gibco, Chagrin Falls, O, USA) supplemented with penicillin (80 U/ml), streptomycin (100 ug/ml), 7.5% calf serum (CS) and 2.5% fetal calf serum (FCS; both Gibco) and were kept in a humidified 5% CO₂ atmosphere. 2.5% Clex (cat. no. C100, Dextran Products, Scarborough, Ont.) was sometimes used in place of FCS. The cells were subcultured every 1-2 days by first washing with Ca⁺⁺- and Mg⁺⁺-free phosphate-buffered saline (PBS, pH 7.4) and then resuspending with the aid of 0.025% trypsin and 1mM ethylenediamine tetraacetic acid (EDTA) in PBS. Single cell suspensions were obtained by vigorously pipetting the cells up and down several times. Cell counts were performed using a Coulter Counter with Channelizer (Coulter Electronics, Hialeah, FLA, USA). F9-AC clone 9 (cl 9) cells were counted using a hemocytometer under phase contrast optics. This was due to the difficulty of obtaining a

Table 2.1: Cells Lines Used in This Study.

<u>CELL LINE</u>	<u>DESCRIPTION</u>	<u>REFERENCE</u>
P19	Euploid, male line of murine EC cells. Forms neurons in RA; striated muscle in DMSO.	McBurney and Rogers (1982); Jones-Villeneuve <u>et al</u> (1982);
O1A1	6-thioguanine- and ouabain-resistant subclone of P19.	McBurney <u>et al</u> (1982); Edwards and McBurney (1983).
D3	DMSO-resistant subclone of O1A1. Unable to form muscle under any conditions; forms neurons in RA.	McBurney <u>et al</u> (1982).
RAC65	RA-resistant subclone of P19. Unable to form muscle or neurons under any conditions.	Jones-Villeneuve <u>et al</u> (1982).
RAC65-D*	Spontaneous partial revertant of RAC65. Unable to form neurons; Forms muscle in DMSO.	(Unpublished).
3 RAP-1	Spontaneously immortal line of fibroblast-like cells derived from RA-treated P19.	Bell <u>et al</u> , (1986).
P19(<u>ras</u> +)1	Subclone of P19 transfected with Ha- <u>ras</u> ^H -1 oncogene. Differentiation same as P19 (with some transformed fibroblast-like cells).	Bell <u>et al</u> (1986).
RASA 1-1-2 and RASA 4363	Transformed lines of fibroblast-like cells derived from P19(<u>ras</u> +)1 and P19(<u>ras</u> +)4 cells (respectively) treated with RA.	Bell <u>et al</u> (1986).

Table 2.1 (continued):

<u>CELL LINE</u>	<u>DESCRIPTION</u>	<u>REFERENCE</u>
F9	Line of nullipotent murine EC cells. Forms only extraembryonic endoderm (in RA).	Strickland and Mahdavi (1978).
-F9-ACC1 9 (cl 9)	Line of spontaneously transformed parietal-like endoderm derived from RA-treated F9 cells.	Hogan <u>et al</u> (1983).
3T3	Line of immortal fibroblasts.	----

- although O1A1 was not used directly in this study, it has been included here to clarify the genealogy of its derivatives.

single cell suspension with these cells.

2.2 In Vitro Differentiation of EC Cells:

P19 cells and their derivatives were differentiated in a manner similar to that previously described (Jones-Villeneuve et al, 1982; Edwards et al, 1983; Edwards and McBurney, 1983; McBurney et al, 1982 and 1983; Jones-Villeneuve et al, 1983; Jones-Villeneuve, 1982; Edwards, 1983). Briefly, 5×10^5 cells were seeded into each 60 mm bacterial-grade Lab-Tek Petri dish (cat. no. D 1901, Miles Laboratories, Naperville, IL, USA.), with or without the appropriate drug in the medium (1% DMSO was used to induce muscle differentiation, and 5×10^{-7} M RA was used to induce neuronal differentiation). After 2 days in suspension, the resulting aggregates were resuspended in fresh Petri dishes with drug-free medium. Following a further 2 days of aggregation, the cells were plated onto 100 mm tissue culture dishes and were allowed to settle out and spread. Differentiated cells subsequently appeared in the attached aggregate. F9 cells were differentiated in a manner similar to that described by Strickland and Mahdavi (1978). Cells were seeded at a density of 1.5×10^4 per 100 mm dish in medium containing 10^{-7} M RA. The medium and drug were changed daily for one week, and the cells were subcultured 1:4 on day 4 or 5.

Table 2.2: Antibodies Used in this Study.

<u>ANTIBODY</u>	<u>SPECIFICITY</u>	<u>REFERENCE</u>
MF-20 (mouse IgG)	Monoclonal anti-muscle-specific myosin.	Bader <u>et al</u> (1982).
TROMA-1 (rat IgG)	Monoclonal anti-cytokeratin-like protein found in both visceral and parietal endoderm.	Boller and Kemler (1983).
TROMA-3 (rat IgG)	Monoclonal anti-cytokeratin-like protein found in parietal endoderm only.	Boller and Kemler (1983).
AEC 3A1-9 (mouse IgM)	Monoclonal anti-SSEA-1 (an EC cell-specific surface marker).	Harris <u>et al</u> (1984).
FITC-goat *	Anti-mouse IgG.	Cedarlane Labs.
FITC-rabbit	Anti-rat IgG.	Cedarlane Labs.
FITC-rabbit	Anti-mouse IgM.	Cedarlane Labs.

* - FITC = fluorescein isothiocyanate-conjugated.

2.3 Indirect Immunofluorescence:

The antibodies used in this study are listed in Table 2.2. The primary antibodies were generous gifts from Drs. D.A. Fischman (MF-20), J. Harris (AEC3A1-9), and R. Kemler (TROMAs-1 and -3). All fluorescein isothiocyanate-conjugated (FITC) secondary antibodies were purchased from Cedarlane Laboratories (Hornby, Ont.). For staining internal antigens (MF-20, TROMAs-1 and -3), cells on coverslips were washed with PBS (pH 7.0) and were immediately fixed in -20°C . methanol (4 min.) and -20°C . acetone (2 min.). Subsequently, they were washed with PBS and incubated at room temperature for 45-60 min. with the primary antibody. The cells were then passed through three 10 min. PBS washes, and were incubated with the second antibody. Following 3 further 10 min. washes, the cells were mounted, cell side down, with a drop of 50% glycerol and 0.2% paraphenylenediamine in PBS. For the surface antigen (AEC3A1-9), the cells were rinsed, as above, and were incubated with the primary antibody for 45 min. at 4°C . while still alive. Three subsequent washes with ice-cold PBS were followed by a similar incubation with the second antibody. Following the final 3 washes, the cells were post-fixed for 15 min. in 5% acetic acid: 95% ethanol (v:v) at -20°C . Following one subsequent 10 min. wash in PBS, the cells were mounted as described above. After staining, the cells were observed, as soon as possible,

using a Leitz microscope with epifluorescence optics.

2.4 Immunofluorescent Cell Counts:

To quantitate the proportions of various cell types present in cultures, cell counts were performed. Cultures at the appropriate stage of development were resuspended using trypsin-EDTA in PBS or 1mM EDTA alone. (EDTA was used alone for cells to be stained with AEC3A1-9 because trypsin destroys the surface antigen recognized by this anti/body). The cells were then plated onto coverslips (which had been previously coated with 1 mg/ml poly-L-lysine (1 min.), rinsed with water, and air dried). The cells were allowed to settle for 10 min., and then immunostaining was performed as described above. Following staining and mounting, random fields of the coverslip were scored. For each field, the total number of cells present was determined under bright field conditions. Subsequently, the same field was observed using fluorescence optics, and the number of positive (fluorescent) cells was recorded. For each slide, 250 to 500 cells were counted.

2.5 Staining with Dolichos biflorus Agglutinin:

Living cells, grown on gelatin-coated coverslips, were stained in situ. These cells were washed with serum-free (SF) medium/PBS (1/1, v/v), and were then stained for 10 min. with 200 ug/ml FITC Dolichos biflorus agglutinin (DBA; cat. no. FL-1031, Vector Labs, Burlingame, CA, USA.). This

was followed by 2-5 min. washes in medium/PBS, and fixation for 10 min. in 95% methanol, 5% acetic acid (v/v). After a final wash in PBS, the cells were mounted and examined as for immunofluorescent staining (Sect. 2.3).

2.6 Frozen Sectioning of Aggregates:

Aggregates grown in 60 mm bacterial-grade dishes (as described in Section 2.2) were collected in 15 ml. centrifuge tubes (cat. no. 25311, Corning Plastics, Corning, NY, USA) and were allowed to settle to the bottom. The medium was then removed and the aggregates were washed with PBS. The aggregates were then fixed for 10-30 min. in -20°C. methanol. Following two additional washes with PBS, the aggregates were incubated for 5 min. in 0.1% aqueous toluidine blue at room temperature. This stained the outer layer of cells in the aggregates and thereby allowed them to be seen during sectioning. The aggregates were then washed 3 times with PBS to remove excess stain. The last wash was used to transfer the aggregates to a 50 ml Corning centrifuge tube (cat. no. 25339). The aggregates were then covered with approximately 2.5 ml of OCT compound (cat. no. 4583, Miles Laboratories), left at room temperature to embed for 20-60 min., and subsequently frozen in a -20°C. freezer. Within a few weeks, the conical blocks were removed from the tubes and were frozen to brass chucks with the aid of a drop of OCT compound. Another drop of OCT was used to cover the aggregates at the tip of the block.

Random 4 um sections were cut using a cryostat and the sections were melted directly onto glass coverslips. Indirect immunofluorescence was performed on the sections in the manner described previously for internal antigens, except that no further fixation was required.

2.7 Production of Uniformly Sized Aggregates:

To produce large numbers of uniformly sized aggregates, a modified hanging drop procedure was used. Briefly, single cell suspensions were diluted to the appropriate density in medium with or without 1% DMSO, as required. This was then distributed in 10 ul drops on the lids of bacterial-grade Petri dishes. These were subsequently inverted over dishes containing a few ml of PBS. The cells were left to aggregate for 3 days, after which the aggregates were individually picked, placed in drug-free medium in a tissue culture dish, and allowed to settle. Medium on the adhering aggregates was subsequently changed every 2 days.

2.8 Production and Assay of Conditioned Media:

Conditioned medium (CM) was initially produced from endoderm derived from RA-treated F9 cells (see section 2.2). After one week in RA, a monolayer of endoderm was produced. At this time, the monolayer was washed 2-3 times with PBS (to remove all traces of RA) and 15 ml of fresh, drug-free medium was placed on the cells. After 2 days,

this CM was removed and centrifuged to remove particulate matter. Originally, the CM was used immediately, but when larger quantities became available, it was stored at 4°C. until needed. Later, F9-AC clone 9 (cl 9) cells were used to condition medium. Originally, the cells (4×10^6) were placed in 100 mm dishes with 15 ml of fresh medium and left for 2 days. Later, 10 confluent 100 mm plates of cells were seeded into 850 cm² tissue culture roller bottles (cat. no. 25140, Corning). After 1 to 3 days at a slow speed, the medium was removed and the cells were washed twice with PBS. The cells were then incubated with serum-free medium (medium plus 1 ug/ml insulin and 5 ug/ml transferrin) and this was harvested every 2 days and treated as described above, yielding serum-free conditioned medium (SF-CM). Later, when it was found that SF-CM was more effective when heat inactivated, this was used following a 10 min. treatment at 56°C. It should be noted that living cells which were growth arrested through either gamma irradiation or treatment with mitomycin C did not give an effective CM, even when confluent cultures were utilized (data not shown).

CM was assayed by placing 5 ml of the medium to be tested in a 60 mm tissue culture dish with the required number of F19 cells (usually 10^5). The appropriate medium was changed every 2 days, and the cells were scored on day 6 or 7. Scoring was done either visually, on the basis of morphology, using an inverted microscope with phase

contrast optics, or immunofluorescent cell counts were done (Section 2.4).

2.9 Plating Efficiencies:

To check the plating efficiencies of EC cells, 100-200 cells per 60 mm tissue culture dish were placed in the medium to be tested. The media were either replaced with fresh medium after two days, or the cells were left undisturbed. At the end of one week, all plates were rinsed with PBS, and were flooded with either 0.1% toluidine blue in ethanol:acetic acid (3:1, v:v) or 1% methylene blue in 70% ethanol. After 10-20 min. these plates were rinsed with tap water, air dried, and scored visually for the number of colonies present. Absolute plating efficiencies of the P19 controls was usually 35-40%, and all plating efficiencies are recorded as a percentage of the controls. All plating efficiencies were done in triplicate.

2.10 Growth Curves and Rates:

Cell growth was checked by plating 10^5 cells in a 60 mm tissue culture dish containing the medium to be tested. Four plates were started for each test. On days 2, 3, 4, and 6, the cells from one plate were resuspended in a known volume of T-EDTA and medium, and were counted. The mean of 5 counts was used for each point. Growth curves were plotted (see Sections 4.3 and 4.9) and the growth rates were calculated from a linear regression line based on the

logarithm of the cell number for days 2, 3, and 4, and are expressed as doubling time.

2.11 Chemicals and Supplies:

The chemicals used in this study were purchased from Fisher Scientific (Fair Lawn, NJ, USA.), with the following exceptions: trypsin for T-EDTA (cat. no. T 8003), human transferrin (cat. no. T 5391), bovine insulin (cat. no. I 5500), RA (cat. no. R 2625), DMSO (cat. no. D 5879), and 1% sterile soybean trypsin inhibitor (cat. no. T 9008, Sigma, St. Louis, MO, USA.), staphylococcal nuclease (cat. no. 107 921) and mitomycin C (cat. no. 107 409, Boehringer-Mannheim Canada, Dorval, Qué.), and sterile lyophilized trypsin for enzymal inactivation (cat. no. 19615, ICN Pharmaceuticals, Cleveland, O, USA.). Ultrafilters were purchased from the Amicon Corp. (cat. no. 10 YM10 76, Danvers, MA, USA).

CHAPTER III

THE ROLE OF AGGREGATION

To study the requirement for aggregation during DMSO-induced differentiation, two approaches were used. The first was a study of muscle differentiation in aggregates of different sizes. Through the use of hanging drops, aggregates with a known number of cells were formed. In this way, it was hoped that any minimum size requirements for aggregates could be established. The second approach was an examination of the internal architecture of the aggregates. This was accomplished through the use of frozen sections stained using indirect immunofluorescence with a variety of antibodies. It was hoped that the exact site(s), if any, of muscle cell differentiation within the aggregate could thus be determined.

3.1 Aggregate Size:

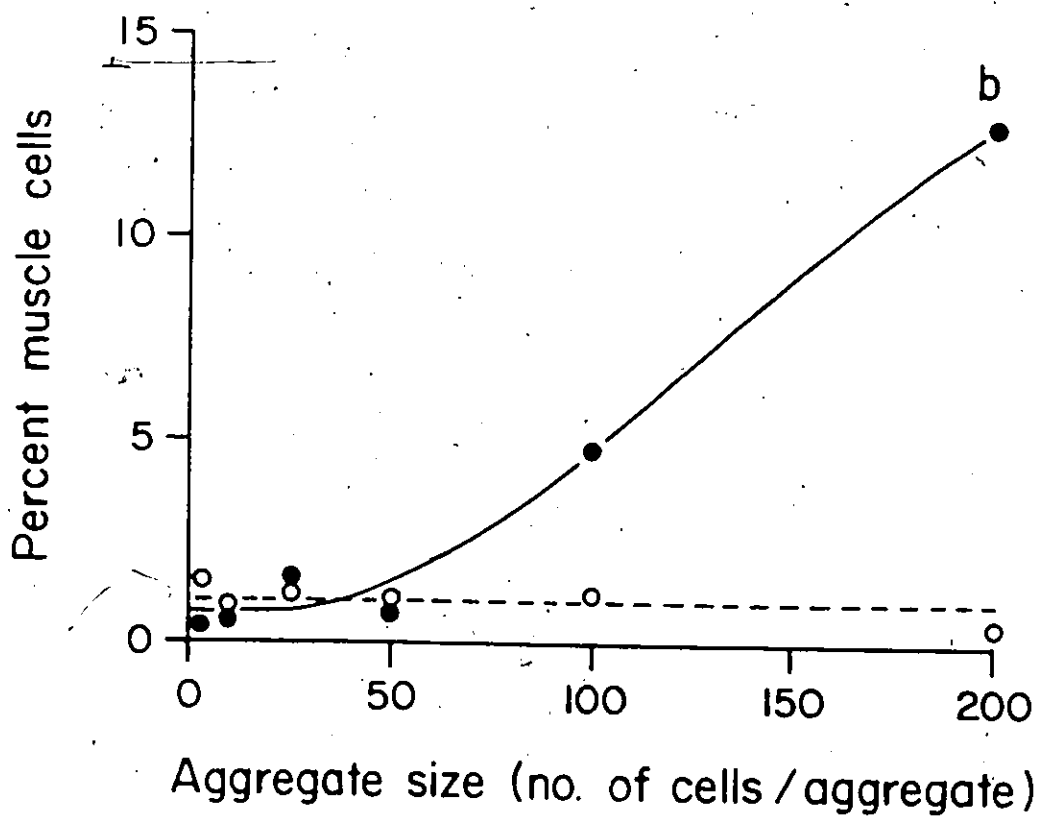
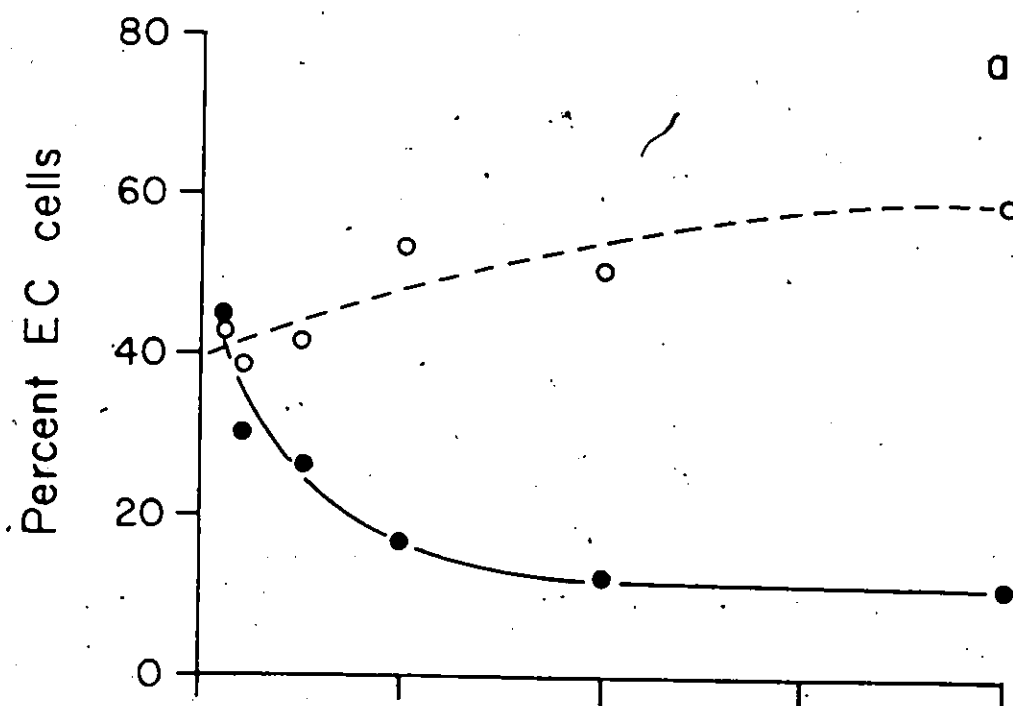
In order to clarify one of the parameters for aggregation during the DMSO-induced differentiation of P19 cells into muscle, a study of the aggregate size requirements for differentiation was initiated. Using the hanging drop technique described previously (Section 2.7), aggregates of known size were formed in both 1% DMSO and drug-free medium by dispensing 10 μ l drops of the medium (containing a known concentration of cells) on the lids of bacterial-grade Petri dishes. Following suspension for 3

days, the aggregates formed in the drops were plated onto tissue culture surfaces, where they were allowed to spread. For MF-20 and AEC3A1-9 staining, the cells were dispersed 7 days after plating. All cells were subsequently plated onto poly-L-lysine coated coverslips, and immunofluorescent cell counts were then performed. Thus, the proportion of cells bearing markers for muscle (MF-20) and EC cells (AEC3A1-9) could be determined in relation to aggregate size.

