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RETINOIC ACID INDUCED DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS

by

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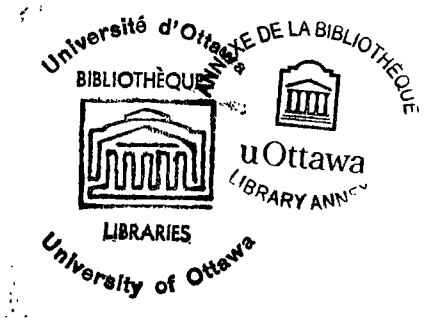

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ABSTRACT

Murine teratocarcinomas are malignant tumours which contain a wide spectrum of differentiated cell types and a population of embryonic-like stem cells. The stem cells, termed embryonal carcinoma cells, can be isolated from the tumours and grown *in vitro* where they may be induced to differentiate into a wide variety of cell types. They can therefore be used to examine the process by which undifferentiated cells become committed to particular developmental pathways.

In an attempt to simplify the differentiation pattern of the embryonal carcinoma cells, I added drugs to the tissue culture medium during the differentiation process. I observed the abundant development of neuron-like cells when embryonal carcinoma cells were aggregated and cultured in the presence of non-toxic concentrations of retinoic acid. I documented this observation with retinoic acid treated cultures of the embryonal carcinoma cell line, P19, which does not differentiate into neurons in the absence of the drug.

The neurons were initially identified by their morphology under the light and scanning electron microscopes. Their identity was confirmed by the presence of neurofilaments in

their cytoplasm and tetanus toxin receptors on their cell surface. In addition, the activities of two enzymes involved in neurotransmission, choline acetyltransferase and acetylcholinesterase, increased coordinately in these cultures. Glial cells, identified by the presence of glial fibrillar protein containing filaments, and a population of fibroblast-like cells were also present. Neither muscle nor epithelial cells were detected in cultures treated with non-toxic concentrations of retinoic acid in excess of 10^{-7} M. Embryonal carcinoma cells, monitored by their ability to form colonies and by the presence of an embryonal carcinoma cell antigen, disappeared prior to the appearance of neurons.

Neurons and glial cells appeared in cultures exposed to retinoic acid for as little as forty-eight hours. Retinoic acid did not change the plating efficiency of P19 cells nor did it have any effect on their growth rate over a forty-eight hour period. The P19 cell population was found to be homogeneous with respect to its ability to respond to retinoic acid. These data suggest that the effect of the drug was to induce the development of neurons and glia rather than to select against cells differentiating along other developmental pathways.

Various retinoids were able to induce the development of neurons with a hierarchy of efficiencies similar to that observed in many other biological systems affected by retinoic

acid. Polyamine metabolism did not appear to be involved in the effect. I have described a mutant clone which does not differentiate in the presence of retinoic acid. This mutant may help elucidate the chain of events triggered by the drug.

The retinoic acid-induced differentiation of P19 cells into neural cells provides a model system for asking questions about the commitment of pluripotent cells to differentiate along an embryonic cell lineage. In addition, these cells will be useful in studying the early events of neural differentiation particularly since the neurons and glial cells appear in a sequence similar to that seen in vivo.

RESUME

Les tératocarcinomes de souris sont des tumeurs malignes constituées de plusieurs types de cellules différenciées et d'une population de cellules souches. Les cellules souches, appelées cellules de carcinomes embryonnaires, peuvent être isolées des tumeurs et mises en culture où elles peuvent être induites à se différencier en une variété de types cellulaires. Elles peuvent alors être utilisées pour étudier le processus par lequel les cellules non-différenciées sont dirigées vers un développement particulier.

Dans le but de simplifier le patron de différentiation des cellulesde carcinomes embryonnaires, j'ai incorporé diverses drogues dans le milieu de culture durant le processus de différentiation. Lorsque les cellules de carcinomes embryonnaires sont sous forme d'aggrégats et cultivées en présence de concentrations non-toxiques d'acide rétinoique, j'observe la présence de plusieurs cellules semblables à des neurones. Cette observation a été soutenue par le fait que les cultures de carcinomes embryonnaires de la lignée cellulaire P19, traitées avec l'acide rétinoique, ne se différencient pas en neurones en l'absence de cette droque.

Les neurones ont été initialment identifiées par leur morphologie à l'aide des microscopes optique et à balayage

électronique. Leur identification a été confirmée par la présence de neurofilaments cytoplasmiques et de récepteurs spécifiques à la toxine tétanique localisés à leur surface cellulaire. De plus, l'activité spécifique de deux enzymes impliqués dans la neurotransmission, la choline acétyltransférase et l'acétylcholinestérase, a augmenté simultanément dans ces cultures. Des cellules gliales, identifiées par la présence de filaments constitués de protéines fibrillaires gliales ainsi qu'une populationde cellules d'aspect fibroblastique ont été également observées. Aucune cellule de type musculaire ou épithéliale a été détectée dans les cultures traité avec un excès d'acide rétinoique à 10^{-8} M, concentration non-toxique. Les cellules de carcinomes embryonnaires, suivie selon leur abilité a former des colonies et par la présence d'un antigène spécifique à ces cellules, ont disparues suite à l'apparition des neurones.

Les neurones et les cellules gliales sont apparues dans les cultures exposées à l'acide rétinoique pour aussi peu que quarante huit heures. L'acide rétinoique n'a pas modifié la capacité de formation de colonies des cellules P19 et n'a eu aucun effet sur leur taux de croissance à l'intérieur de la période étudiée de quarante huit heures. La population de cellules P19 s'est avérée homogène en respect de son abilité à répondre à l'acide rétinoique. Ces résultats suggèrent que l'effet de la droque est d'induire le développement des neurones et des cellules gliales plutôt que d'éliminer les cellules ayant d'autres potentiel de développement.

Divers composés rétinoiques ont induit le développement de neurons avec une gamme d'efficacités similaire à celle observée dans d'autres systèmes biologiques affectés par l'acide rétinoique. Le métabolisme de polyamines ne semble pas être impliqué dans ces effets. J'ai décrit un mutant incapable de se différencier en présence de l'acide rétinoique. Ce mutant peut aider à éludider la série d'événements initiés par cette droque.

La différentiation induite par l'acide rétincique chez les cellules P19 en cellules neuronales procure un modèle pour l'étude de la différentiation des cellules pluripotentes vers une lignée cellulaire embryonnaire spécifique. De plus, ces mêmes cellules seront utiles pour l'étude des événements initiaux de différentiation des neurones puisque les neurones et les cellules gliales apparaissent selon une séquence similaire à celle observée <u>in vivo</u>.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

DMSO	dimethyl sulfoxide
EC	embryonal carcinoma
EDTA	ethylene diamine tetraacetic acid
GFP	glial fibrillar protein
PBS	phosphate buffered saline
RA	retinoic acid

Chapter I

INTRODUCTION

The complex events which occur during mammalian embryogenesis are difficult to study in the intact embryo because many processes take place simultaneously in an embryo comprised of a small number of cells. A model system which could be easily manipulated, provide large amounts of experimental material, and mimic the developing embryo as closely as possible would simplify the study of embryonic development. Murine teratocarcinomas fulfil at least some of these requirements (Graham, 1977). These malignant tumours contain not only differentiated cells from all three germ layers of the embryo, but also a population of undifferentiated embryonic-like cells. These undifferentiated cells can be grown in large numbers as cell lines in tissue culture and will differentiate *in vitro* into a spectrum of cell types similar to that seen in the tumour. The purpose of this introduction is to discuss the experimental approaches which have been used to study teratocarcinomas and the undifferentiated cells derived from them. The following section contains an outline of the early development of normal mouse embryos in order to provide a frame of reference for the ensuing discussion of teratocarcinomas.

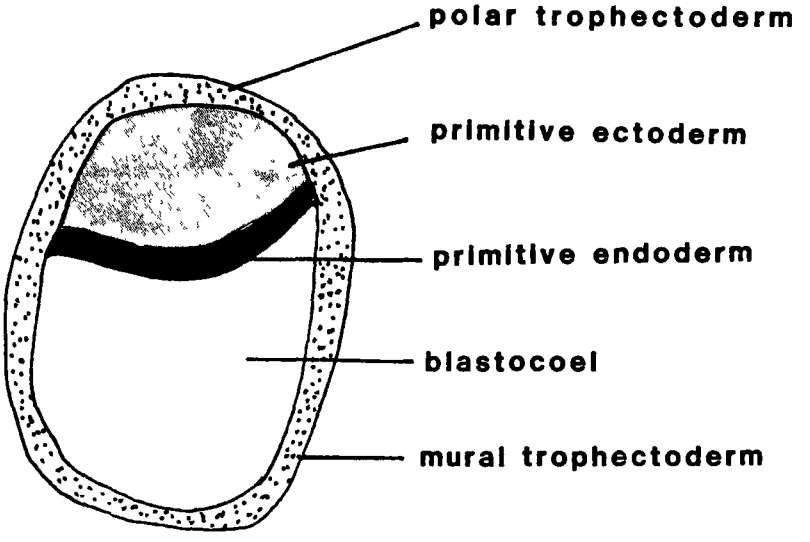
1.1 EARLY DEVELOPMENT OF MOUSE EMBRYOS

After fertilization, the zygote divides synchronously to form a embryo of 8 totipotent cells (Tarkowski and Wroblewska, 1967; Kelly, 1977). The embryo then undergoes a process of compaction whereby the cells move closer to each other and come into extensive contact (Ducibella and Anderson, 1975). The cells become polarized with the appearance of intercellular tight junctions and later zona occludens at the outside surface. This creates a permeability barrier between the inside and the outside of the embryo, which is now called a morula (Ducibella et al, 1975; Ducibella and Anderson, 1975). Gap junctions between the cells also appear at this stage (Magnuson et al, 1977; Lo, 1980; Goodall and Johnson, 1982). At the 16 to 32 cell stage, the outside cells begin to differentiate and the inner cells are displaced to one end by the formation of a fluid-filled blastocoel in the interior of the embryo.

At the 64 cell stage, which occurs about 3 1/2 days after fertilization, two cell populations can be distinguished; trophectoderm cells in a layer around the outside of the blastocyst and the inner cell mass cells (ICM) in a plano-convex disc at one end of the blastocoel. The polar trophectoderm cells adjacent to the ICM are diploid and eventually form the ectoplacental cone. In contrast, the mural trophectodermal cells surround the blastocoel and undergo transformation into giant cells containing large amounts of

endoreplicated DNA. By implantation of the blastocyst into the uterine wall at 4 1/2 days, the cells of the ICM have differentiated into primitive endoderm on the blastocoelic surface and the primitive ectoderm in the interior and trophectodermal surface (fig 1.1a). The primitive endoderm grows peripherally until it covers the entire inner surface of the blastocoel. That portion which remains associated with the ICM is termed visceral endoderm and the remainder forms the parietal endoderm. Both cell types have characteristic morphological and biochemical properties (reviewed in Graham, 1977). After implantation, the primitive ectoderm, covered by visceral endoderm, grows downwards to form the egg cylinder which eventually fills the blastocoel. By 7 days, the primitive ectoderm is clearly divided into a dorsal extra-embryonic region and a ventral embryonic region (fig 1.1b). Gastrulation begins at about 7 1/2 days when the primitive streak mesoderm appears at the posterior end of the embryonic ectoderm. More detailed reviews of embryonic development may be found in Snell and Stevens (1966) and Rossant and Papaioannou (1977).