Figure 3.1 shows the results of staining with AEC3A1-9 (3.1a) and MF-20 (3.1b). As the number of cells in the DMSO-treated aggregates increased, so did the proportion of cells which had differentiated into muscle. The proportion of undifferentiated cells declined drastically in DMSO-treated aggregates, but remained high in untreated aggregates of all sizes, demonstrating a requirement for DMSO. Figure 3.1 also shows that there was a threshold size which aggregates had to exceed in order to differentiate effectively into muscle. This threshold was approximately 50 cells per aggregate (starting size). In aggregates larger than this, muscle differentiation (as indicated by MF-20 staining) occurred readily and the percentage of EC cells (AEC3A1-9 positive cells) remaining dropped drastically. Below a starting size of 50 cells per aggregate, a large proportion of the cells remained undifferentiated. Since the proportion of muscle cells increased, there appeared to be some intrinsic difference between smaller and larger aggregates. This suggested that



Figure 3.1: Differentiation in aggregates of different sizes, as marked by (a) AEC3A1-9 (anti-EC) and (b) MF-20 (anti-muscle-myosin). Aggregates were treated with 1% DMSO (●—●) or were left untreated (○—○) in hanging drops for 3 days. The aggregates were then allowed to settle in tissue culture dishes and, after a further 7 days, were dispersed, allowed to settle on coverslips, and were stained with the appropriate antibody. Lines drawn by eye.



muscle cells form randomly throughout the interior of DMSO-treated aggregates. (In larger aggregates, more cells are present, and therefore there is a greater chance of having some that will form muscle).

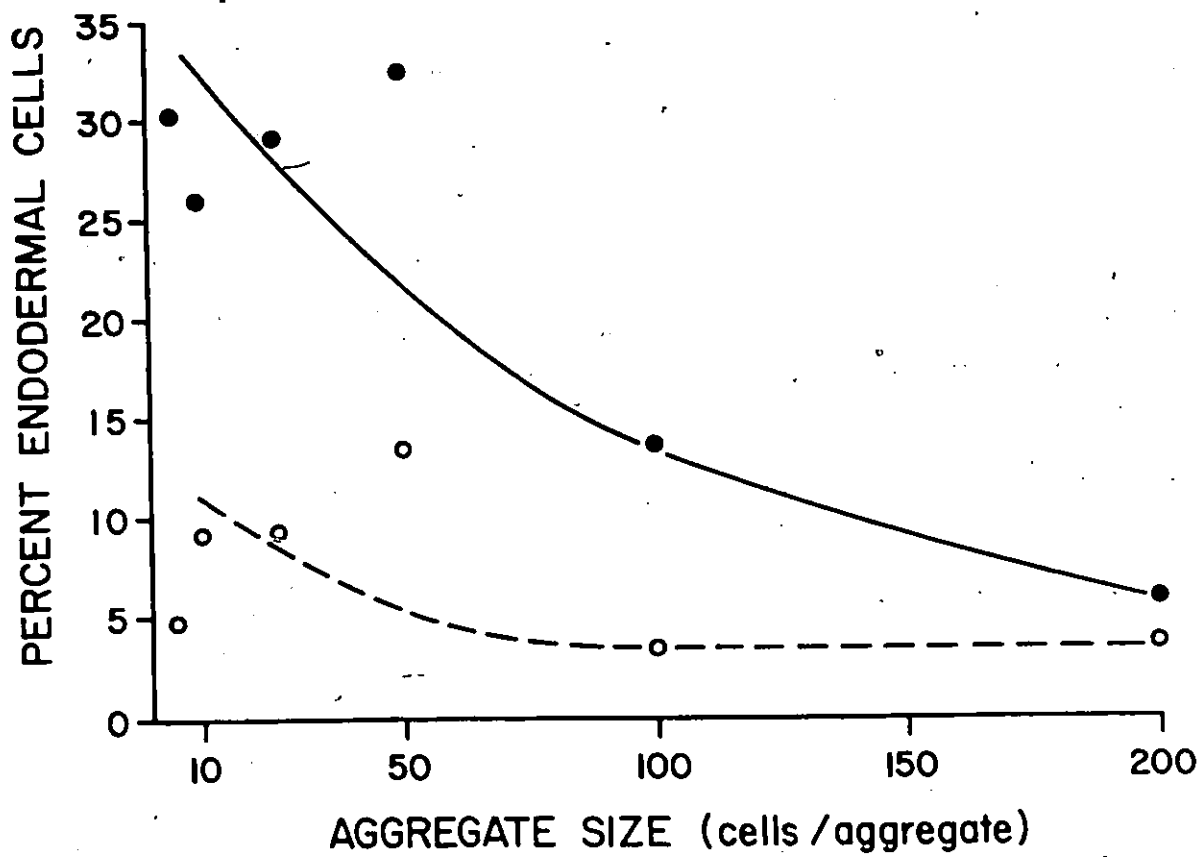
In order to ascertain if the proportion of all differentiated cell types increased with increasing aggregate size, the proportion of TROMA-1 positive (extraembryonic endoderm-like) cells was determined. For TROMA-1 immunostaining, the cells were dispersed 3 days after plating, rather than the 7 days used for MF-20 and AEC3A1-9 because these cells appeared (and subsequently disappeared) earlier than muscle. Therefore, the proportion of cells was determined near their peak.

The results of the TROMA-1 staining are presented in Figure 3.2. Contrary to the results observed with MF-20, it was noted that a larger amount of extraembryonic endoderm appeared to form in smaller aggregates. The proportion of endodermal cells formed decreased with increasing aggregate size. Again, this was dependent on DMSO treatment, as a lower level of endoderm was formed in untreated control cultures. Therefore, at least one differentiated cell type appeared in greater proportion in smaller rather than larger aggregates.

Endoderm has been shown to differentiate preferentially around the periphery of aggregates in other EC cell systems, notably F9 (Martin and Evans, 1975; Hogan and Taylor, 1981; Rosenstrauss et al, 1983). This experiment

Figure 3.2: Differentiation in aggregates of different sizes treated with 1% DMSO (●—●) or left untreated (○- - -○) in hanging drops for 3 days. The aggregates were then allowed to settle in tissue culture dishes and, after a further 3 days, were dispersed, allowed to settle on coverslips, and were stained with TROMA-1. Lines drawn by eye.





suggested that extraembryonic endoderm formed on the periphery of aggregates, rather than on the interior, in P19 cells as well. Thus, the decreasing surface area to volume ratio would cause a decrease in the proportion of endodermal cells.

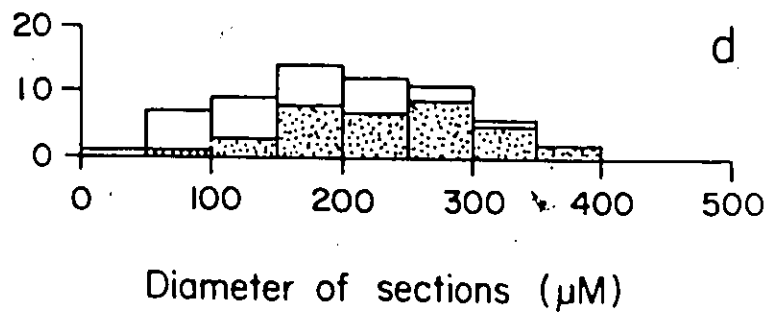
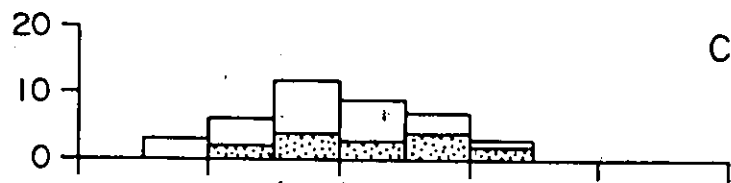
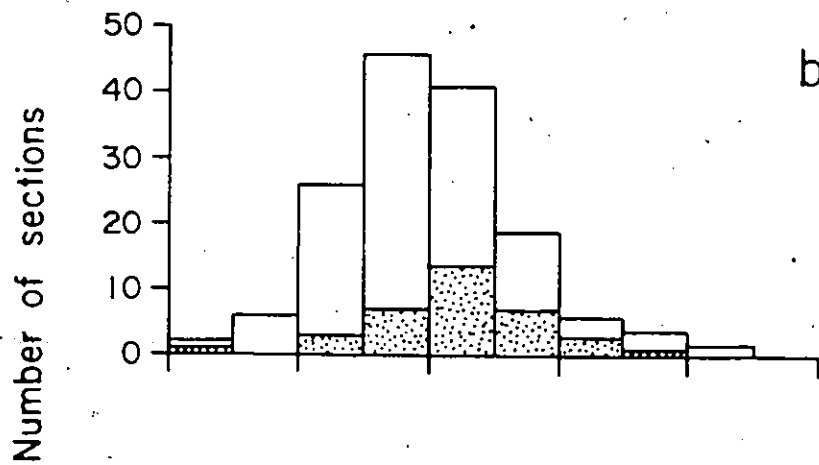
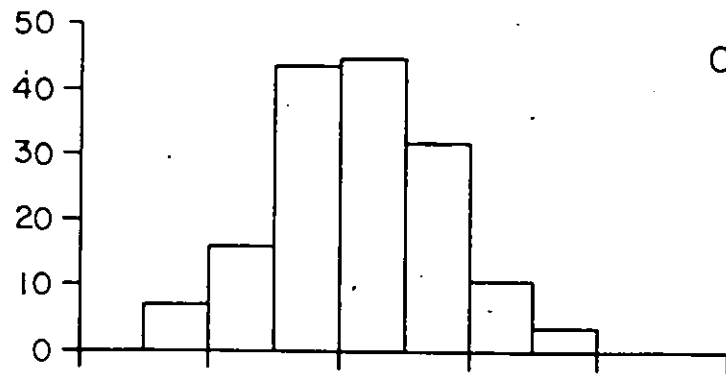
3.2 Location of Muscle Cells in Aggregates:

In order to determine if muscle cells did form only in the interior of DMSO-treated aggregates, a study of the location of muscle cells within these aggregates was initiated. This was done using random frozen sections of DMSO-treated aggregates stained with the muscle-myosin-specific antibody MF-20. The location of MF-20 positive cells within DMSO-treated aggregates was checked on different days. In this way the internal pattern of differentiation within the aggregates could be studied and any preferential site(s) for the formation of muscle could be located.

Muscle myosin-containing cells were first detected in DMSO-treated aggregates 5 days after the beginning of aggregation. Of 159 4-day-old aggregate sections examined, no MF-20 positive cells were seen. This is consistent with the results of previous workers (Edwards, 1983; Edwards et al., 1983).

The mean radius of the sections examined (Fig. 3.3) was about 100 μ m. This figure also shows that, while the sections studied on days 4 through 9 were of approximately

Figure 3.3: Diameters of (a) 4, (b) 5, (c) 6, and (d) 9 day old aggregate sections stained with MF-20. All aggregates were treated with 1% DMSO. Shaded areas indicate those sections containing at least one MF-20 positive cell. Mean radii of sections = 100 μ m.



the same size, the proportion of sections containing muscle increased with time.

As shown in Fig. 3.4, muscle first appeared 5 days after the beginning of aggregation. MF-20 positive cells were not randomly distributed throughout the aggregate, however, but were most commonly found just inside the periphery, approximately 20 μ m beneath the surface (see also Fig. 3.6). With a random distribution of cells within the aggregate, a linear decrease in the number of cells from the periphery towards the centre would be expected (see Fig. 3.5). With time, muscle-myosin-containing cells became more dispersed within the aggregates (Figs. 3.4c and 3.6). By day 9, many of the aggregates had areas which were contracting rhythmically and the muscle cells were usually arranged in groups (Fig. 3.7).

Therefore, muscle-myosin positive cells did, in fact, appear on the interior of the aggregate. These cells were not randomly distributed throughout the interior, but seemed to appear preferentially within narrow area just inside the periphery. It should be noted that the entire area delineated by these boundaries did not usually form muscle, but that patches of muscle were formed uniquely within the boundaries. Thus, muscle cells were found to form within a defined area of the aggregate. If extraembryonic endoderm did, indeed, form on the periphery of the aggregate, as postulated in the previous section, this might possibly indicate the presence of some link

Figure 3.4: Distance of MF-20 positive cells from the periphery of sections of (a) 5, (b) 6, and (c) 9 day old aggregates. All aggregates were treated with 1% DMSO. Mean distance from periphery = 20 μ m.

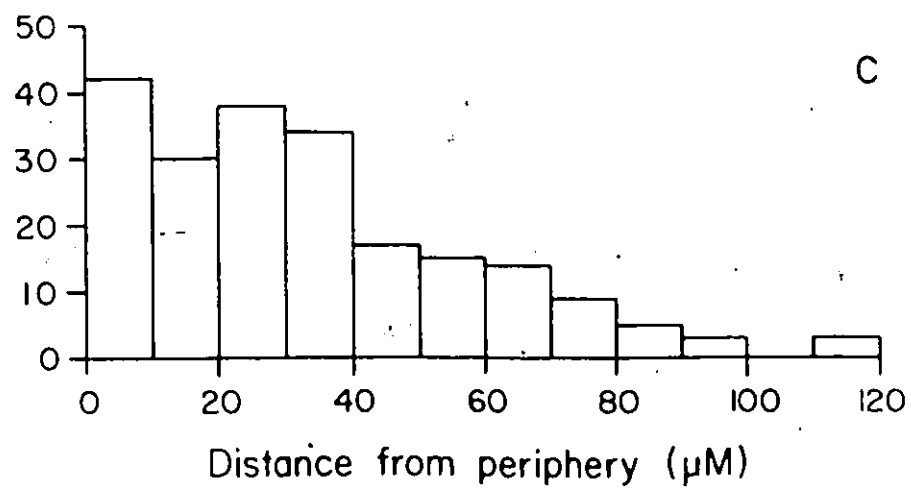
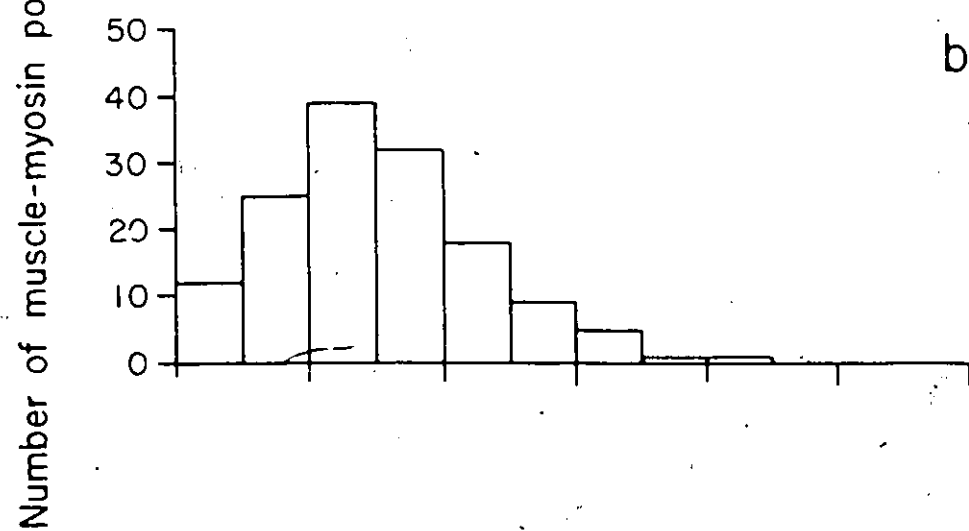
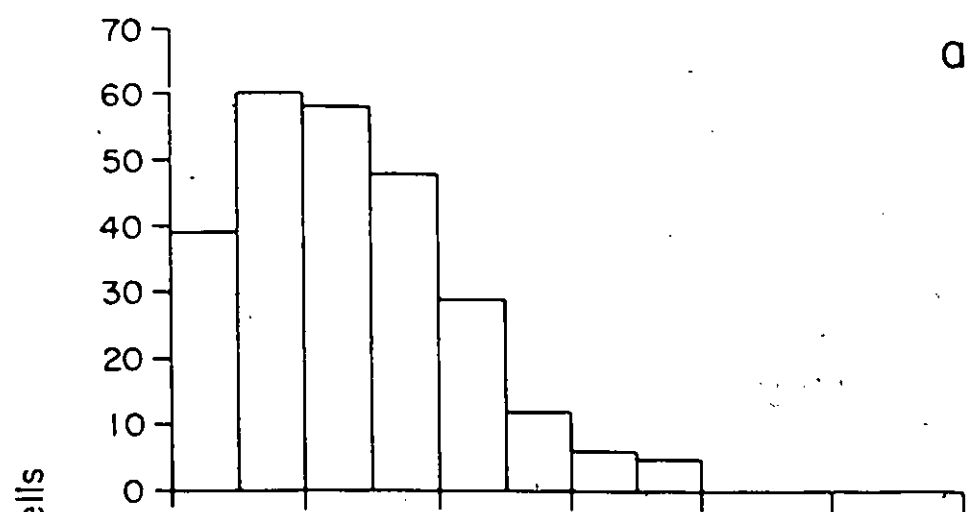


Figure 3.5: Proportion (b) of the total area of a hypothetical circular section (a; radius = 100 μm) within different concentric regions (A-J). This illustrates the proportion of cells which would be found in each region if the distribution were random.

$$\% \text{ of total area} = \frac{\pi(r_o)^2 - \pi(r_i)^2}{\pi(r_T)^2} \times 100$$

where r_o = radius to outer edge of region.

r_i = radius to inner edge of region.

and r_T = radius of section (= 100 μm).

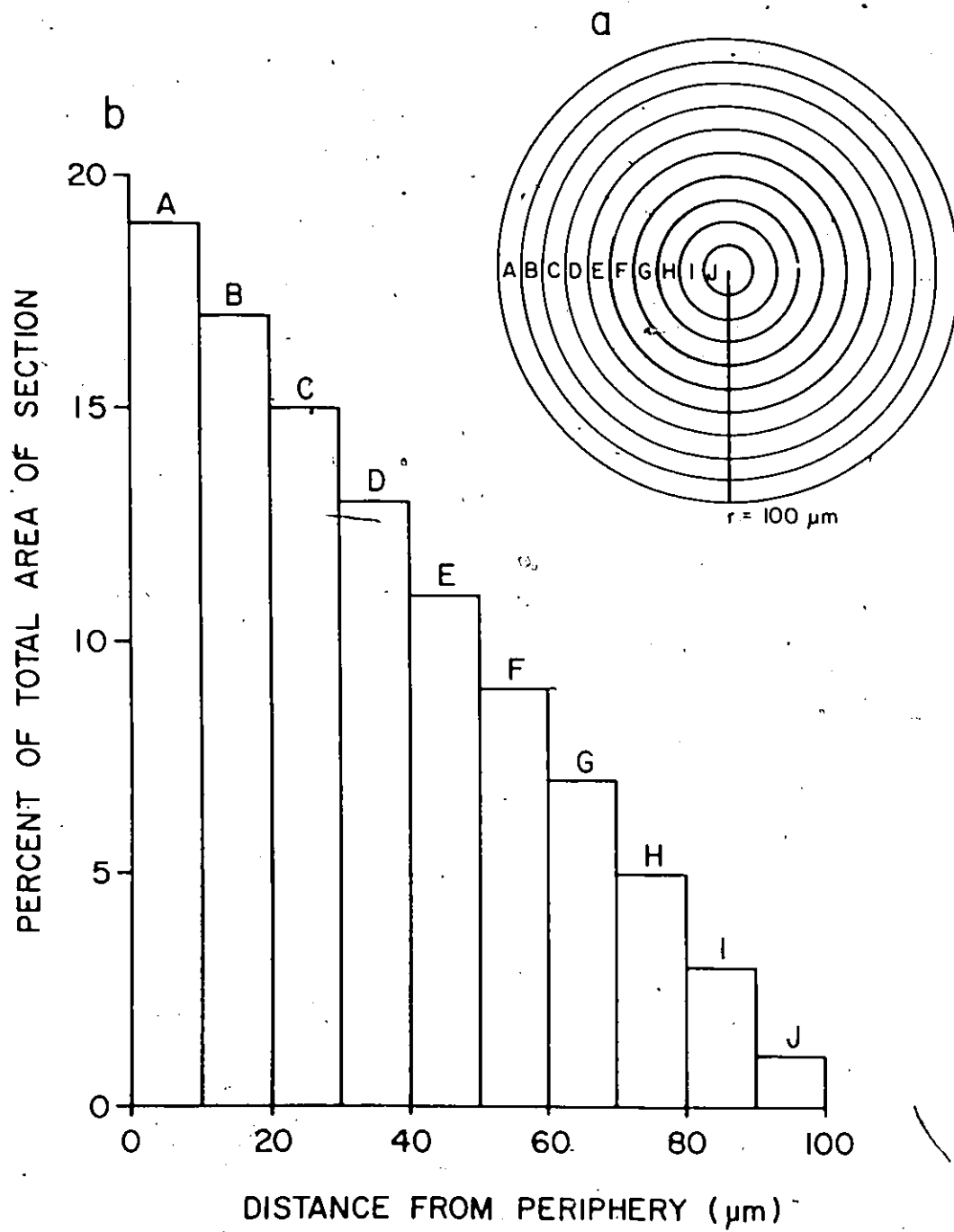


Figure 3.6: Photomicrographs of a section of a 5 day old DMSO-treated aggregate stained with MF-20. (a) phase and (b) fluorescence photographs of the same field. Bar = 50 μ m.