a


polar trophectoderm
primitive ectoderm
primitive endoderm
blastocoel
mural trophectoderm

b

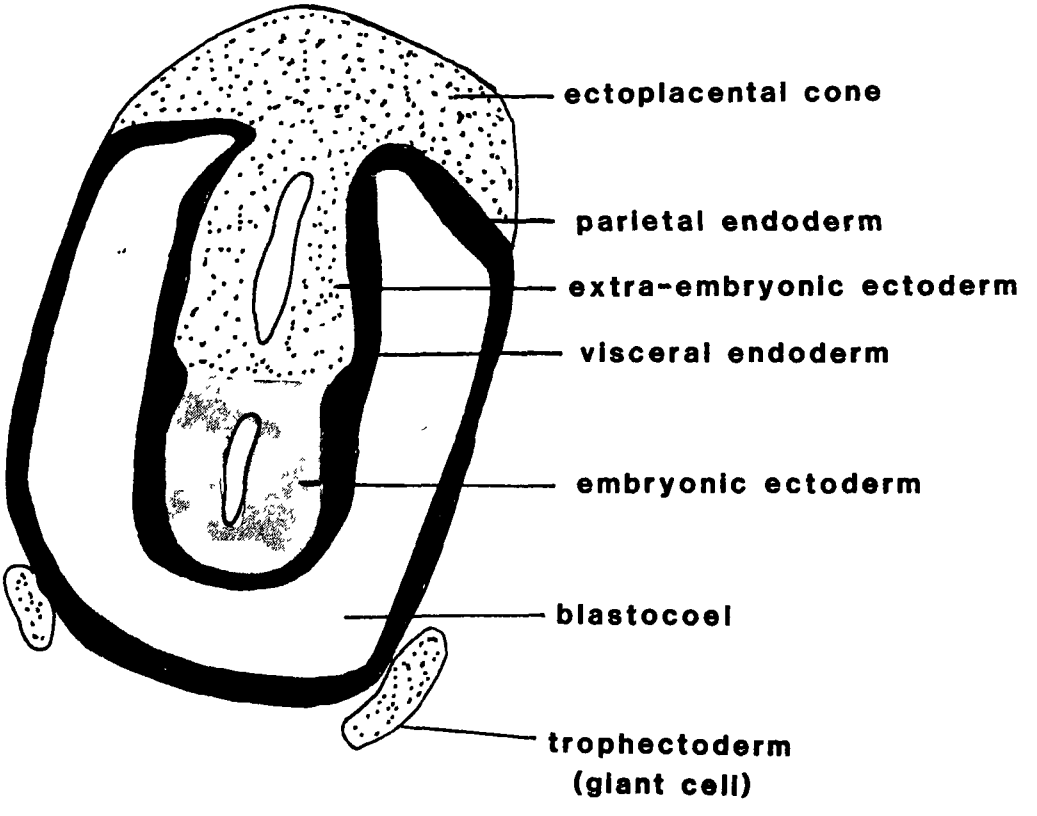

ectoplacental cone
parietal endoderm
extra-embryonic ectoderm
visceral endoderm
embryonic ectoderm
blastocoel
trophectoderm
(giant cell)

Figure 1.1. Diagrams of sections of mouse embryos at the 4 day (a) and 7 day (b) stages. At the 7 day egg cylinder stage, embryonic ectoderm and extra-embryonic ectoderm are separated by a constriction. Visceral endoderm surrounds the egg cylinder, parietal endoderm is located on the blastocoelic surface away from the egg cylinder, and the mural trophectoderm has given rise to giant cells. The shading indicates the relationships between the tissues in a and b. See text for further details. Redrawn from Rossant and Papaioannou (1977).

The origin of the embryonic cell lineages is well understood in many organisms where the embryo is available for experimental manipulation. However, in mammals where this complex determination process takes place in the uterus, much less is known. Although much descriptive information can be obtained by sectioning fixed embryos, this approach provides only a static picture (Snell and Stevens, 1966), which may be misleading. For example, the extra-embryonic ectoderm was believed to originate from the ICM. However, experimental reconstitution of blastocysts from genetically different donors and analysis of their subsequent development after implantation into a foster mother has demonstrated that trophectoderm is the origin of extra-embryonic ectoderm (Rossant and Papaioannou, 1977). Similar manipulations of the postimplantation embryo are not possible since they can not be obtained from the uterus. Attempts at isolating preimplantation embryos and growing them *in vitro* have been successful until the early somite stage (Hsu et al, 1974). Unfortunately, it is difficult to obtain large amounts of experimental material because the embryos are very small, only a small proportion survive in culture, and because there are problems in obtaining synchronously dividing embryos. The differentiation of murine teratocarcinomas provides a model system which overcomes some of the disadvantages of working with embryos.

1.2 TERATOCARCINOMAS IN VIVO

In 1954, Stevens and Little described testicular tumours which arise spontaneously in about 1% of male strain 129 mice. These tumours consist of various types of differentiated cells and an undifferentiated cell type. Stevens and Pierce (1975) have defined the following terms: embryonal carcinoma (EC) cells refer to the undifferentiated multipotential stem cells of the tumour; teratocarcinomas are those tumours which consist of EC cells and differentiated cells from all three germ layers of the embryo; and teratomas are benign tumours containing only differentiated cells. Pierce and Dixon (1959) and Pierce et al (1960) showed that the EC cells were responsible for new tumours when injected subcutaneously into a host mouse. Kleinsmith and Pierce (1964) showed that a single EC cell from an embryoid body was capable of forming a subcutaneous tumour consisting of many differentiated cell types and EC cells, thus confirming that the EC cells were the stem cells of the tumour. Intraperitoneal injection of tumour cells led to an ascites tumour consisting of free floating structures termed embryoid bodies which superficially resembled 5-6 day embryos (Stevens, 1960). Teratocarcinomas may be propagated by either subcutaneous injection or as an ascites tumour. Ovarian teratomas arise spontaneously in the LT strain of mice when ovarian eggs are parthenogenetically activated and become disorganized after the blastocyst stage (Stevens and Varnum,

1974). Only a small proportion of these tumours are transplantable teratocarcinomas.