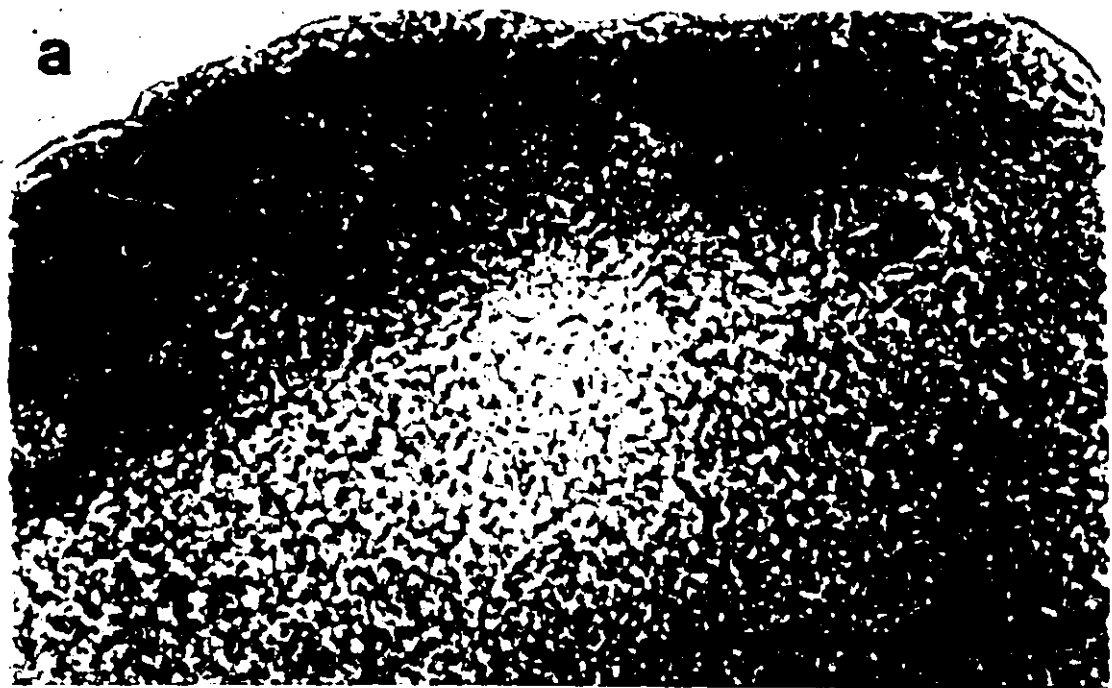
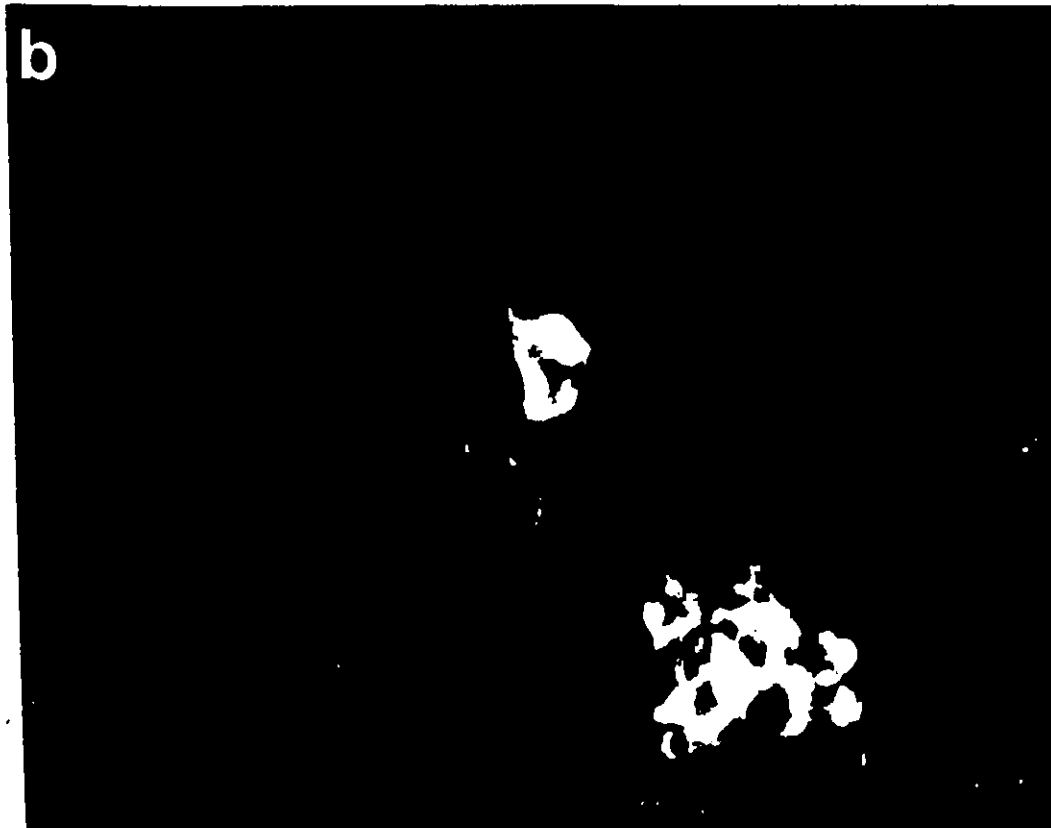
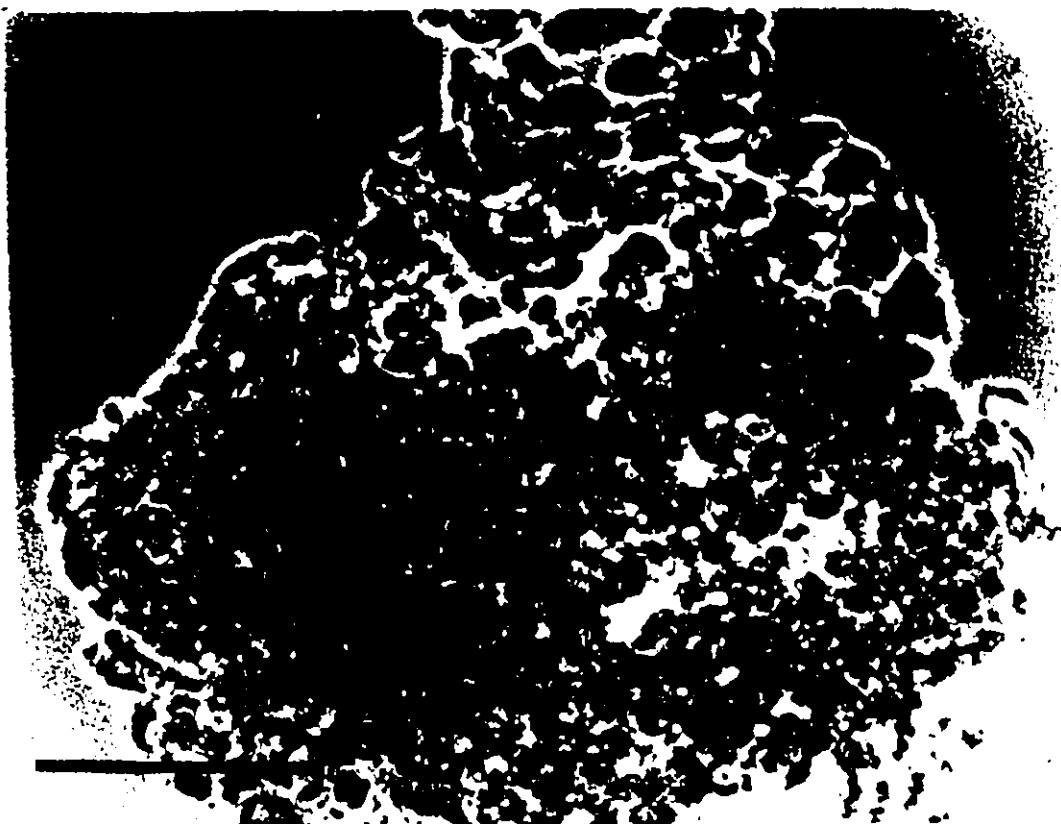


Figure 3.7: Photomicrographs of a section of a 9 day old aggregate stained with MF-20. (a) phase and (b) fluorescence photographs of the same field. Bar = 50 μm .



between the formation of the extraembryonic endoderm-like cells and the formation of muscle immediately beneath the periphery.

3.3: Location of Endoderm in Aggregates:

In addition to locating muscle in DMSO-treated aggregates, the location of endodermal cells was also determined. This was done by staining frozen sections of aggregates with TROMA-1.

As suggested by the aggregate size data, endoderm was found around the periphery of the aggregates and appeared to be only a single cell layer thick. This coating of endoderm was well developed by day 4 (Figs. 3.8 and 3.10). The extent of this endodermal sheath was measured from the beginning of aggregation through day 5 in both DMSO-treated and untreated aggregates. This was accomplished by staining frozen sections of aggregates of different ages with TROMA-1. The proportion of each section surrounded by an endodermal sheath was estimated using an eyepiece which was marked off into 36° arcs (i.e. 10% of a circle; see Fig. 3.9).

The results of this study are shown in Figure 3.10. This shows that the endodermal sheath around DMSO-treated aggregates becomes quite extensive, rapidly covering a large proportion of each aggregate. This sheath begins to appear by the second day of aggregation, and most aggregates are surrounded by endoderm by days 4 and 5. In

Figure 3.8: Photomicrographs of a section of a 4 day old aggregate stained with TROMA-1. (a) phase and (b) fluorescence photographs of the same field. Bar = 50 μ m.

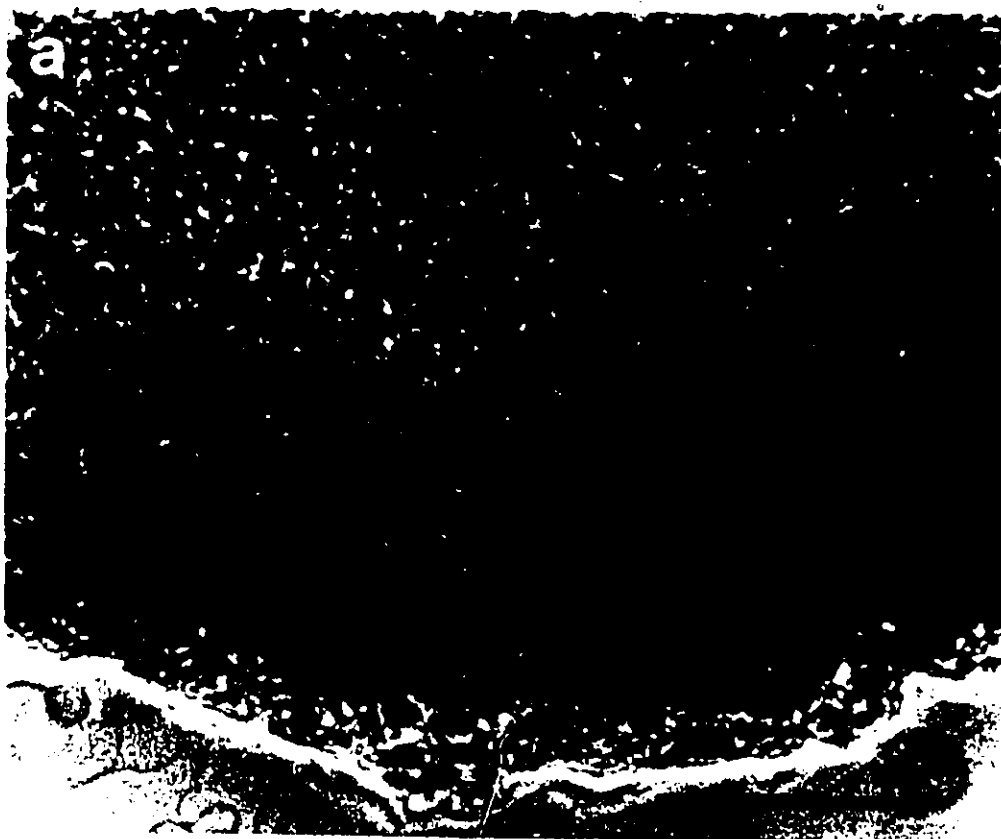


Figure 3.9: Diagram of the eyepiece reticule used to measure the proportions of aggregate sections surrounded by TROMA-1 positive cells. Each angle = 36° (10% of a circle).

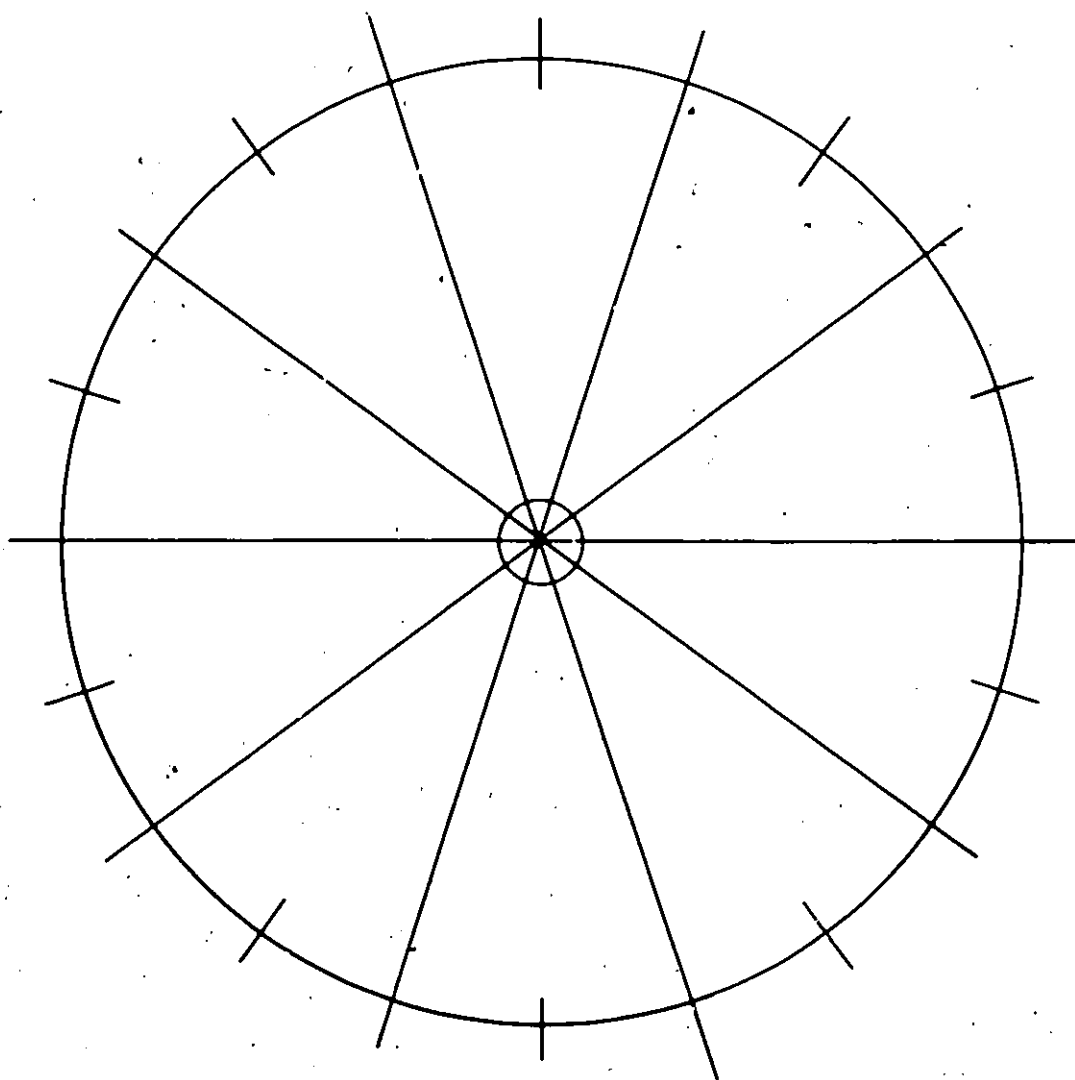
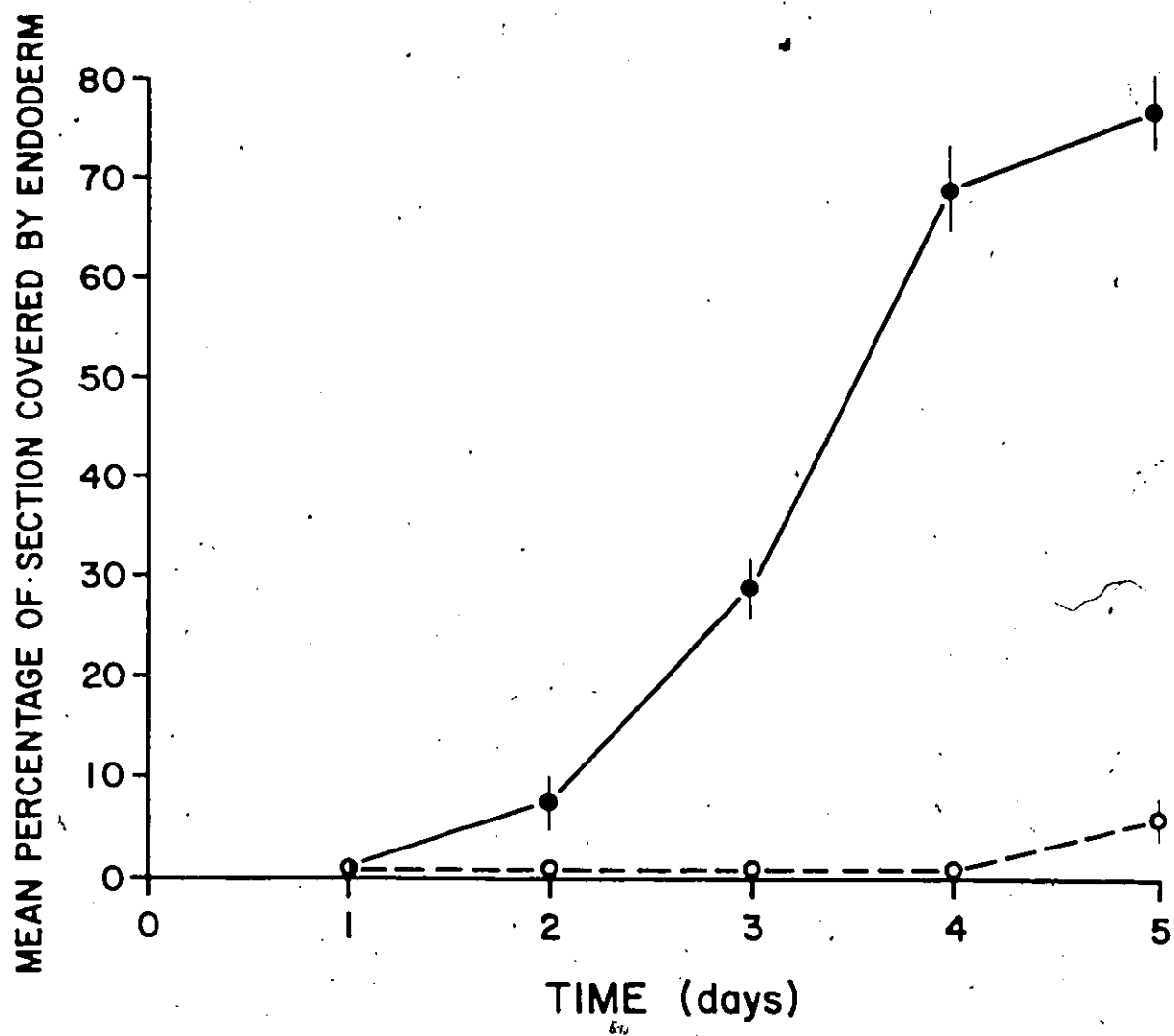


Figure 3.10: Proportion of aggregate sections surrounded by TRDMA-1 positive cells. ●—● aggregates treated with 1% DMSO and ○— — — —○ without any drug ($\pm$ SEM). At least 50 sections were scored for each point.



contrast, untreated aggregates contained little or no endoderm, and remained, for the most part, completely uncoated.

In this manner, the location of the extraembryonic endoderm was established to be, as expected, on the exterior surface of the aggregate. As well, it shows that endoderm formed 1-2 days before the appearance of muscle. This suggested that a temporal as well as spatial relationship existed between the formation of endoderm and the formation of muscle in DMSO-treated aggregates.

3.4 Discussion:

Since aggregation is absolutely required for DMSO induction of muscle in P19 cells, I began a study of the role that aggregation played during DMSO-induced differentiation. This began with a study of one of the parameters of aggregation (aggregate size).

The examination of aggregate size showed that there was a minimum threshold size which DMSO-treated aggregates had to exceed for muscle differentiation to ensue. This threshold was about 50 cells per aggregate, starting size. Very small aggregates were incapable of forming myosin positive cells, and the proportion of undifferentiated cells was high. As the size of the aggregates was increased, the proportion of undifferentiated cells decreased and muscle began to form a higher proportion of the cell types present. Not all differentiated cell types

formed with greater efficiency in larger aggregates. The proportion of extraembryonic endoderm-like cells which stained with TROMA-1, in fact, decreased with increasing aggregate size. Differentiation into both muscle and endoderm was dependent upon treatment of the aggregates with DMSO. In untreated aggregates, very little of either cell type appeared, and the majority of the cells remained undifferentiated.