Teratocarcinomas from other mouse strains can be produced by transplanting early embryos to extra-uterine sites in syngeneic mice, usually under the testes or kidney capsules (Stevens, 1970b; Damjanov et al, 1971a). Some of these embryos become disorganized and produce teratocarcinomas. The host environment can determine whether a teratocarcinoma or teratoma develops; certain strains of mice such as C57Bl and AKR are nonpermissive for teratocarcinomas (Solter et al, 1975) as are athymic mice (Solter and Damjanov, 1979). Embryos from the 2 cell stage to the eight day egg cylinder stage will form teratocarcinomas with the highest frequency being obtained with seven to eight day embryos (Stevens, 1968; Stevens, 1970b; Damjanov et al, 1971b). Generally older embryos give rise only to teratomas (Iles, 1977). Transplantation of twelve to thirteen day old genital ridges to extrauterine sites also leads to teratocarcinomas (Stevens, 1970a).

Spontaneous testicular tumours are likely derived from the primordial germ cells of the fetal testis (Pierce and Beals, 1964). When Stevens (1967) grafted genital ridges from both normal and sterile mice to the testes of normal mice, only the normal grafts gave teratocarcinomas. This observation supports the hypothesis of a germ cell origin for these tumours. It is possible that grafted embryos also

give rise to teratocarcinomas through parthenogenetic development of primordial germ cells. However, this possiblity is not supported by the experimental evidence. Mintz et al (1978) demonstrated that 6 day old embryos from both sterile and normal mice, grafted to the testes of normal mice, gave rise to the same proportion of teratocarcinomas, thus eliminating the possibility that, at least in this case, the origin of the tumours was from primordial germ cells developed after grafting of the embryos. Teratocarcinomas derived from embryos have both male and female karyotypes in constrast to those tumours derived from primordial germ cells which are always male. Some strains of mice which can form teratocarcinomas from grafted embryos do not give rise to germ cell derived tumours (Graham, 1977). These data suggest that another pluripotent cell type of the embryo, possibly embryonic ectoderm, may give rise to teratocarcinomas. Diwan and Stevens (1976) have successfully obtained teratocarcinomas by grafting 6 day embryonic ectoderm into the testes of adult mice.

1.3 EMBRYONAL CARCINOMA CELL LINES

Lines of embryonal carcinoma cells can be established *in vitro* from teratocarcinomas by either dissociating cells from embryoid bodies or solid tumours and culturing them on layers of non-dividing feeder cells (Kahan and Ephrussi, 1970; Martin and Evans, 1975b; McBurney, 1976) or by allow-

ing embryoid bodies to attach to the surface of a plastic tissue culture dish (Rosenthal et al, 1970; Evans, 1972; Bernstine et al, 1973; Lehman et al, 1974). In either case, clusters of EC cells arise amid the differentiated cells and by subculturing, homogenous populations of the rapidly dividing EC cells can be obtained and cloned. These lines of EC cells can be maintained in culture by frequent subculturing to ensure that they remain in exponential growth. Finch and Ephrussi (1967) showed that subclones derived from EC cells after 25 to 50 generations in culture could still give rise to teratocarcinomas with the same range of differentiated cells. Recently, methods for generating EC cell lines directly from early embryos have been developed thus obviating the need for the lengthy in vivo grafting procedure (Evans and Kaufman, 1981; Martin, 1981; Axelrod and Bennett, 1982).

The cells of the embryo develop in a reproducible manner within a precise organizational framework. The differentiation of EC cells is not as rigidly constrained since they do not develop into actual organ structures. However, certain conditions are necessary for EC cells to differentiate maximally in vitro, including a requirement for a certain level of cell density. This has been achieved by culturing cells in dense monolayers (Nicolas et al, 1975), in large attached clumps (McBurney, 1976) or as aggregates in suspension (Martin and Evans, 1975a). The outer cells of aggregates of

pluripotent EC cells differentiate into primitive endoderm (Martin et al, 1977) in a manner analogous to the formation of embryoid bodies *in vivo*. Replating the aggregates leads to an outgrowth of endoderm followed by the appearance of many differentiated cell types including neurons, beating muscle, keratinizing epithelium, cartilage, and adipose tissue (Martin and Evans, 1975a and b). Some lines of EC cells, which have lost the capacity to differentiate, fail to develop any endodermal layer when aggregated (Martin and Evans, 1975a; Martin, 1980).

Many morphological and biochemical markers have been used to assess the differentiation of EC cells *in vitro*. EC cells have a characteristic morphology; they have sparse cytoplasms, relatively large nuclei with prominent nucleoli, spherical mitochondria, little endoplasmic reticulum and Golgi, and numerous dispersed ribosomes which give the cells a uniform appearance (Pierce and Beals, 1964; Lo and Gilula, 1980). EC cells have high levels of alkaline phosphatase (Berstine et al, 1973) and lactate dehydrogenase (Graham, 1977) which may be detected histochemically. Using morphology to distinguish differentiated cells may not be adaquate for many cell types, although nerve and muscle are examples of cell types which can be identified easily with both the light and electron microscopes. A number of biochemical markers have been used for more accurate identification of differentiated cell types appearing *in vitro*. Antibodies

used in indirect immunofluorescence assays provide specific markers for some cell types and allow individual cells to be examined. Antibodies to intermediate filament proteins are an example of antisera which have been used in this way (Jones-Villeneuve et al, 1982; Paulin et al, 1982). Specific isozymes for aldolase (nerve), creatine phosphokinase (muscle), and phosphoglycerate mutase (muscle) are useful, providing the cells differentiate fully in culture (Adamson, 1976). Some cell types can be identified by a constellation of markers. For example, parietal endoderm is characterized by production of plasminogen activator, laminin, and collagen type IV (Graham, 1977; Strickland et al, 1980).

EC cells can thus differentiate into many different cell types whose appearance can be monitored by biochemical criteria <u>in vitro</u>. The question then arises as to how closely these tumour cells resemble the cells of the early embryo. The best evidence that EC cells can differentiate normally comes from blastocyst injection experiments. Brinster (1974) showed that 129 derived teratocarcinoma cells transferred to a blastocyst from an albino mouse resulted in a mouse with agouti hair. Mintz and Illmensee (1975) extended this observation using EC cells from ascites embryoid bodies. Chimeric mice resulted which had many developmentally unrelated tissues, including the germ cells in one mouse, derived from the EC cells. Illmensee and Mintz (1976) generated chimeric mice from single EC cell transfers, thus

confirming that a single EC cell is capable of developing into many tissue types. EC cells which have been maintained in tissue culture are also capable of contributing to embryonic development after transfer to blastocysts (Papaioannou et al, 1975). Using this approach it may be possible to obtain strains of mice carrying specific mutations by using EC cells with these mutations in blastocyst transfer experiments (Dewey et al, 1977b; Dewey and Mintz, 1980). The conclusion from the blastocyst transfer experiments was that EC cells are capable of differentiating like embryonic cells when they are supplied with the proper set of environmental cues. Although EC cells are malignant, they lose this property upon differentiation (Pierce et al, 1960; Adamson and Graham, 1980). Thus they can be grown in tissue cultures in large numbers due to their tumourgenic properties but their differentiation into mature cell types can be used as a model for normal embryonic development.

There is some controversy over which embryonic cell type EC cells most closely resemble. It has been shown that EC cells can differentiate into extra-embryonic endoderm as well as embryonic tissues but that they do not give rise to trophectodermal cells (Graham, 1977). The embryoid bodies derived from EC cells resemble the structures formed when ICM are isolated and cultured. These observations suggest that EC cells closely resemble ICM cells. Isolated ICM can generate pluripotent cell lines when cultured in EC condi-

tioned medium (Evans and Kaufman, 1981; Martin, 1981). However, since ICM cells are the progeniters of the embryonic ectoderm, it is possible that these pluripotent cells could have arisen from embryonic ectodermal cells. Analysis of proteins present in EC cells, ICM cells, and in primitive ectoderm (Martin et al, 1978), showed that EC cells share a protein with embryonic ectoderm that is not found in ICM cells. Dewey et al (1978) have demonstrated that ICM and EC cells differ in protein profiles seen on two dimentional gels. Evans et al (1979), also using two dimensional gels, showed that EC cells resemble embryonic ectoderm of the 6 to 7 day embryo more closely than the ICM cells of the earlier embryo. These observations suggest that EC cells are the equivelent of embryonic ectoderm. It is possible that both ICM cells and embryonic ectoderm are capable of giving rise to EC cells and that different EC cell lines may represent slightly different developmental stages. This idea is supported by the observation that several EC cell lines with female karyotypes are at different stages of X chromosome inactivation (McBurney and Adamson, 1976, Martin et al, 1978, McBurney and Strutt, 1980).

1.4 EXPERIMENTS USING EMBRYONAL CARCINOMA CELLS

The following section contains a discussion of ways in which EC cells have been used to examine developmental phenomena.

1.4.1 Cell surface molecules

Many investigators have looked for markers which are shared by EC cells and embryonic cells. The cell surface has been of particular interest because of its potential role in cell-cell interactions during embryogenesis.

Immunological techniques have been used to obtain syngenic and monoclonal antisera against cell surface molecules of EC cells. An antiserum raised against F9 EC cells in syngeneic mice serves to illustrate this approach (Artzt et al, 1973). This antiserum reacts with F9 cells and other EC cell lines, sperm, and with embryos from the 2 cell stage to the blastocyst (Jacob, 1977). Only the embryonic ectoderm of the 6 to 9 day old embryo is positive. F9 antigen may be important in cell- cell interactions. Fab fragments of anti F9 antibodies reversibly inhibit compaction of the morula (Kemler et al, 1977), cell to cell adhesion of ICM and EC cells in culture (Nicolas et al, 1981), and modulate gap and tight junctions (Dunia et al, 1979), all without affecting cell division (Jacob, 1977). F9 antigen is undetectable on embryos homozygous for some recessive genes of the T complex (Kemler et al, 1976) and it has been suggested that the an-

tiserum may recognize a wild type product of this locus. (Recessive mutations in the T complex are lethal in the homozygous form at various stages of embryonic development) (Bennett, 1975). Several other antisera have been raised against EC cells (Stern et al, 1975; Gachelin, 1976; Dewey et al, 1977a; Webb, 1980). Monoclonal antibodies are a powerful tool because they detect only one antigenic specificity. Using a monoclonal antibody, Solter and Knowles (1978) have defined a stage specific antigen (SSEA-1) which appears on 8 cell stage embryos and is present in highest concentrations on primitive ectoderm. The major conclusion from this work is that EC cells share some antigens with embryonic cells that are not present on most adult or other tumour cells. Conversely, some cell surface antigens such as H-2 and beta-2 microglobulin are absent from EC cells and appear only when they differentiate (Jacob, 1977; Croce et al, 1981).

The carbohydrate content of EC cell surface molecules differs from that of differentiated cells as assessed by lectin binding (Reisner, et al, 1977; Fujimoto et al, 1982), and fucosylglycopeptide analysis (Muramatsu et al, 1978). Grabel et al (1979, 1983) detected a lectin like component on EC cells which recognized oligomannosyl residues presumably found on a complementary receptor on neighbouring EC cells. A Ca^{++}-dependent adhesion system which is shared by early embryonic and EC cells has also been described (Takei-

chi et al, 1981; Ogou et al, 1982). These studies confirm that EC cells and their differentiated progeny show differences at the cell surface. The challenge is to ascertain what role these differences play in differentiation.