In addition to examining aggregate size, frozen sections of aggregates were used, combined with indirect immunostaining, to examine the internal architecture of developing DMSO-treated aggregates. In DMSO-treated aggregates, muscle myosin positive cells first appear at 5 days. No positive cells were seen in the 159 4-day-old sections examined, and only a small proportion of the 152 5-day-old sections contained muscle. The work with frozen sections stained with MF-20 also showed that muscle appeared predominantly on the inside of treated aggregates, but not throughout the interior, as might have been predicted with the aggregate size results. Instead, muscle appeared in a relatively narrow area just beneath the periphery of the aggregate. This could be pictured as a sort of hollow sphere within a sphere. In this way the cells covering the periphery and in the centre (and thus not in the appropriate location) did not differentiate into muscle.

The use of frozen sections stained with TROMA-1 showed

that the extraembryonic endoderm-like cells formed around the outside of DMSO-treated aggregates, effectively covering much of the periphery. This sheath of endoderm was well developed by day 4, at least one day prior to the appearance of muscle. As well, it showed why the proportion of extraembryonic endoderm would decrease with increasing size. This would be due to the decreasing surface area to volume ratio as aggregate size was increased. In this way, the proportion of TROMA-1 positive cells would decrease. Formation of endoderm around the periphery of aggregates has been demonstrated in other EC cell systems (Martin and Evans, 1975; Hogan et al, 1981; Rosenstrauss et al, 1983), but this is the first indication of a similar localization in F19 cells.

These results on the role of aggregation in DMSO-induced muscle differentiation lead to several conclusions. First, there is a minimum threshold size which aggregates must exceed to allow for efficient muscle differentiation. From the results obtained with the frozen sections and MF-20 staining, it appeared that this is because muscle forms inside the aggregate. In aggregates smaller than the threshold size, insufficient aggregate "interior" would be present. Only in larger aggregates, that is only in aggregates with a starting size of greater than 50 cells, would enough of the appropriate internal milieu be present to allow for the differentiation of muscle. In addition, this differentiation is completely

dependent on the presence of DMSO. In the absence of this inducing drug, little differentiation occurred in aggregates of any size. As well, I have shown that the endodermal sheath covering aggregates develops rapidly in DMSO-treated aggregates, and is well formed by days 4 and 5, prior to the appearance of the MF-20 positive cells. Little endoderm forms in control aggregates during the same period. Therefore, muscle myosin positive cells appear immediately below a previously formed endodermal sheath covering the aggregate.

Since the differentiated cell types formed in different locations within the aggregate, it suggests that there must be some intrinsic difference between the interior and the exterior of a DMSO-treated aggregate. Furthermore, it has been shown that muscle does not form throughout the interior of the aggregate, but only in a relatively narrow area just beneath the periphery. Muscle myosin-containing cells appear to be more randomly distributed only in older aggregates.

This appearance of myosin-containing cells immediately beneath a previously formed endodermal sheath may be merely coincidental. These facts, however, suggest that the endodermal sheath surrounding DMSO-treated aggregates is causally involved in the differentiation of the muscle immediately below it. If true, this may be the first indication of a primitive type of pattern formation in aggregates of EC cells. It also appears to be an extension

of the positional regulation hypothesized for extraembryonic endoderm formation both in EC cell aggregates and mammalian embryos (see Hogan *et al.*, 1981; Rosenstrauss *et al.*, 1983), in that muscular as well as endodermal differentiation depends upon the location of the cells. In addition, this would be an indication of tissue interaction in the EC cell system, perhaps indicating that higher levels of organization may be studied with this system. The hypothesis that the endodermal sheath could be implicated as an inductor of muscle differentiation is consistent with the work of several classical embryologists, who have found that the endoderm of amphibian embryos contained the ability to induce heart formation (see Jacobson, 1961, for review; Jacobson and Duncan, 1968; Fullilove, 1970).

Originally, I postulated that there were three possible ways in which this endodermal coating could affect the interior of the aggregate. (i) There could be the production of some soluble factor (a diffusible morphogen) by the endoderm which induced muscle differentiation in the adjacent cells beneath. (ii) The endodermal sheath could form a basement membrane which elicits muscle differentiation in the cells beneath. In fact, parietal extraembryonic endoderm is known to form a thick basement membrane known as Reichert's membrane (see Clark *et al.*, 1975; Hogan *et al.*, 1980 and 1982; Howe and Solter, 1980; Jetten *et al.*, 1979). As well, visceral endoderm is also

known to form a thin basement membrane (see Adamson and Grover, 1983). This, combined with the fact that extracellular materials have been shown to be closely linked to inductive and inhibitory processes during migration and cytodifferentiation of various cell types (see Toole, 1981; Hay, 1981; Yamada et al., 1982; Grotendorst et al., 1982; Darmon et al., 1982; Reichard-Brown and Akeson, 1983; Sanders, 1983; Edelman, 1984; Rizzino, 1983; Grabel et al., 1983; Chua and Maylie-Pfenninger, 1983; Fridman et al., 1985), may suggest that a basement membrane formed by endoderm may be implicated in the differentiation of muscle beneath. (iii) The endodermal sheath could restrict the passage of some factor(s) in the medium or serum which normally inhibits muscle differentiation. This alternative is suggested by findings that, in some systems, serum deprivation elicits the spontaneous differentiation of EC cells (Rizzino, 1982 and 1983).

The next chapter deals with an examination of the possibility that a soluble factor is produced by extraembryonic endoderm which can induce the differentiation of P19 cells in vitro.

CHAPTER IV
INDUCTION OF DIFFERENTIATION OF EC CELLS
BY CONDITIONED MEDIA

In the preceeding chapter, it was shown that the formation of TROMA-1 positive cells resembling extraembryonic endoderm preceeded the formation of MF-20 positive (muscle) cells in DMSO-treated aggregates. These endodermal cells differentiated rapidly, and formed a sheath surrounding the aggregates. Subsequently, muscle cells were detected just below this sheath. I postulated, based upon the spatial and temporal relationship of endoderm and muscle, that the appearance of these endoderm-like cells was causally related to the differentiation of muscle in DMSO-treated aggregates.

One possible way in which endoderm could affect the formation of muscle beneath was if the endoderm produced a soluble factor(s) (a diffusible morphogen) which induced muscle differentiation. Therefore, I decided to try to identify such a soluble factor(s) released by extraembryonic endoderm. This chapter is concerned mainly with the findings of these experiments.

It should be noted that the experiments reported herein were repeated several times. The figures shown are from representative experiments, however, due to the variability between batches of CM. Where the results of more than one experiment are shown, the experiments were

done simultaneously with a single large batch of CM.

4.1 Conditioned Medium:

As a first step towards identifying a soluble factor(s) released by extraembryonic endoderm, monolayers of this cell type were produced by treating F9 EC cells with 10^{-7} M RA for one week (section 2.2). In this way, a monolayer of extraembryonic endoderm was formed (Strickland and Mahdavi, 1978). Following differentiation, the endodermal monolayer was washed twice with PBS, and drug-free, serum-containing medium was placed on the cells. After 2 days, this conditioned medium (CM) was placed on sparsely populated plates of P19 cells in monolayer (as described in Sect. 2.8). The CM was changed every 2 days, and within 6-7 days, the cells were found to have differentiated almost completely into healthy, epithelial-like cells which morphologically resembled extraembryonic endoderm (Fig. 4.1). The treated P19 cells soon became highly vacuolated, and died. It appeared that, indeed, a soluble factor(s) which induced P19 cell differentiation was being released into the CM by the F9 endoderm.

When stained in situ, the treated P19 cells were found to be positive for TROMA-1, but negative for TROMA-3, AEC3A1-9, and Dolichos biflorus agglutinin (DBA; Table 4.1). Since AEC3A1-9 stains a surface antigen present on the surface of undifferentiated EC cells, but not their

Figure 4.1: Photomicrographs of P19 monolayers after 7 days
(a) in CM and (b) in fresh medium. Note the
patch of endoderm²-like cells which form in open
areas of the untreated cultures (arrow).
Bar = 200 μ m.

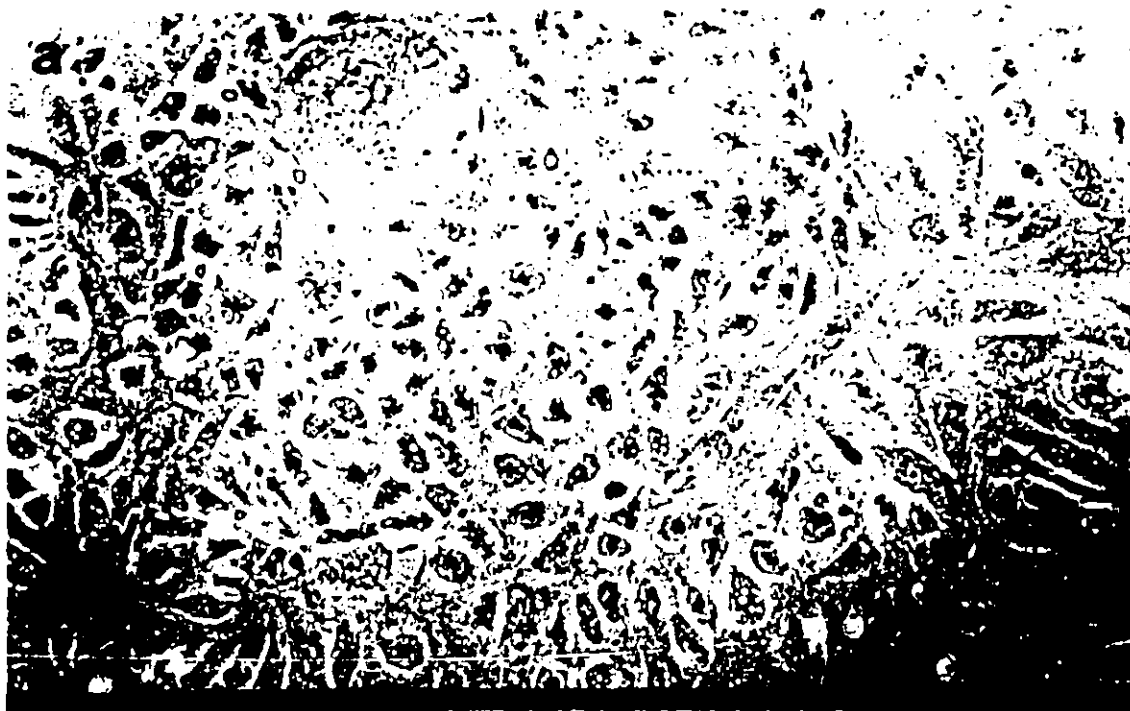


Table 4.1: In situ Staining of Cells With Antibodies and DBA.

<u>CELL LINE</u>	<u>STAIN</u>			
	<u>TROMA-1</u>	<u>TROMA-3</u>	<u>AEC 3A1-9</u>	<u>DBA</u>
cl 9	+	+	-	+
P19 (EC) ^a	- ^c	-	+	+
P19 (CM) ^b	+	-	-	- ^d

^a - P19 cells left without passage for 6 days.

^b - P19 cells treated with CM for 6 days.

^c - Some positive cells in patches; majority negative.

^d - Some bright patches seen in association with cells



differentiated derivatives (see Harris et al., 1984), the lack of staining with AEC3A1-9 confirmed that the cells had differentiated. Positive staining with TROMA-1 tended to suggest that the cells were extraembryonic endoderm. TROMA-1, however, stains a variety of (primarily) epithelial cell types in both adult and embryonic animals (see Silver et al., appendix, 1983; Boller and Kemler, 1983). TROMA-3 reacts with cytokeratin-like filaments in all the same tissues as TROMA-1, with a few exceptions. Notable among these is that TROMA-3 does not stain the intermediate filaments present in visceral extraembryonic endoderm (Boller and Kemler, 1983). This, in itself, would seem to indicate that the cells formed are composed of visceral extraembryonic endoderm. This seems to be contradicted by the lack of (or very weak) DBA staining, however. According to Noguchi and colleagues (1982), Dolichos biflorus agglutinin stains all types of endoderm, both embryonic and extraembryonic. The apparent absence of DBA receptors may be due to the highly flattened morphology of the cells, and the resultant dispersal of the receptors, but this is unclear. These cells may be primitive endoderm, as this cell type is recognized only weakly by DBA, or visceral endoderm, in which the distribution of DBA receptors has been described as patchy at best (Noguchi et al., 1982). In fact, the highly vacuolated appearance of the cells (Fig. 4.1) also seems to suggest that they are, indeed, visceral endoderm (see Hogan et al., 1981).

From the rapidity with which the tissue forms, the staining patterns with TROMAs-1 and -3, the morphology, and the fact that, to my knowledge, no other TROMA-1 positive cell type has been identified in P19 cultures, I have tentatively identified this as extraembryonic (possibly primitive or visceral) endoderm. While this identification is not conclusive, for simplicity the terminology has been maintained throughout.

4.2 Evidence for the Production of a Factor(s):

While it appeared that a factor was present in CM, it was distinctly possible that something other than a differentiation-inducing substance was causing the differentiation observed in P19 monolayers. It was possible (1) that CM was merely medium from which some component had been depleted, (2) that the F9 cells were somehow sequestering the RA used to induce them and were releasing it into the CM, or (3) that a pre-committed sub-population of P19 cells was being selected from the general population.

In order to test for the possibility that depletion was occurring, CM was diluted with varying amounts of fresh, serum-containing medium, and these mixtures were assayed for their ability to induce differentiation in P19 cells. Figure 4.2 shows the resulting dose response curve (see also Fig. 4.3). With increasing doses of CM, the proportion of TROMA-1 positive cells increased. The proportion of

Figure 4.2: Formation of TROMA-1 positive cells by P19 cells in response to ●—● CM and ○— — —○ PBS. Cells were grown for 8 days in the appropriate medium, were dispersed, plated on coverslips and stained. Note that all cells died in 100% PBS. Lines drawn by eye.

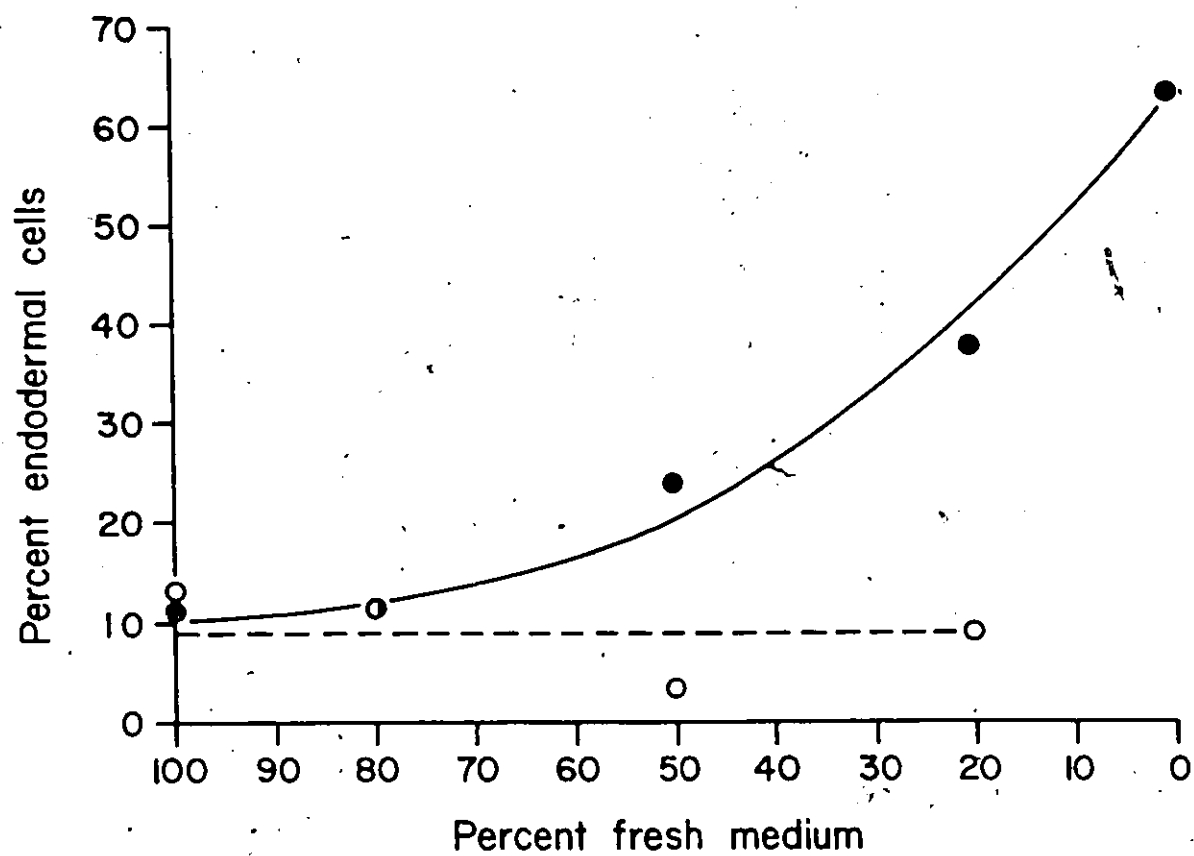


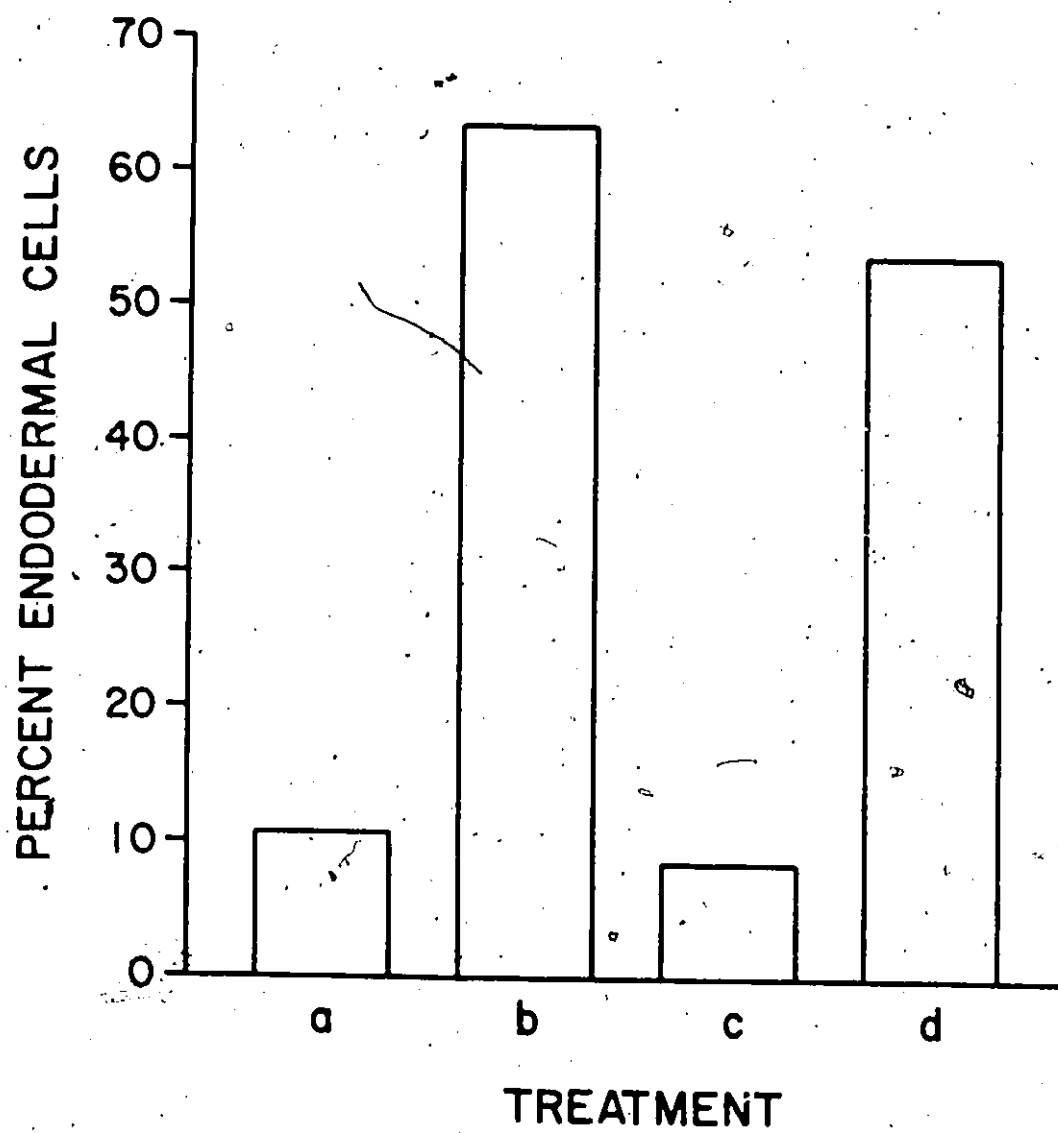
Figure 4.3: Photomicrographs of P19 cells grown for 9 days in (a) 0%, (b) 20%, (c) 50%, (d) 80%, and (e) 100% CM diluted with fresh medium. Note the patch of endoderm-like cells in the untreated culture (arrow). Bar = 200 μ m.