1.4.2 Gene expression

EC cells provide a system for studying the mechanisms of differential gene expression during development. Although the specific genes and gene products which regulate differentiation are largely unknown, several groups have examined either endogenous genes which are expressed only after differentiation of the EC cells (Croce et al, 1981), or the expression of exogenous viral or plasmid genomes after their integration into the EC cell DNA (reviewed in Levine, 1982, also see Heubner et al, 1981; Stewart et al, 1982; Niwa et al, 1983; and Gautsch and Wilson, 1983). The inactivation of one of the X chromosomes in somatic female cells, which involves the turning off of transcription of almost an entire chromosome, may also be studied using lines of EC cells with two active X chromosomes (McBurney and Strutt, 1980; Featherstone, 1980; Paterno and McBurney, in preparation).

1.4.3 Determination

Although there are many cell lines which differentiate in culture along specific developmental pathways, EC cells are particularly valuable because they differentiate into many

distinct cell types. EC cells can therefore be used to study determination, the process by which a cell and its progeny become committed to a particular differentiation pathway. The main obstacle in studying determination with pluripotent EC cells has been the complexity of their differentiation patterns and the lack of control that the experimenter had over the process. One way to surmount these problems is to manipulate the cells so that they differentiate into only one or a few related cells types.

Several groups have added drugs to differentiating cultures of EC cells in order to achieve this goal. Strickland and Mahdavi (1978) reported the appearance of extra-embryonic ectoderm in monolayer cultures of F9 EC cells treated with retinoic acid (RA). Addition of cAMP to these cultures led to the development of parietal endoderm (Strickland et al, 1980). Hogan et al (1981) demonstrated that aggregation of F9 cells during treatment with RA led to the development of visceral endoderm rather than parietal endoderm. The RA treated F9 cultures thus are potentially useful for studying determination events leading to the differentiation of EC cells into extra-embryonic tissues.

Speers et al (1979) added hexamethylene bis acetamide, polybrene, and dimethylacetamide to cultures of the pluripotent EC cell line PCC4, and observed the appearance of epithelial-like and fibroblast-like cells respectively. Paulin et al (1979) also observed the appearance of a flat

adhesive cell type in EC cultures which were treated with hexamethylene bis acetimide.

Growth of EC cells in defined medium without serum leads to differentiation of some EC cell lines into parietal endoderm (Rizzino, 1983). Darmon et al (1981) grew pluripotent EC cell lines in defined medium and obtained neurons and fibroblasts.

Recent work in our laboratory has been concerned with the differentiation of EC cells into embryonic cell types. We have reported that high doses of RA induce several lines of EC cells to differentiate into neurons and glia (Jones-Villeneuve et al, 1982) while dimethyl sulfoxide (DMSO) and low doses of RA lead to production of muscle (McBurney et al, 1982; Edwards and McBurney, 1983). The effect of RA in inducing differentiation of neurons is the subject of this thesis. A short discussion of the differentiation of neural tissue in the embryo is presented in the next section. I shall also summarize the biochemical and cellular actions of RA.

1.5 NEURAL DIFFERENTIATION IN THE EMBRYO

The nervous system of mammals originates from the embryonic ectoderm after the appearance of the primitive streak mesoderm. Neuralation begins at 7 to 7 1/2 days in the mouse with the appearance of a groove on the dorsal surface of the gastrulating embryo. The lateral margins turn

up as the groove widens and eventually fuse beginning at the anterior end. This process forms a stucture called the neural tube (Snell and Stevens, 1966). Neurons and glial cells of the central nervous system (CNS) differentiate from the neuroepithelium of the neural tube. The neural crest, a transient structure which develops on the dorsal surface of the neural tube, gives rise to most of the periperal nervous system. I shall discuss the differentiation of the CNS.

The cells of the CNS may be divided into three classes; the neurons, the glial cells, and fibroblasts. Glial cells are classified as astrocytes, oligodendrocytes, ependymal cells and microglia. There are a number of markers which can be used for identification of the various neural cell types (reviewed by Fields, 1979 and Schachner, 1982). Tetanus toxin binds specifically to neurons of both the central and peripheral nervous systems. Neurons also contain three specific intermediate filament proteins. Astrocytes, which are divided into protoplasmic and fibrous subclasses, are recognized by the presence of the intermediate filament protein, glial fibrillar acidic protein (GFAP). Oligodendrocytes synthesize CNS myelin and are thus identified by the presence of galactocerbroside, the major glycolipid of myelin. The ependymal cells, characterized by beating cilia, line the ventricles of the brain and also the spinal cord in a palisade arrangement. The microglial cells, derived from mesoderm, are small migratory cells with phagocytic proper-

ties. Fibroblasts are characterized by the presence of fibronectin and also Thy-1, a cell surface molecule also seen on some neurons and T lymphocytes.

The very early events in differentiation of neurons and glia in the CNS are still unclear. This is largely due to the complexity of the developing brain, its inaccessiblilty, and a lack of markers for the differentiating cell types. At some point, neurons and glia probably share a common precursor, but it remains uncertain at which point their differentiation pathways diverge. The developmental relationships between the various types of glial cells is also under investigation. Recently, there has been progress in this area, partly because monoclonal antisera recognizing antigens on specific neural cell types have become available.

Some investigators have used embryonic or neonatal brain to generate monoclonal antisera on the assumption that differentiating cells contain specific antigens characteristic of their developmental stage. Sommer and Schachner (1981) have isolated monoclonal antisera which react with murine oligodendrocytes at different stages of differentiation. Another interesting antigen, designated C1, is detected on the primitive radial glial cells of 10 day embryos. Later it is expressed on glia which do not undergo extensive morphological change and upon ependymal cells (Schnachner, 1982a). Extensive reviews on these monoclonal antisera are found in Schachner (1982b) and Mirsky (1982).