AEC3A1-9 (EC) cells present decreased with increasing doses of CM (data not shown). In the control experiment, fresh medium was diluted with varying amounts of PBS. This, presumably, would have the effect of diluting (depleting) the medium, but would have kept it isotonic. As seen in Figure 4.2, the differentiation into TROMA-1 positive cells at all dilutions was only equivalent to the background differentiation found in whole medium. To test (specifically) for the possibility of serum depletion in CM, P19 cells in monolayer cultures were grown in CM with 2.5% fetal calf and 7.5% calf sera added (Fig. 4.4). The amount of serum added was equivalent to the amount added to fresh medium, and was in addition to any serum remaining in the CM. As can be seen (Fig. 4.4) CM supplemented with 10% sera gave a level of differentiation approximately equal to 90% CM, or the dilution factor produced by the addition of the sera.

Since any deficiencies in the CM would presumably have been replenished by the added fresh medium, the first experiment argued against the possibility that medium depletion was responsible for the effects shown by CM. It did not completely exclude the possibility, however. Since fresh medium "wholly depleted" with PBS gave only background levels of differentiation, depletion of the medium could be ruled out as a cause of differentiation in CM. Since the addition of sera to CM did not interfere with differentiation-inducing potential of CM, serum

Figure 4.4: Proportion of TROMA-1 positive cells formed in P19 cell monolayers treated with (a) fresh medium, (b) CM, (c) fresh medium with 10^{-7} M RA, and (d) CM with 2.5% CS and 7.5% FCS added. All cells were grown for 6 days in the appropriate medium, dispersed, allowed to settle on coverslips, and stained.

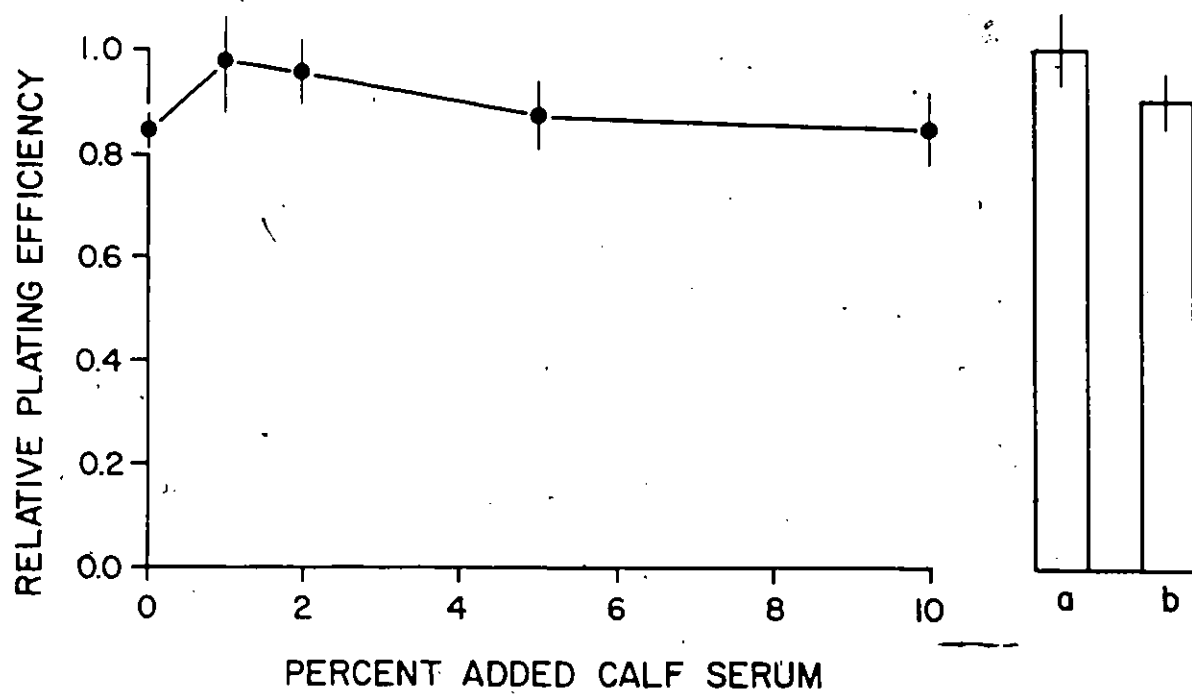


depletion could be specifically ruled out. It should be noted that a background level of 10% differentiation is rather high for these cells. This is a result of growing the cells without passage for 6-7 days. When P19 cells become overgrown, there is some spontaneous differentiation of the cells into endoderm in small pockets (see Fig. 4.3).

To test the possibility that RA was being released into CM by the conditioning cells, 10^{-7} M RA was added to fresh medium. This was the full dose used to induce the F9 cells to differentiate. The results of this are shown in Figure 4.4. Fresh medium supplemented with added RA gave a level of endodermal differentiation equivalent to background. That RA played no role in the differentiation observed in P19 monolayers is also supported by the fact that an effective CM can be formed by cl 9 cells (which had not been exposed to RA) in serum-free medium (which lacked even the trace retinoids found in serum). Therefore, neither residual RA nor depletion of the serum was responsible for the effects observed with CM.

To test for the possibility of the selection of a pre-committed sub-population of P19 cells, the plating efficiencies of P19 in fresh medium and in CM were checked. In addition, the plating efficiencies were determined with varying amounts of calf serum added to CM, in order to further check for serum depletion. It should be noted that, in this particular experiment, the test media were removed after 2 days and fresh medium was placed on all the plates.

Figure 4.5: Plating efficiencies of P19 exposed for 48 hours to CM with different amounts of added CS, (a) in fresh medium, and (b) in CM with 2.5% CS and 7.5% FCS added. All plating efficiencies are relative to (a) ($\pm$ SEM).



This was left until the resulting colonies were fixed and stained (day 7).

Figure 4.5 shows the plating efficiency of P19 cells exposed to CM and CM with added sera for 48 hours. There was little difference between the plating efficiencies in fresh medium, in CM with added sera, or in CM with different amounts of CS added. This, combined with the healthy appearance of the cells treated with CM (see Fig. 4.1), would seem to indicate that selection (at least within the first 48 hours) of a population of cells within P19 pre-committed to form extraembryonic endoderm was not responsible for the effects of CM. This was also further evidence that serum depletion was not implicated.

In addition to testing the plating efficiency of CM with different doses of CS added, the plating efficiency of P19 in different doses of CM was determined. In these experiments, however, the test medium was left on the cells for the entire course of the experiment (7 days). In this case (Fig. 4.6), the plating efficiency of P19 dropped dramatically in higher doses of CM. While, at first glance, this appears to contradict the results of the previous experiment, on closer examination it does not. In both cases, the colonies which did form were composed almost entirely of undifferentiated EC cells (Fig. 4.7a). Those cells which differentiated did not form visible (macroscopic) colonies, but only small (microscopic) colonies of a few differentiated cells (Fig. 4.7b).



Figure 4.6: Plating efficiencies of P19 cells in different amounts of CM diluted with fresh medium. All cells were maintained in the appropriate medium until scored (7 days). All plating efficiencies relative to 0% CM ($\pm$ SEM).

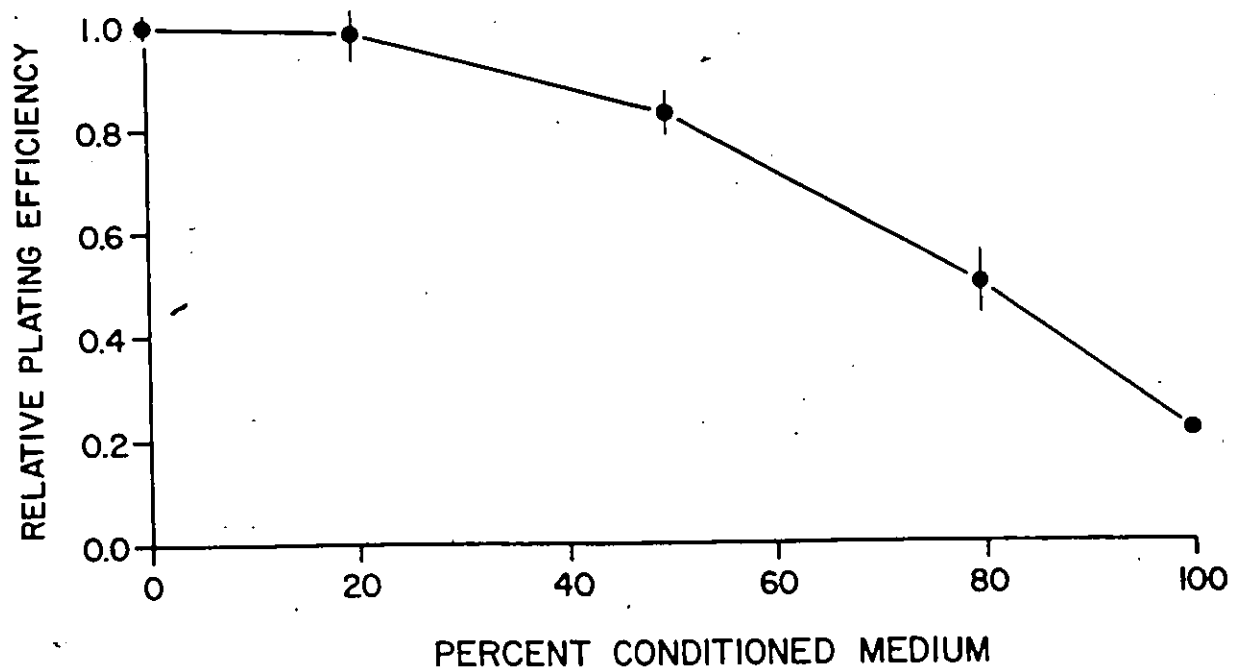


Figure 4.7: Photomicrographs of (a) macroscopic colony of EC cells and (b) microscopic differentiated colony of P19 cells, both grown in 100% CM. Bars = 100 μ m.

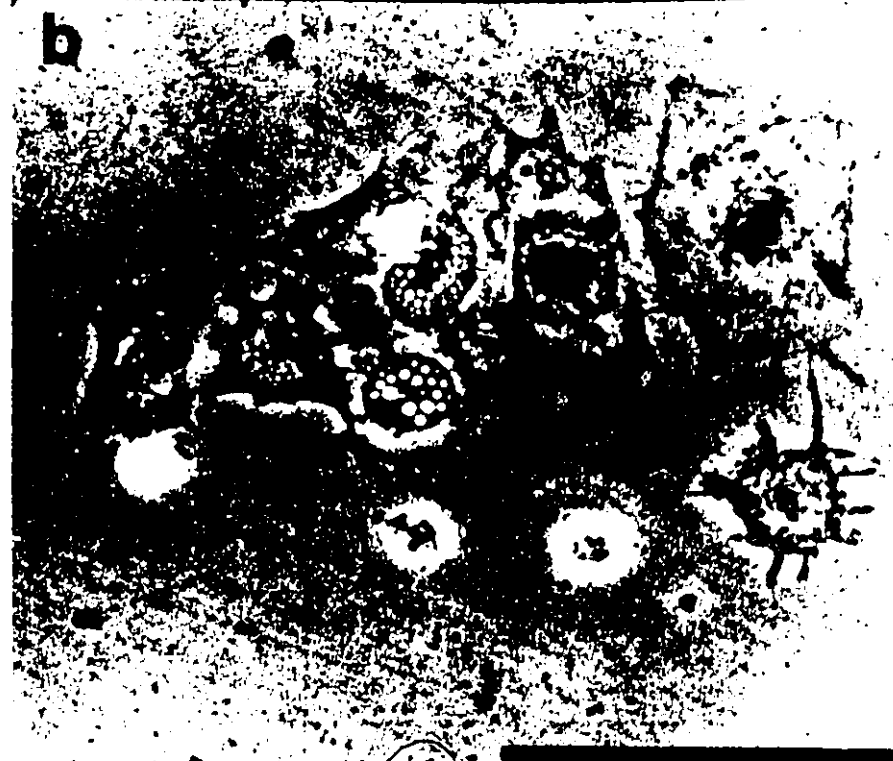
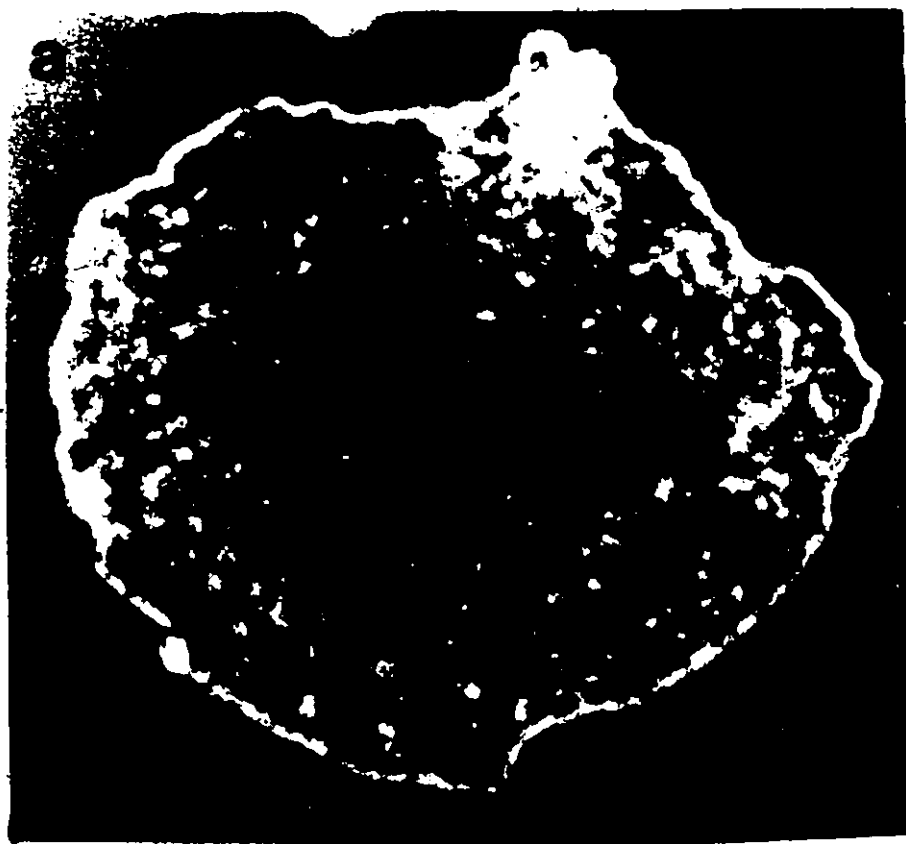
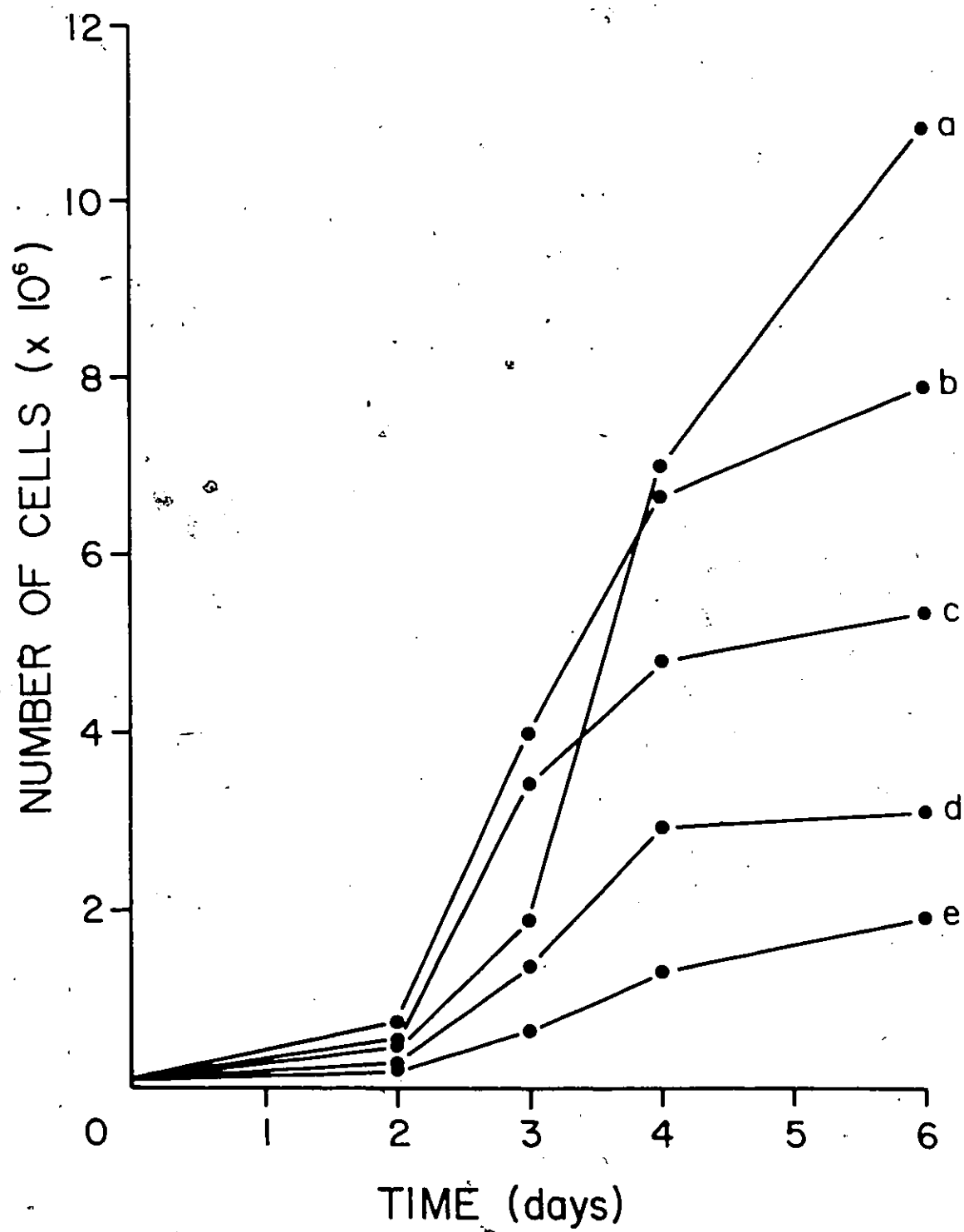


Figure 4.8: Growth of P19 cells in (a) 0%, (b) 20%, (c) 50%, (d) 80%, and (e) 100% DM.



4.3 Growth Rate of P19 in CM:

The growth rate of P19 was examined in different concentrations of CM. As can be seen (Fig. 4.8), the growth of P19 cells was greatly decreased in increasing doses of CM. This was particularly true after 3 or more days in CM. Even 20% CM decreased the growth of the cells. The population in control plates began to level off after 4 days, presumably due to overcrowding of the plates. Therefore, doubling times of the cells were calculated using linear regression lines based on the logarithm of the number of cells on days 2, 3, and 4. This avoided any differences due to plating efficiencies in the early phases of the experiments, and the saturation effect near the end. The results of this are shown in Table 4.2. As can be seen, the doubling times of the cells also decreased with increasing doses of CM.

While the decrease in plating efficiency in higher concentrations of CM may appear to indicate some sort of selective pressure which acts only after exposures longer than 48 hours, on closer inspection it seems to be due to the rapid differentiation of the cells. The fact that all the macroscopic colonies seen in CM were composed of EC cells seems to indicate that only those cells that were resistant to CM formed colonies. This resistance may be due to a) the presence of mutant cells which are resistant to CM, or b) a stochastic process whereby some clones become

Table 4.2: Doubling Times of P19 Cells in Different Doses of CM.

<u>Dose (% CM)^a</u>	<u>Doubling Time (hours)</u>
100	18
80	14
50	15
20	15
0	12

^a— CM diluted with fresh medium.

rapidly too dense to be affected by CM (see Section 4.6). The presence of microscopic colonies of a few differentiated cells may indicate that differentiation in most clones occurred before (macroscopic) colony formation could occur. Indeed, decreased colony formation has been used as a marker of differentiation, in that only undifferentiated cells form colonies (Rosenstrauss and Levine, 1979; Campione-Piccardo et al, 1985). Thus, the decrease in growth rate may be more a manifestation of the rapid differentiation-inducing effects of CM than of selection. This was also suggested by the reduced growth rate of P19 cells seen in increasing doses of CM (Table 4.2; Fig. 4.8). In addition, the supposition that selection is occurring assumes that a pre-committed population of cells exists in the P19 line. This is contradicted by the fact that P19 cells are clonally related and are capable of forming both mesodermal (striated muscular) and neurectodermal derivatives, depending on the inducer. Therefore, the existence of a pre-determined population of cells appears doubtful. These facts seem to preclude selection as a mechanism.