The neural tube of the CNS can be divided into a number of zones (Boulder Committee, 1970). The inner ventricular zone contains mitotic cells, the subventricular zone contains dividing cells which are presumably limited in differentiation potential, the intermediate zone consists of non-mitotic cells and committed cells, and the outer marginal zone is comprised mainly of migrating cells and axons.

The differentiation of neurons and specific neuronal populations within the CNS may be divided into several stages; the generation of neuronal precursors by successive waves of mitosis in the ventricular zone, their post-mitotic migration to their final location in the developing brain, their aggregation with other neurons, and the differentiation and migration of processes with the formation of connections to other neurons (Cowan, 1978). In general, neurons belonging to a specific layer of the brain are generated and withdraw from mitosis at about the same time. A striking example of this is seen in the monkey visual cortex where the neurons for the inner layers form first and so on with the most superficially-destined neurons appearing last (Rakic, 1974).

It was long thought that the ventricular zone of the neural tube was composed of only one primordial cell type which first generated neurons and then gave rise to glial cells after neurogenesis was complete (Jacobson, 1978). However, using an immunoperoxidase technique combined with electron microscopy, Levitt et al (1981) have demonstrated

that both GFAP positive and GFAP negative cells are present in the ventricular zone of fetal monkeys. This work does not identify a common precursor for glia and neurons, but it does show that at least some cells produced in the ventricular zone are determined to be glia and that they coexist with neuronal precursors. It also demonstrates that glial cells begin to differentiate before they cease cell division.

The first differentiated glial cells observed during development are the radial glial cells, processes of which traverse the developing brain wall from the cell body located in the ventricular zone. It has been suggested that these glial cells may function as a guide to migrating neurons, thus providing a way of establishing patterns of neurons within the brain (Rakic, 1972). Radial glial cells, after a period of mitotic inactivity, divide and may generate astrocytes. Other astrocytes may be derived directly from the GFAP positive cells of the ventricular zone.

The origin of oligodendrocytes is also under investigation. Raff et al (1983) have recently isolated a cell type from neonatal rat optic nerve which can differentiate into either a fibrous astrocyte or an oligodendrocyte. This cell type is characterized by the presence of A2B5 antigen, recognized by a monoclonal antiserum (Eisenbarth, 1979; Schachner, 1982b). This finding is consistent with previous observations that A2B5 antigen is located on immature

astrocytes and oligodendrocytes, but since this antigen can also be detected on some neurons it is not a unique marker for the precursor cell described by Raff et al (1983).

Neural tissue has been studied *in vitro* as a way of simplifying the complexity encountered *in vivo*. The study of cell lineages may be approached *in vitro* by isolating cells at different stages of development and allowing them to form colonies. The cellular composition of the colonies may help to identify immature cells which cannot be distinguished morphologically and may also allow some quantitation of immature cell types. Federoff and Doering (1980) have examined the astrocyte cell lineage in this manner.

Primary explants of developing neural tissue allow closer manipulation of the cellular environment. Cultures derived from disaggregated neural tissue are valuable in answering questions about the time of determination to specific cell lineages (Abney et al, 1981), and the importance of the microenvironment in such processes as the cytodifferentiation of processes (Cowan, 1978).

1.6 RETINOIC ACID

Retinoic acid (RA) is a derivative of retinol, more commonly known as vitamin A. Vitamin A and its derivatives, termed retinoids, are ultimately derived from the plant compound, beta-carotene. The parent structure of the retinoids consists of a trimethylcyclohexenyl ring, a dimethyl substi-

tuted tetraene chain, and a polar end group which is a carboxyl group in the case of RA (fig 1.2) (Pawson, 1981).

Vitamin A was shown to be essential for life in 1909. Since then its importance to growth, vision, reproduction, and glycoprotein biosynthesis has been elucidated (Lotan, 1980). RA, a natural metabolite of retinol, is important to the growth functions of vitamin A (Lotan, 1980), particularly with respect to the differentiation of epithelial tissue and the suppression of epithelial cancers (reviewed by Bollag and Matter, 1981).

Retinoids derived from dietary sources are stored in the liver as retinyl esters and secreted into the blood as retinol bound to a retinol binding protein which then forms a complex with serum albumin for transport (Lotan, 1980). Uptake into target cells is via the cell membrane, probably by a specific membrane receptor. Different cytoplasmic binding proteins, which are specific for retinol and retinoic acid, are present within many cell types (Chytil and Ong, 1979). In the target cell retinol may be metabolized to RA, which can then bind to the cellular retinoic acid binding protein (cRABP). It has been suggested that retinoic acid and retinol are transported to the cell nucleus complexed to their binding proteins, where they may modify gene activity in a manner analogous to steroid hormones (reviewed in Chader et al, 1981).

RA has other biochemical effects. Like retinol, a hydroxyl containing metabolite of RA can after phosphorylation, combine with a nucleotide sugar and subsequently transfer the sugar to a membrane glycoprotein (De Luca , 1977). Specific cell surface glycoproteins have been shown to altered in retinoid treated cells and glycolipid biosynthesis may also be affected (Lotan, 1980).

On the cellular level, RA has diverse biological effects including inhibiting proliferation of various tumour cell lines (Jetten et al, 1979a; Lotan, 1980), antagonizing the effects of tumour promotors (Verma et al, 1978; Fish et al, 1981), reversal of keratinization in epithelial cells (Sporn et al, 1976; Wilkoff et al, 1976), and regression of carcinogen-induced skin tumours (Lasnitzlei, 1976; Chopra and Wilkoff, 1977). RA can also affect pattern formation in developing and regenerating limbs (Maden, 1982; Tickle et al, 1982). The underlying mechanisms of these effects may lie in the interaction of RA with its binding protein or could be a result of glycoslyation of cell surface proteins. Two excellent reviews of of the literature concerning RA may be found in Lotan (1980) and De Luca and Shapiro (1981).