4.4 Use of cl 9 Cells to Condition Medium:

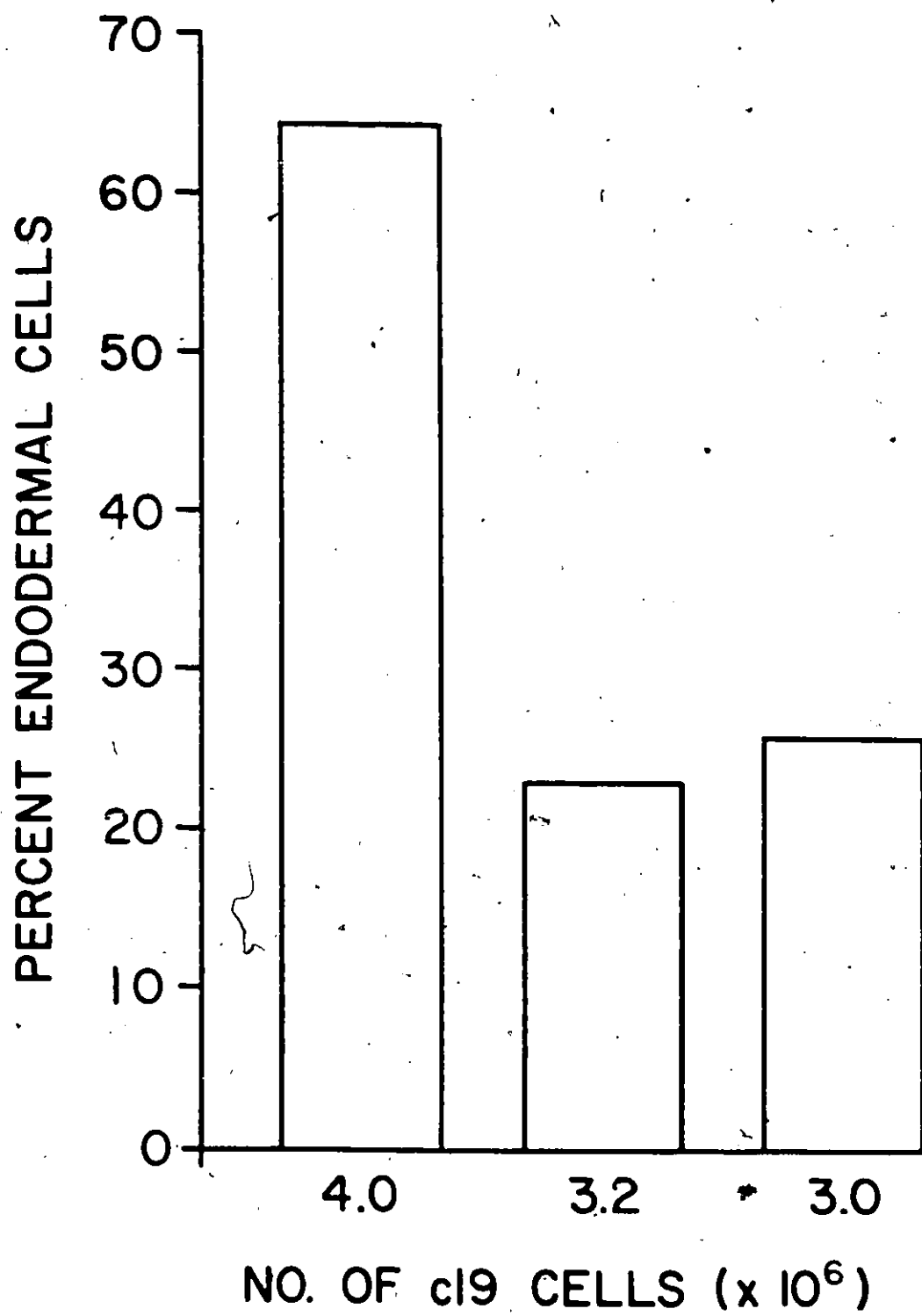
In order to increase its availability (and avoid the necessity of differentiating F9 cells every time), CM was made using a permanent line of parietal-like extraembryonic endoderm cells. This line, spontaneously transformed and

immunoselected from RA-treated F9 cells, (see Howe and Solter, 1981) is designated F9-AC clone 9 (F9-AC cl 9; herein designated cl 9). It was first necessary to test the CM produced by these cells. Therefore, different numbers of cl 9 cells were seeded into 100 mm dishes containing 15 ml of serum-containing medium. These CMs were then tested on P19 monolayers.

It appeared that CM from cl 9 cells did induce differentiation in P19 cells. They appeared to make a less effective CM than freshly made F9 endoderm. Indeed, while approximately $2.75 - 3 \times 10^4$ F9 endodermal cells per 100 mm dish formed a very efficient CM, it was found necessary to seed at least 4×10^4 cl 9 cells per 100 mm dish to form a reasonable CM. Even a slight reduction in the starting number of cells gave a relatively ineffective CM (Fig. 4.9).

Since cl 9 cells can grow in serum-free medium (SFM), whereas freshly made F9 endoderm died rapidly under these conditions, they became a valuable asset in experiments where SF-CM was required. Since the growth of cl 9 cells was greatly reduced in SFM, it was necessary to obtain a confluent culture before placing SFM on the cells. Both serum-containing (SC-CM) and serum-free conditioned media (SF-CM) from cl 9 cells were used in the subsequent experiments. Since storage was improved following heat inactivation, SF-CM was heat inactivated at 56°C for 10 min. before use.

Figure 4.9: Differentiation-inducing effectiveness of CM produced by seeding different numbers of cl 9 cells per 100 mm dish with 15 ml. of medium. Percent of TROMA-1 positive cells produced when these media were assayed on P19 cells in monolayer culture.



4.5 Range of Cell Types Producing the Factor:

When the effects of CM from F9 endoderm were discovered, undifferentiated F9 cells were checked for the production of the factor(s) present in CM. By seeding 2.75×10^4 cells / 100 mm dish, a CM produced by F9 EC cells was produced. This had no visible effect on P19 cells in monolayer (data not shown). When it was discovered that c19 cells needed to be plated at higher density, however, F9 EC cells were again tested by plating 4×10^4 cells / 100 mm dish. In addition, P19 EC cells, and 3T3 fibroblasts were also tested for the ability to make CM at this density (Table 4.3). A differentiation-inducing CM was produced by P19 and F9 EC cells, but not by 3T3 cells. The 3T3 cells did appear to retard the growth of P19 cells slightly (possibly due to the factor described by Okai, 1984), but the P19 cells remained morphologically undifferentiated. A control plate of 15 ml of medium which was incubated for two days without any cells showed the same effect as fresh medium.

Whether the substance(s) produced by F9 and P19 EC cells are the same as those produced by extraembryonic endoderm has not been ascertained. The differentiation-inducing effects appear to be identical, however.

Table 4.3: Production of CM by Different Cell Lines.

<u>CELL LINE</u>		<u>Differentiation of</u> <u>P19 Monolayer</u>
F9 (endoderm) ^b		+
c1 9	=	+
F9 (EC)	=	+
P19 (EC)	=	+
3T3	=, ^d	-

- - P19 monolayers scored visually for the presence of a monolayer of endoderm (see Fig. 4.1) after 6 days in CM.
- ^b - Approximately 2.75×10^6 cells seeded per 100 mm dish.
- = - 4×10^6 cells seeded per 100 mm dish.
- ^d - Some slowing of growth observed, but no visible differentiation.

Figure 4.10: Photomicrographs of P19 in CM; densely packed area (left column) versus sparsely packed area (right column) of the same dish. Shown on days 1 (a, b), 2 (c, d), 5 (e, f), 6 (g, h), and 7 (i, j). Note the patches of EC cells remaining in crowded areas after 7 days in CM (arrows). Bar = 200 μ m.

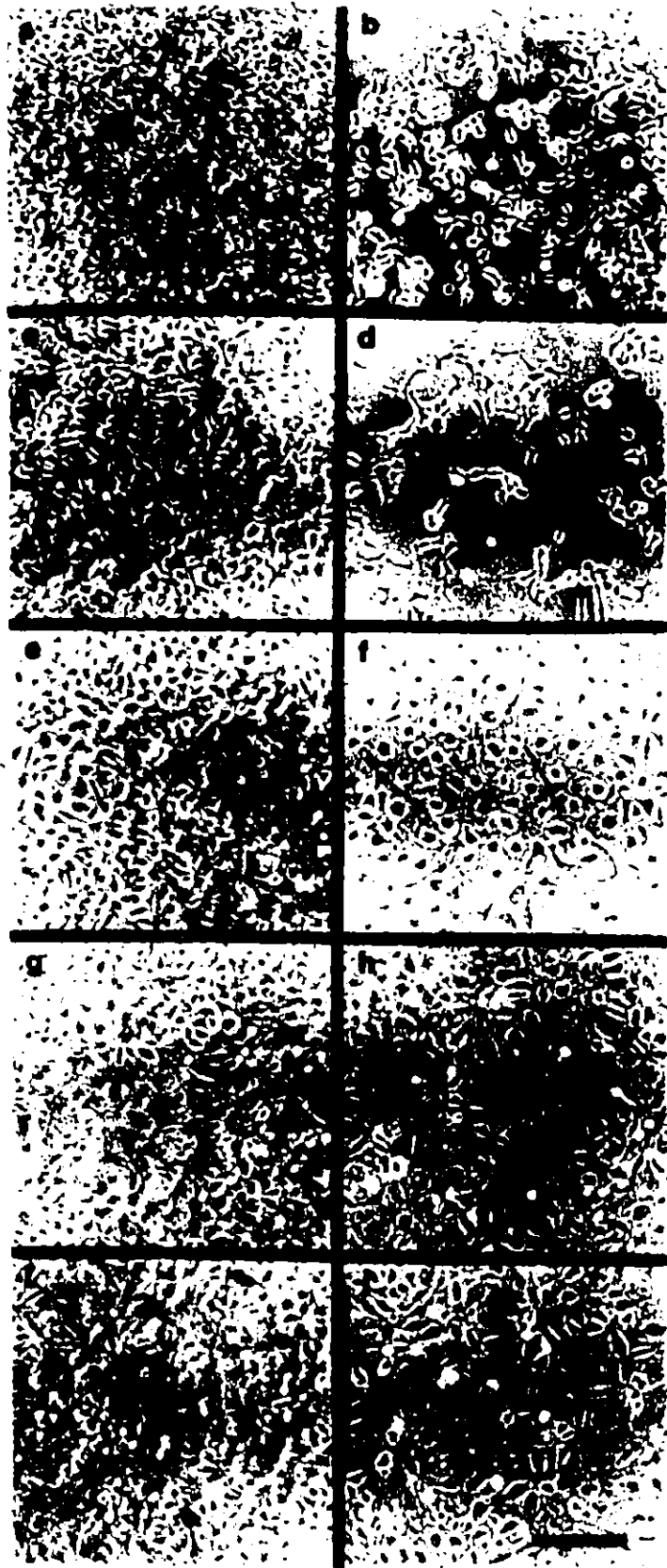
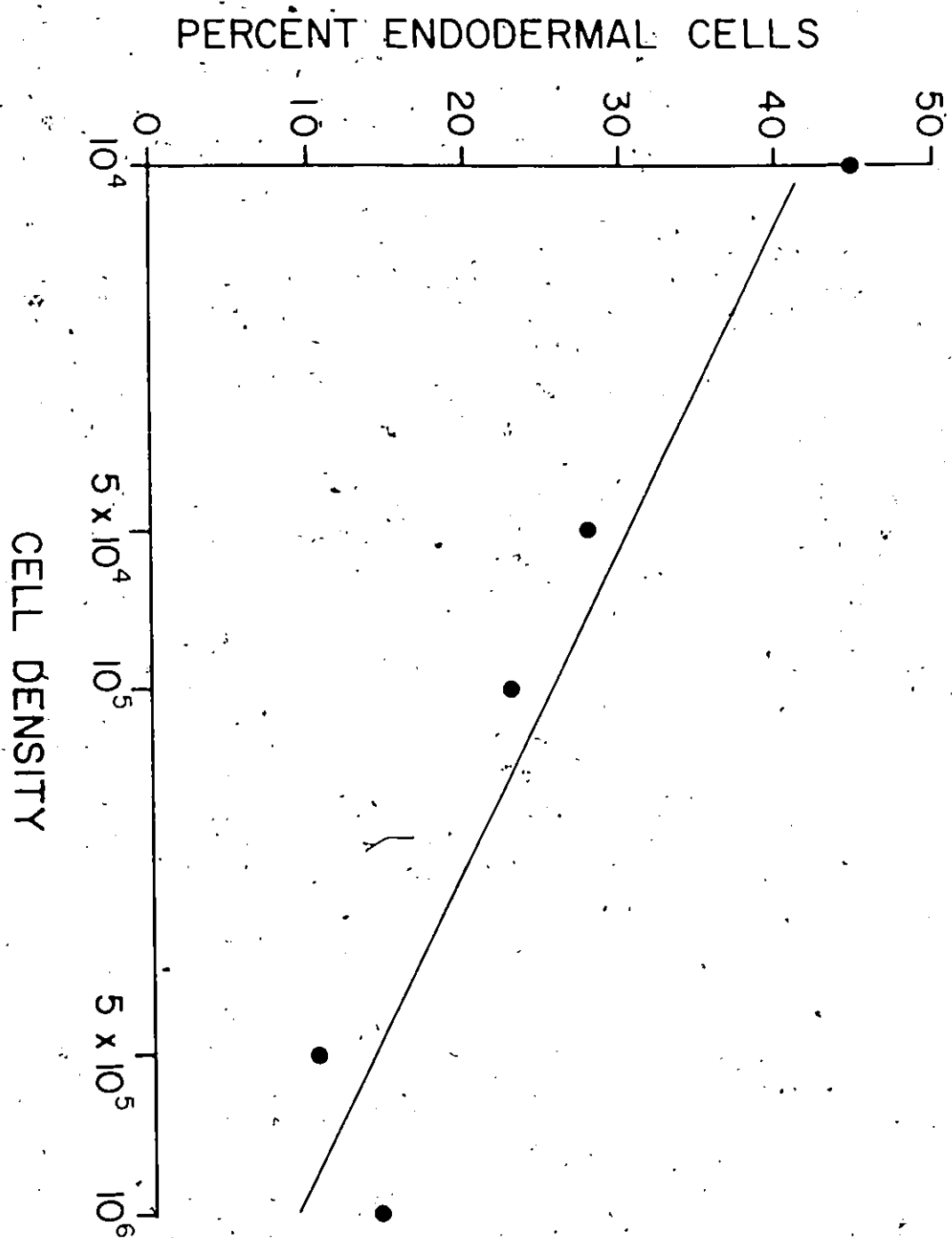


Figure 4.11: Density dependence of induction of P19 cell differentiation by CM. Proportion of TROMA-1 positive cells formed after 6 days in CM as a function of the number of P19 cells seeded per 60 mm plate. Linear regression.



4.6 Density Dependence of the Effect:

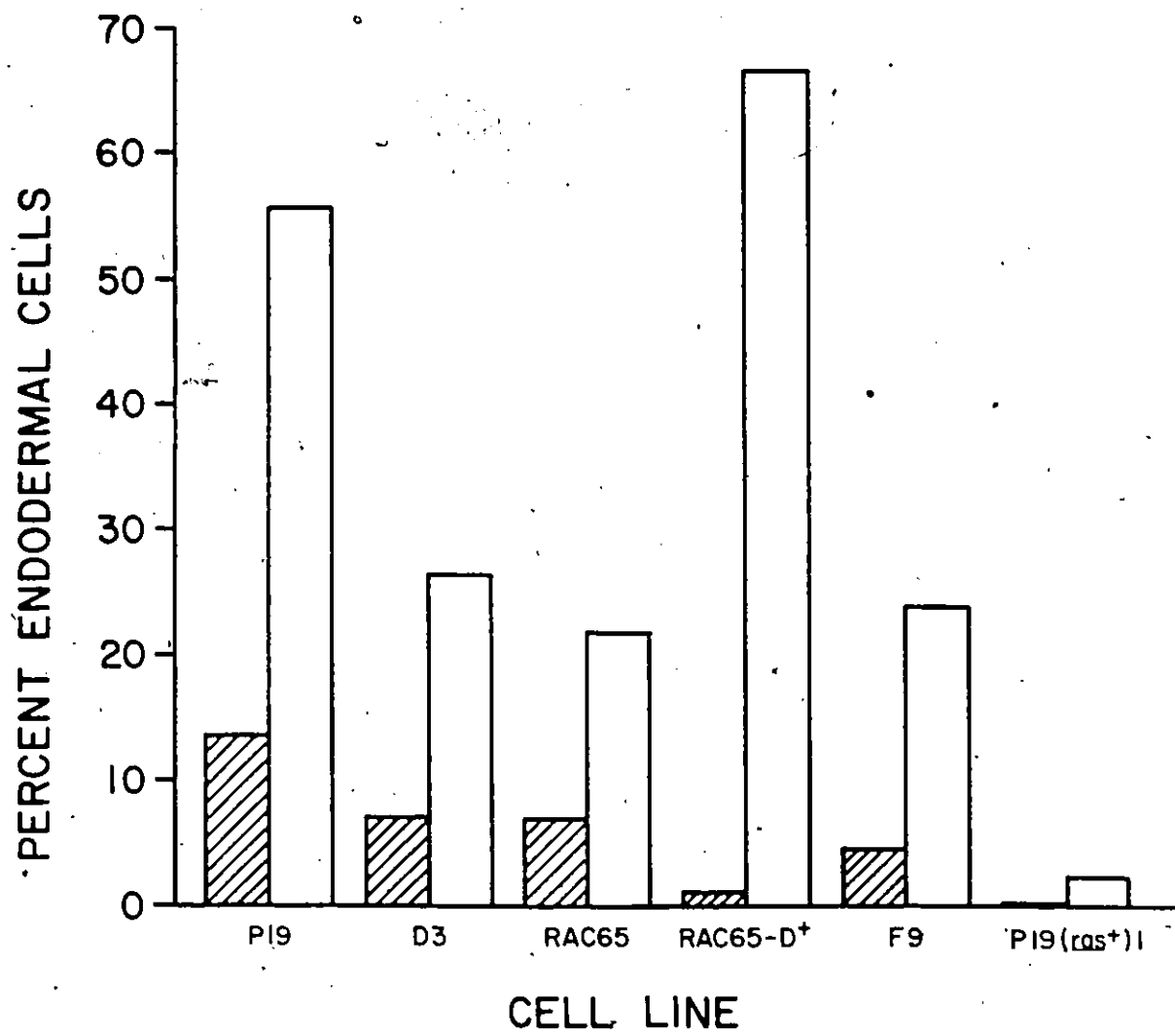
During the course of other experiments, it was noted that sparsely populated areas of the culture dishes appeared to differentiate faster and more effectively than more densely populated areas of these same dishes (Fig. 4.10). It appeared that perhaps the differentiation induced by CM was dependent upon cell density. In order to test this, different numbers of P19 EC cells were plated into CM (Fig. 4.11). A definite decrease in the proportion of endodermal cells produced was seen as the cell density increased.

This could, indeed, be the result of the inhibition of differentiation observed in dense cultures. Alternatively, this result could be explained by a requirement for a threshold amount of morphogen per cell. At higher densities, less morphogen would be present per cell, and fewer cells would subsequently differentiate. Since a difference in both the rate and amount of differentiation was seen between crowded and sparse areas of the same dishes, however, it would appear that, although only a limited amount of morphogen may be present (see Fig. 4.9), there is a prohibitive effect in overcrowded areas.

4.7 Range of Cell Types Affected:

In addition to verifying the effects of CM on P19 cells, several other cell lines were tested in CM. The cell

Figure 4.12: Amount of differentiation in different cell lines treated for 6 days with CM versus parallel untreated cultures (hatched areas). 10^5 cells seeded per plate.



lines used have been described previously (see Table 2.1).

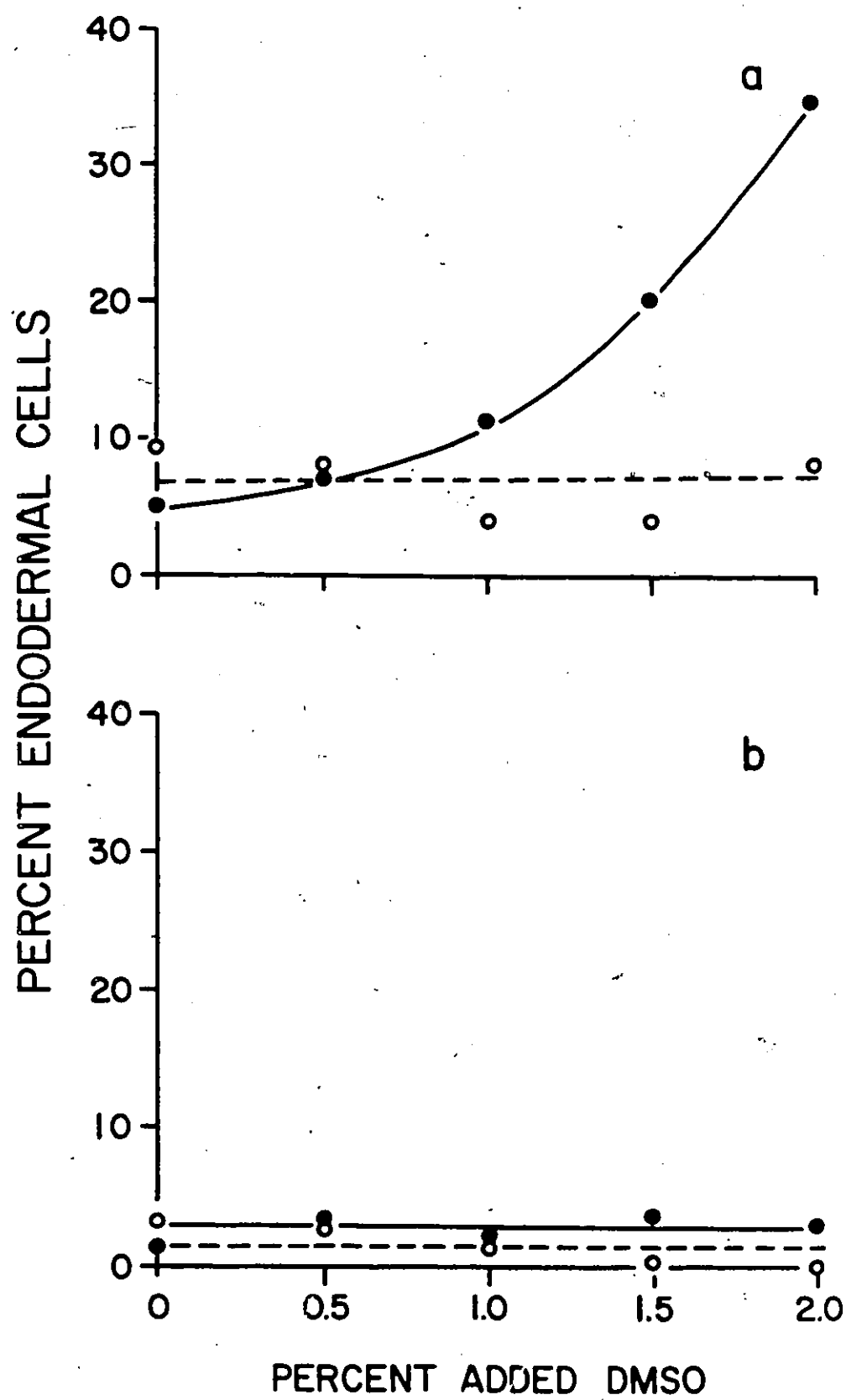
As can be seen in Fig. 4.12, D3 (DMSO-resistant), RAC65 (RA-resistant), and F9 cells were less efficiently induced to differentiate into endoderm. P19(ras⁺)1 (a line of P19 transfected with the Ha-ras oncogene) showed even less differentiation into TROMA-1 positive cells. In fact, F9 appeared to remain largely undifferentiated, while D3 and RAC65 appeared to form flattened fibroblast-like cells which were not TROMA-1 positive. P19(ras⁺)1 appeared to form predominantly the same elongated fibroblast-like cells seen when this line is treated with RA (Bell et al., 1986). Since differentiation into TROMA-1 positive cells was the effect of interest, however, these responses were not pursued.

The most notable effect was shown by RAC65-D⁺. This line is a spontaneous partial revertant of RAC65 which makes large amounts of cardiac muscle when treated with 1% DMSO, but will not form neurons in high doses of RA (unpublished observation). In CM, RAC65-D⁺ showed higher levels of differentiation into endoderm than P19. Therefore, the ability to differentiate into TROMA-1 positive cells in response to CM appeared to be linked to the ability to form muscle in response to DMSO.

4.8 Synergistic Effect of CM and Other Inducers:

Given the original working hypothesis, that the formation of endoderm around the exterior of DMSO-treated

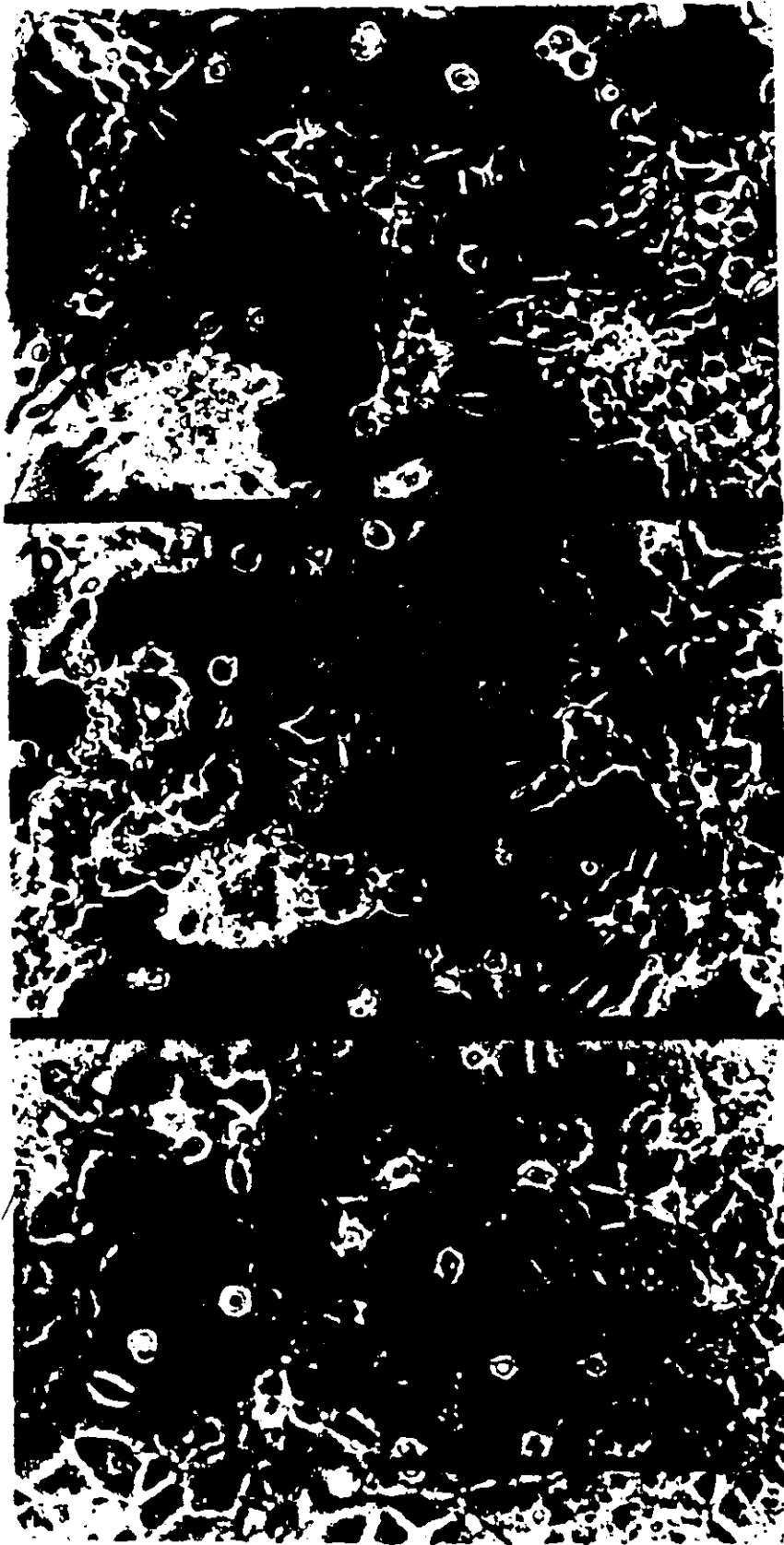
Figure 4.13: Effect of CM and DMSO on F19 cells. Proportion of TROMA-1 cells produced after 6 days (a) in CM + 1% DMSO and (b) in 1% DMSO alone on F19 cells (●—●) and on D3 cells (○—○). Lines drawn by eye.



aggregates is somehow implicated in the differentiation of muscle beneath, and that those cell lines which are capable of responding to DMSO with muscle differentiation also respond to CM, it seemed plausible that DMSO might influence the effect of CM. In order to test this, P19 and D3 cultures were treated with CM in combination with different doses of DMSO. The CM (produced by using fewer cl 9 cells to condition the medium) had little differentiation-inducing activity. This was used in order to enhance any effects produced by DMSO. DMSO enhanced the differentiation-inducing activity of CM on P19 cells (Fig. 4.13a). D3 cells remained unaffected by increasing DMSO concentrations. As well, DMSO alone had no effect on either cell line (Fig. 4.13b). Only in combination with CM did DMSO show any effect, and then only on P19 cells.

In light of the synergistic effect observed when CM was combined with DMSO, a combination of RA and CM was tested on P19, D3, and RAC65 cells. Again, a low dose of CM was used, and RA was tested in concentrations ranging from 5×10^{-9} M to 5×10^{-7} M. Both P19 and D3 produced large numbers of neurons (and other cell types) at all the concentrations of RA tested (data not shown). RA-treated P19 cells in monolayer cultures will normally form a few rare neurons at the two highest RA concentrations, but not to the extent seen in these cultures. Usually, only fibroblast-like cells are seen (Jones-Villeneuve et al, 1982), and often, most of the cells appear to remain

Figure 4.14: Photomicrographs of (a) F19, (b) D3, and (c) RAC65 cells in monolayer, treated with CM + RA. (a) and (b) in 5×10^{-7} M RA, and (c) in 10^{-7} M RA. Bar = 20 μ m.



undifferentiated. D3 cells react similarly to F19 when treated in monolayer. Interestingly, RAC65 cells exposed to both CM and RA showed some differentiation into neurons. These were relatively rare in 5×10^{-7} M, but the number appeared to increase as the RA concentration was raised (see Fig. 4.14).

Therefore, it appears that the morphogen present in CM is capable of acting synergistically with the other inducers commonly used in this system. DMSO appeared to enhance the effect of CM on F19 cells, and CM, in turn, appeared to enhance the effect of RA on all three cell lines.

As a final note, when aggregates of RAC65 were treated with different amounts of a high dose of CM diluted with fresh medium, neurons were formed in the two highest concentrations (80 and 100% CM). In 20 and 50% CM, all the cells remained undifferentiated. F19 aggregates formed neurons in all concentrations of CM tested, but remained undifferentiated in fresh medium. The effects of aggregation in CM seemed to be quite variable, however. With other batches of CM, little differentiation of F19 was observed. Due to the variability of the batches of CM, purification of the factor involved may be necessary before its effects in conjunction with aggregation can be fully discerned.

4.9 Effect of CM on Transformed Cells:

As indicated in an earlier section (1.3), cancer can be viewed as a problem of development. The study of EC cell differentiation is especially pertinent to this because, whereas the undifferentiated cells are malignant, the differentiated cell types are, in fact, benign. Therefore, it is of particular interest in the case of an inducing agent (such as CM), to see if this inducing agent will also inhibit the growth of transformed cells. Two measures of growth were checked on the transformed cell lines cultured in CM. The cell lines used in this study were 3 RAP-1, RASA 1-1-2, and RASA 4363. 3 RAP-1 is an immortal but anchorage dependent line of fibroblast-like cells derived from RA-treated P19 cells. RASA 1-1-2 and RASA 4363 were derived from RA-treated P19(*ras*⁺)1 and P19(*ras*⁺)4 cells, respectively (Bell *et al.*, 1986). These cells are both immortal and anchorage independent.

Plating efficiencies of these cell lines (Fig. 4.15) decreased with increasing doses of CM. As well, the colonies that did form were smaller in higher doses of CM (data not shown).

As a second measure of growth, the growth of parallel cultures of these cell types was monitored over a period of 3 days. The growth of the three lines was reduced by even small amounts of CM in the medium (Fig. 4.16).

These cells all appeared healthy, even though their

Figure 4.15: Plating efficiencies of 3 RAP-1 (●—●), RASA 1-1-2 (○- - -○), and RASA 4363 (△-----△) in different doses of CM diluted with fresh medium. All plating efficiencies are relative to the plating efficiency of that line in 0% CM ($\pm$ SEM).

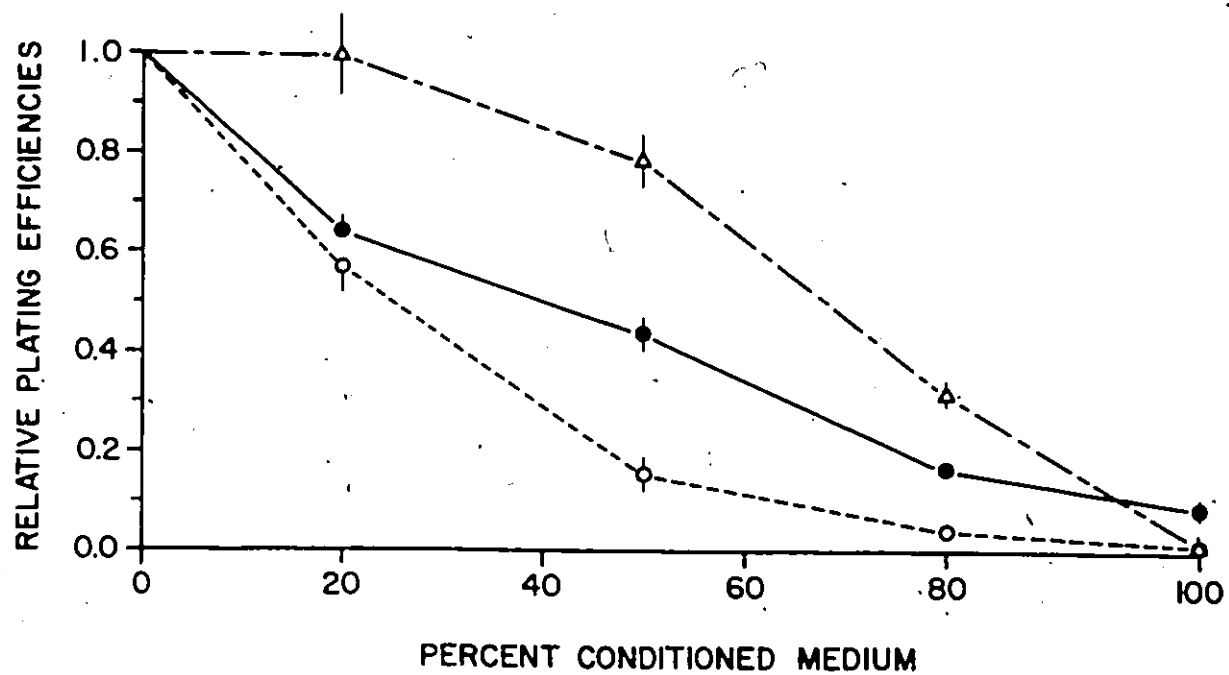
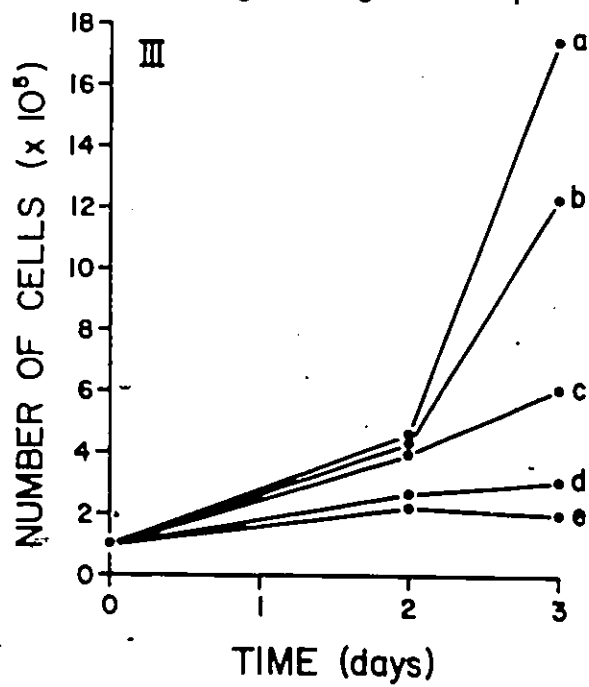
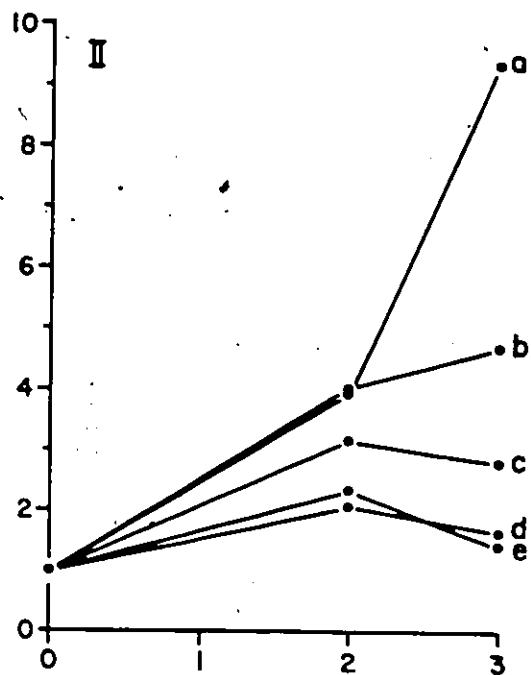
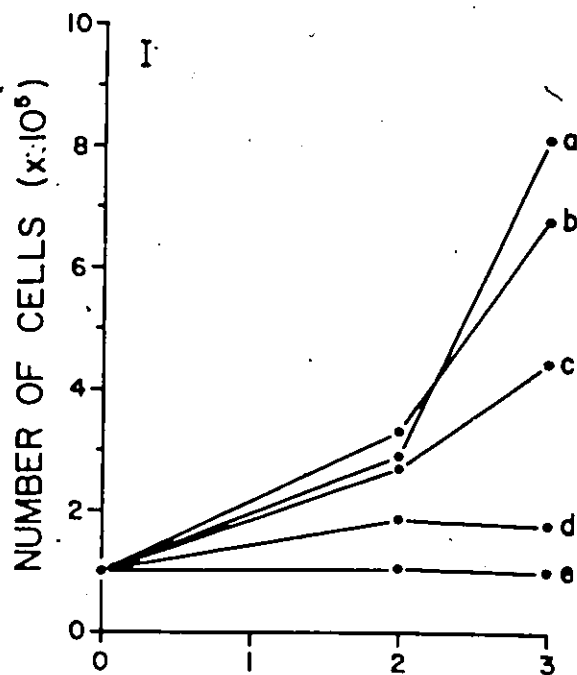


Figure 4.16: Growth of 3 RAP-1 (I), RASA 4363 (II), and RASA 1-1-2 (III) in (a) 0%, (b) 20%, (c) 50%, (d) 80%, and (e) 100% CM diluted with fresh medium.



growth was severely restricted. No signs of morphologically recognizable differentiation was seen in the cells. Therefore, it appears that CM not only induces the differentiation of pluripotent EC cells, but it also dramatically restricts the growth of at least 3 other cell lines, of which two are transformed.

4.10 Partial Characterization of the Factor in CM:

To initiate the characterization of the factor(s) responsible for the apparent induction of differentiation of P19 cells, three approaches were used. This consisted of i) an attempt to heat inactivate the factor, ii) an attempt to inactivate the factor with enzymes, and iii) a partial determination of the size range of the factor through positive pressure ultrafiltration.

Heat inactivation of the factor present in CM was performed by incubating SC-CM from cl 9 cells at 100°C for various periods of time (Fig. 4.17a). A drastic drop in the potency of CM was observed after treatment for 10 minutes.

This seemed to indicate that the factor could, indeed, be inactivated in this manner. When SF-CM was tested at 100°C, however, it was noted that inactivation by boiling of the factor was not possible (Fig. 4.17b). Even 30 min. at 100°C would not inactivate the factor.

Inactivation attempts at 56°C for up to 30 min. failed to inactivate SC-CM (data not shown). Serum-free CM was also stable at 56°C for up to 30 min. (Fig. 4.17c). In

Figure 4.17: Heat inactivation of (a) SC-CM at 100°C, and SF-CM at (b) 100°C and (c) 56°C. All media assayed on monolayer cultures of P19 cells for 6 days.

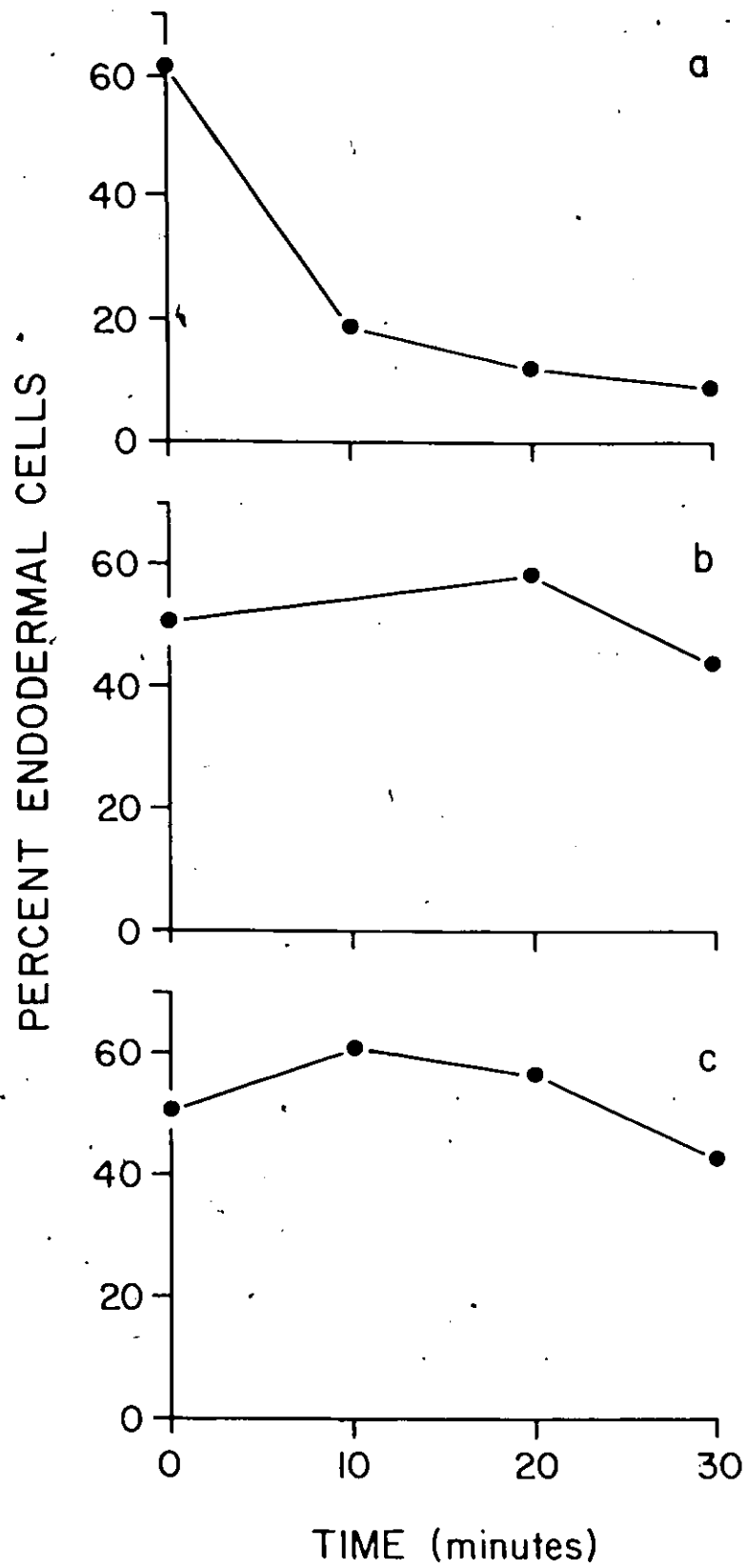
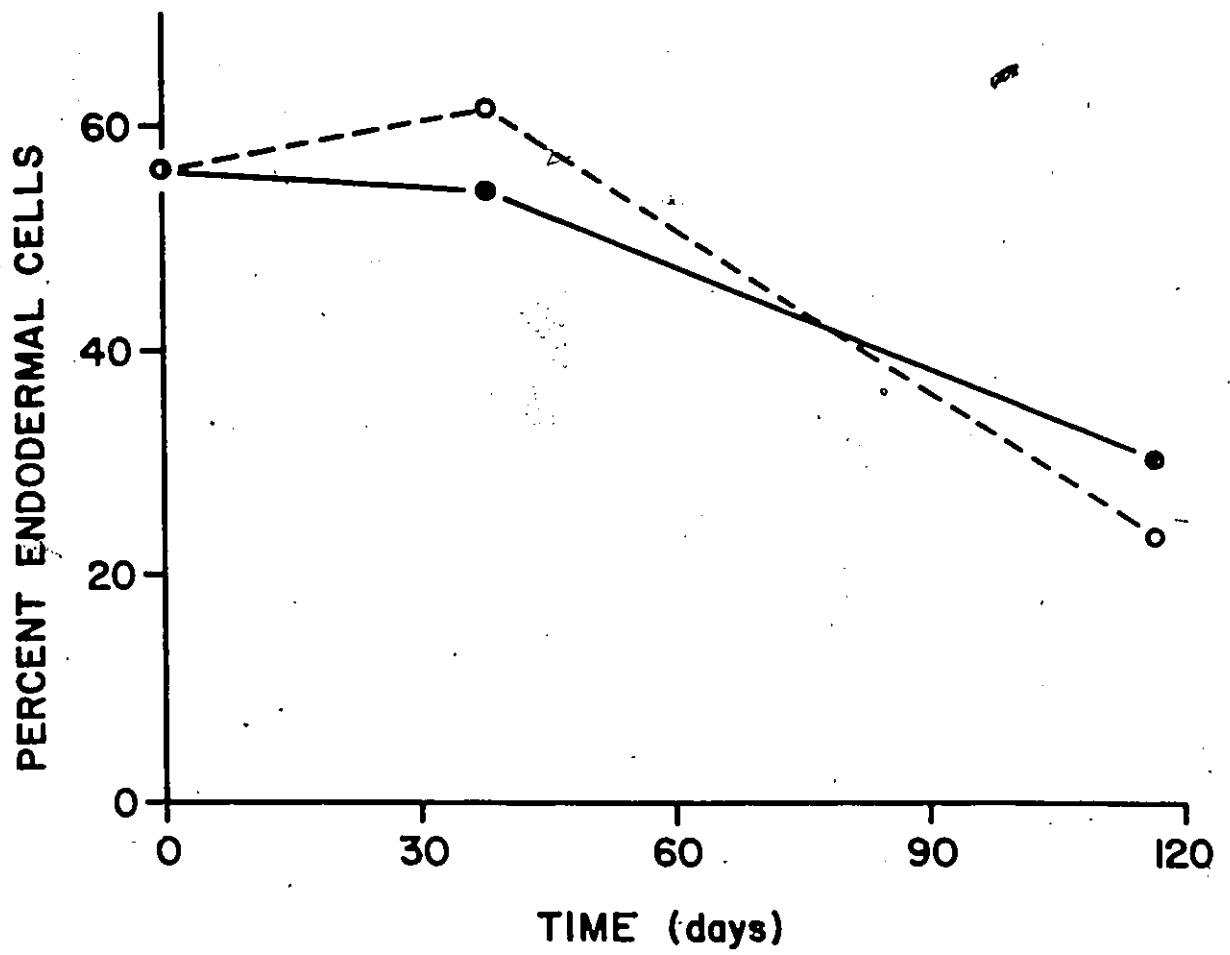


Figure 4.18: Differentiation-inducing efficiency of CM following storage at 4°C (●—●) and -20°C (○—○). Media assayed on F19 cells in monolayer cultures for 6 days.



fact, heat treatment of SF-CM at 56°C seemed to improve the storage stability of the CM (data not shown).

It should be noted that when SC-CM was incubated in a boiling water bath, a great amount of turbidity was seen in the medium. This, presumably, was due to the denaturation of the serum proteins present in the medium. No such flocculent precipitate was seen when SF-CM was treated in a similar manner.

In addition, it was found that the activity of SC-CM was stable for at least one month at 4° or -20° (Fig. 4.18). Some degradation did occur when the medium was stored for 4 months at either temperature. SF-CM lost its differentiation-inducing activity very quickly when stored at either 4° or -20°C. This loss of activity could be retarded by the addition of serum and/or by heat treating the CM at 56°C for 10 minutes (data not shown).

Some other characteristics of CM were observed during the course of these experiments. During attempts to find a means to sterilize CM, it was noted that the active factor present in serum-containing-CM from cl 9 would not pass through a Nalgene filter unit with a pore size of 0.45um (data not shown). It was believed that this was due to a) the factor being very large or b) the factor adsorbing onto the filter. When serum-free-CM was assayed after passage through a filter, however, it was found that none of its potency was lost (data not shown).

The observations that i) SC-CM (but not SF-CM) could

be inactivated by boiling, ii) that SF-CM (but not SC-CM) could be filtered without loss of activity, and iii) that loss of activity upon storage could be retarded by the addition of serum to SF-CM suggest that, in fact, the differentiation-inducing factor(s) present in CM is associated with some component of serum. This serum component would be greatly removed from serum upon filtration, and would precipitate out of boiled serum. In addition, serum may either inactivate a factor-degrading substance also present in SF-CM, or it may protect the factor from the action of this hypothesized substance. If such a substance is present in serum, it does not inhibit the action of the CM factor, and may suggest a way of partially purifying this factor.

As a further attempt at characterization, inactivation of CM was attempted using enzymes. Aliquots of single batches of SF-CM were incubated overnight at 37°C. One aliquot remained untreated, one contained 0.05% (w/v) trypsin, and one contained 0.01% (w/v) staphylococcal (micrococcal) nuclease. Following incubation, 0.05% soybean trypsin inhibitor was added to the trypsin-containing aliquot, and each of the aliquots was made 7.5% with CS and 2.5% with Clex. Parallel aliquots of fresh medium were treated identically to this and served as controls. The addition of trypsin inhibitor to those media containing trypsin was essential, as even with the addition of 10% sera, the potency of the trypsin was such that all cells



Table 4.4: Attempted Enzymal Inactivation of SF-CM.

<u>TREATMENT</u>	<u>PERCENT ENDODERMAL CELLS</u>	
	<u>FRESH MEDIUM</u>	<u>CM</u>
---	12.1	47.0
Trypsin b.c	14.2	48.2
Staphylococcal Nuclease b.d	11.8	40.6

▲ - Percent endodermal cells from immunofluorescent cell counts with TROMA-1.

♢ - Treated at 37°C overnight.

c - 50 mg/ml.

d - 10 mg/ml.

Table 4.5: Ultrafiltration of CM.

<u>MEDIUM</u>	<u>DIFFERENTIATION OF P19 MONOLAYER</u>
FRESH	-
WHOLE CM	+
CM FILTRATE [▯]	+
CM RETENTATE [▯]	+

- - Cells scored visually for the presence of a monolayer of endoderm (see Fig. 4.1) after 6 days in CM.
- ▯ - Fractions after passage of 90% of medium through an ultrafilter with a 10 Kd cutoff.

were rapidly killed without addition of the inhibitor.

Neither exhaustive tryptic digestion nor digestion with staphylococcal nuclease had any effect on the activity of CM (Table 4.4). This suggests that the active factor present in CM is neither a trypsin-sensitive protein nor a free nucleic acid.

As a final step in the initial characterization of the morphogen present in CM, a partial determination of its size was done using positive pressure ultrafiltration. Using a filter with a nominal molecular weight cutoff point of 10Kd, pressurized nitrogen was used to pass at least 90% of the SF-CM through the filter. The filtrate, the retentate, and unfiltered CM retained from the original batch all had equal differentiation-inducing activity (Table 4.5), suggesting that the factor had a molecular weight of less than 10K.

CHAPTER VDISCUSSION AND CONCLUSIONS

In DMSO-treated aggregates an endodermal sheath is formed prior to the appearance of and immediately above the muscle cells forming beneath. Based on this observation, I speculated that this endodermal sheath might somehow be at least partly responsible for the formation of muscle.

In addition to the production of a soluble factor by this endoderm (which was subsequently discovered), two other possibilities were raised. These were that a) there was a restriction of diffusion by the endodermal cells, thereby preventing the passage of something normally present in the medium, and b) that the differentiation beneath the endoderm was somehow induced by a basement lamina formed by this endoderm. Both of these possibilities are suggested by the findings of other workers (see Sect. 3.4).

In fact, neither of the alternative possibilities were examined in this study and their contribution(s), if any, to the formation of muscle remain unknown. It is important to note, however, that none of the three possibilities mentioned (diffusible morphogen, restriction of diffusion, and basement membrane formation) can be regarded as mutually exclusive. Indeed, it may be one or any combination of these factors which induces muscle formation in DMSO-treated aggregates. As well, it may be that the

presence of DMSO is absolutely required for the formation of muscle, even though it does not seem to be required for the prior differentiation of endoderm. Since endoderm may form without DMSO, if some product(s) of endoderm can induce muscle differentiation, DMSO may not be required.

I have found that media conditioned by extraembryonic endoderm contain a differentiation-inducing substance (or substances) which induces the differentiation of pluripotent P19 cells into a cell type morphologically resembling extraembryonic endoderm. The differentiation-inducing effects of CM were not due to depletion of either medium or serum, or to residual RA, and were probably not due to the selection of a subpopulation of P19 cells. CM also decreased both the growth rate and the plating efficiency of P19 cells. In addition, CM decreased the growth rate of three transformed cell lines (without noticeably affecting their morphology).

The factor(s) are produced by densely populated cultures of cl 9 cells, P19 and F9 EC cells, and RA-treated F9 cells. The production of this factor is dependent on the number of cells used to condition the medium. A reduction in the number of cells caused a dramatic reduction in the effectiveness of the CM. The differentiation-inducing effect of CM was enhanced by the presence of DMSO. The effect of RA was, in turn, enhanced by the presence of CM.

The characterization of the active factor, though incomplete, revealed a) that the factor(s) had an apparent

molecular weight of less than 10 000, b) that the factor was probably neither a protein nor a nucleic acid, and c) that the active factor(s) present in CM may be associated with one or more components of serum.

At this juncture, the question arises as to what the morphogen in CM is. A variety of substances are known to be secreted by extraembryonic endoderm. These include collagen and a variety of large glycoproteins from parietal endoderm, mostly as components of Reichert's membrane (Clark et al., 1975; Jetten et al., 1979; Howe and Solter, 1980; Hogan, 1980; Hogan et al., 1982) as well as plasminogen activator (Strickland and Mahdavi, 1978; Solter et al., 1979). Visceral endoderm is known to secrete alpha fetoprotein and transferrin and a thin basement membrane (Dziadek and Adamson, 1978; Hogan, 1980; Hogan et al., 1981). All of the reported substances are far too large to have been the factor in CM. As well, most are proteins or glycoproteins, and would have presumably been degraded by trypsin and/or denatured by boiling.

EC cells are also known to secrete a variety of substances, including the so-called "F9 antigen" (McCormick et al., 1982), uvomorulin (Vestweber and Kemler, 1984), and a recently described embryonal carcinoma-derived growth factor (ECDGF; Heath and Isacke, 1984). None of these compounds appear to be candidates for the active factor(s) of CM for several reasons: 1) all of these substances are too large, ranging from 17.5 Kd for ECDGF to on the order

of 80 Kd for uvomorulin and the F9 antigen, 2) all are at least partly proteinaceous, and 3) all are reportedly secreted only by the undifferentiated cells and not by their differentiated derivatives. Finally, none appear to have effects similar to those reported herein for CM. Therefore, none of the substances known to be secreted by either extraembryonic endoderm or EC cells appear to be candidates for the differentiation-inducing factor(s) observed in CM.

One reported study does describe proteoglycans secreted by both F9 EC cells and their RA-treated derivatives (Kapoor and Prehm, 1983). One of the molecules studied (PGM-2) was completely shed into the medium by both differentiated and undifferentiated cells. The amount secreted, however, increased at least 3-fold upon differentiation. This molecule, which consisted of a large protein core with several covalently linked oligosaccharide chains, was much larger than the 30 Kd ultrafiltration cutoff used to concentrate their CM, however. Although it was not studied by Kapoor and Prehm (1983), the free sulphated glycosaminoglycan (GAG) which composed most of the attached sugar had an approximate molecular weight of 10-12 Kd. If it can exist in the free form, this GAG, which reportedly resembles keratan sulphate, may be a possible candidate for the factor. Only the proteoglycan was studied by Kapoor and Prehm (1983), however, and GAG's seldom exist as free molecules, but are usually covalently attached side

chains in proteoglycan molecules (Hascall and Hascall, 1981).

Of the substances recently reported to be secreted by a variety of other cell types, none appear to exhibit properties identical to that of the morphogen described herein. These include a factor produced by feeder cells which inhibits EC cell differentiation (Smith and Hooper, 1983; Koopman and Cotton, 1984), a factor from confluent 3T3 cells which retards proliferation (Okai, 1984), and some differentiation-inducing factors from a murine fibroblast line (Tomida et al, 1984) and mouse yolk sac (Labastie et al, 1984). The first factor can be excluded from consideration simply because of its effect, which is the opposite of that observed with CM. The factor from 3T3 cells can also be excluded, since a CM from 3T3s in this study revealed no differentiation-inducing activity, although some retardation of growth was seen.

The differentiation-inducing activities observed by Tomida and colleagues (1984) and Labastie and colleagues (1984) both relate to blood cell lineages, and affect only cells pre-committed to these lineages. In addition, the factor described by Tomida et al (1984) has a molecular weight larger than 10 K, and is sensitive to both heat and trypsin.

The term "morphogen" is frequently used in this study. This is used in the sense of a diffusible, differentiation-inducing substance, (see Yamada, 1967;

Berrill and Karp, 1976; Saunders, 1982) and is based upon the initial, and major, biological effect observed. It should be noted that the growth retardation observed in transformed cells may be caused by either this morphogen or some other factor present in CM.

Explanations for several phenomena observed in cultures of F19 and other EC cells may be drawn from this study. These include the low level of spontaneous differentiation seen in crowded monolayer cultures and aggregates, an explanation of how "positional regulation" works, and what the exact role of DMSO may be in the induction of muscle in aggregates.

The spontaneous low level of differentiation of F19 cells in monolayer is probably a result of the fact that EC cells, at high density, secrete a differentiation-inducing factor similar or identical to the morphogen produced by endoderm. (The dramatic reduction in the potency of CMs produced by seeding slightly fewer cells could be an indication that the active principle(s) present in CM are only produced when the cells reach a minimum level of crowding, perhaps by means of a cell contact mechanism.) Higher levels of differentiation in monolayer cultures would be prevented by two mechanisms. When the cells are at low density, insufficient morphogen would be produced for any effect to be seen. At higher densities, the crowding of the cells would inhibit the differentiation of the cells (see Section 4.6). Inhibition of differentiation in crowded

cultures could also explain why differentiation into endoderm in overcrowded cultures of P19 cells occurs preferentially in open areas of the plate.

As well, the production of the morphogen by crowded EC cells may explain the small number of extraembryonic endoderm-like cells seen in untreated aggregates of P19. The close crowding of the cells would cause a localized increase in the amount of morphogen present. Due to the inhibition of differentiation in dense cultures, only those cells on the outside of the aggregate would be in a location favourable for differentiation into endoderm. This effect of cell density may therefore represent an explanation for the positional regulation of differentiation espoused by several workers (Hogan et al, 1981; Rosenstrauss et al, 1983). According to this theory, endoderm forms around the exterior of aggregates due to some vaguely defined "positional information". The morphogen required for differentiation into endoderm is present in the aggregates, but only those cells which are not too crowded (i.e. those on the outside of the aggregate) can respond to it.

Whether the morphogen is involved in the differentiation of muscle (as originally hypothesized) has not been clearly established, but two lines of evidence seem to indicate that it may be involved. The first evidence was the ability to form TROMA-1 positive (endodermal) cells in response to CM. From my work with

aggregates, the ability to form endoderm appears to be linked to the ability to form muscle. P19 cells, which form muscle when aggregated in DMSO, formed a large proportion of TROMA-1 positive cells when treated with CM. The cell lines incapable of forming muscle (F9, D3, and RAC65) were also less susceptible to the differentiation-inducing effect of CM. A partial revertant of one of these lines which had regained muscle-forming potential, RAC65-D⁺, formed a large proportion of TROMA-1 positive cells.

The best line of evidence linking CM (and thereby the endoderm which produces it) with muscle formation was the synergistic effect of CM and DMSO. DMSO, which is normally used to induce muscle differentiation, augmented the differentiation-inducing effect of CM on P19 cells. D3 cells, which do not form muscle when aggregated in DMSO, remained unaffected in all doses of CM and DMSO. Therefore, the ability to form muscle appeared to be linked to the ability to differentiate in response to CM.

Thus, a model for the formation of muscle in aggregates can be proposed. Aggregation (bringing the cells into close contact) would be the first step toward the formation of an endodermal sheath. The differentiation-inducing factor would be produced by the tightly packed EC cells of the aggregate, and some endoderm would begin to form around the outside, since this is where the cells are the least crowded. (This hypothesis is supported by the appearance of at least a few endodermal

cells in untreated aggregates.) DMSO, acting in conjunction with positive feedback from the endodermal cells already present (and the tightly packed EC cells), would cause a dramatic increase in the number of endodermal cells. In this way the aggregate would become completely coated with endoderm, which would stimulate the differentiation of muscle beneath. Untreated aggregates would not receive the DMSO enhancement of endodermal differentiation and therefore would rarely achieve the conditions (endodermal sheath formation) necessary for muscle differentiation. One of the reasons why D3 cells (and possibly RAC65 cells as well) are incapable of forming muscle may be their inability to form sufficient endoderm, due to their resistance to both CM (Fig. 4.12) and the DMSO enhancement of the differentiation-inducing effect of CM (Fig. 4.13).

If this model were correct, DMSO would operate merely as an enhancer of a factor(s) normally produced by aggregates of P19 cells by augmenting its effect. Since DMSO enhances the differentiation-inducing effect of CM, and since DMSO is known to facilitate the internalization of some substances (Stroughton and Fritsch, 1964), it may be that the active principle present in CM must be internalized by the cell in order to act. Therefore, it may be that the sole role of DMSO in the induction of muscle formation is the augmentation of the effect of a natural factor(s), perhaps through the facilitated internalization of this factor(s).

It should be noted that this model does not assume direct implication of CM in muscle formation, but only indirectly, through enhancement of endoderm formation. As discussed previously, some other effects of endoderm formation and/or another effect of DMSO and/or CM may be implicated in muscle formation itself.

Indeed, if this morphogen is endogenously produced in embryonic as well as EC cells, it may be this substance which is responsible for one of the first differentiation steps which occurs - that of extraembryonic endoderm. After the formation of the first differentiated cells, the positive feedback effect of the morphogen would result in more extensive differentiation of endoderm and thus, (perhaps indirectly) other tissues as well.



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