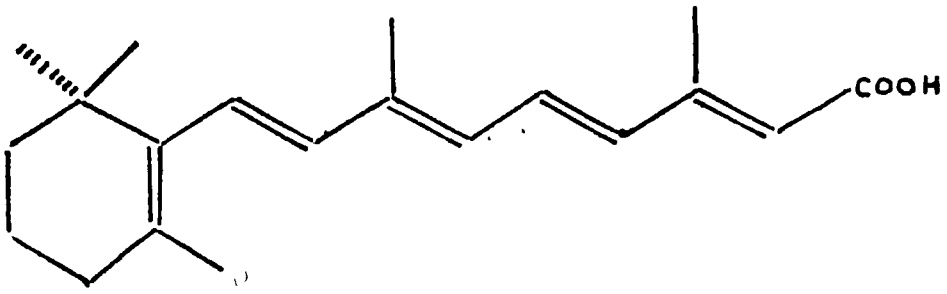


Figure 1.2. Structure of all trans retinoic acid.

1.7 THESIS PROJECT

EC cells provide a model system with which to study the commitment of pluripotent cells to differentiate along particular developmental pathways. However the differentiation pattern of pluripotent EC cells is complex and it is difficult to examine the events necessary for commitment to a specific differentiation pathway. By adding drugs to the culture medium during differentiation of the EC cells, I hoped to reduce the spectrum of cell types formed by interfering with some of the determination events leading to particular developmental pathways. Early in the work, I observed that EC cells differentiated in neurons, glial cells, and fibroblasts in the presence of non-toxic concentrations of RA. The rest of my project has consisted of persuing this observation. I have collaborated with a number of people in order to bring a greater number of techniques to bear on this problem. Dr. V.I. Kalnins and Mr. K. Rogers provided some of the antibodies for the intermediate filament work and Mr. Rogers demonstrated both the immunofluorescent and photographic procedures involved in that phase of the project. Dr. J.B. Harris provided the monoclonal antiserum against EC cell antigen and performed the experiments involving the quantitation of this antigen on differentiating cells. Mr. M. Rudnicki did his fourth year honours project under my supervision and is responsible for the assays for tetanus toxin binding and choline acetyl transferase.

The thesis takes the following form; chapter 2 contains details of the experimental methods which were used, chapter 3 documents the observation obtained with RA, and chapter 4 describes some experiments aimed at elucidating the mechanism of action of RA in this system. Appendix A consists of a paper describing the effects of DMSO in inducing P19 cells to differentiate into cardiac and skeletal muscle. It is included because I was involved in the electron microscope work and as a basis for discussion contained in chapter 5, the conclusions.

Chapter II

MATERIALS AND METHODS

2.1 CELL LINES AND CULTURE TECHNIQUES

The cell lines C145A12 (McBurney and Strutt, 1979), OC15S1 (McBurney. 1976), and P10 (McBurney and Strutt, 1980) are pluripotent lines of EC cells which differentiate into a variety of cell types when aggregated *in vitro*. All other cell lines are subclones of the P19 cell line. The P19 line of EC cells was isolated from a teratocarcinoma induced in the C3B/He strain of mice. These cells are euploid with a normal male karyotype (McBurney and Rogers, 1982). P19S18 is a cell line derived from a single P19 cell. P19S18C1A1 is a ouabain resistent and 6-thioguanine resistent subclone of P19S18 (McBurney et al, 1982). All cells were cultured in alpha minimal essential medium (Stanners et al, 1971) (Gibco Laboratories, Grand Island, NY), supplemented with 2.5% fetal calf serum and 7.5% calf serum (Flow Laboratories, Mississauga, Ontario). They were maintained at 37°C in a 5% CO_2 atmosphere.

Differentiation of all the cell lines was carried out as follows: cells in exponential growth were treated with Ca^{++} and Mg^{++}- free phosphate buffered saline (PBS) containing 0.025% trypsin and 1mM EDTA to remove them from the surface

of the tissue culture dish. They were plated at a concentration of 10^5 per ml into a bacteriological grade Petri dish (Martin and Evans, 1975a) where they aggregated spontaneously. The medium was replaced after 3 days and 2 days later the aggregates were plated into tissue culture dishes. The drugs used in the experiments were added at the initiation of the aggregation phase and remained in the medium usually for the duration of the experiment. Aggregates were scored for neurons at 7 to 8 days.

2.2 RETINOIC ACID PREPARATION

RA and the other retinoids were prepared as stock solutions at 10^{-2} M in 95% ethanol. The stock solution was diluted directly into the culture medium to obtain the desired concentration, usually 5×10^{-7} M. In experiments where RA was removed from the culture medium, the aggregates were washed 3 times with serum-free medium before resuspension in serum- containing medium. 13-cis-RA and the TMMP retinoids were kind gifts from Hoffman- Laroche Inc. (Nutley, N.J.).

2.3 GROWTH EXPERIMENTS

Cells were grown for either 48 hours or 8 days in the presence or absence of 5×10^{-7} M RA. In the 48 hour growth experiments, the cells were seeded at 10^5 per ml in 2 ml Linbro wells and counted after 48 hours with a Coulter Counter (Coulter Electronics Inc., Hialeah, Florida). For

the 8 day experiments, the cells were initially plated at 10^5 per ml into 2 100 mm tissue culture dishes. After 24 hours the cells in one dish were counted and discarded. At 48 hours, the cells from the remaining dish were counted and used to seed 2 more 100 mm dishes at a concentration of 10^5 per ml. These dishes were counted at 3 days and 4 days respectively and the cells from the 4 day dish were used to set up 2 more dishes. The process was repeated for 8 days and allowed us to keep the cells at optimal density for growth during the entire experiment.

2.4 ELECTRON MICROSCOPY

2.4.1 Transmission electron microscopy

P19 cells were fixed in 4% gluteraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1.5 hours at room temperature, washed in the same buffer and postfixed, on ice, in 1% osmium tetroxide. After dehydration, on ice, in increasing concentrations of acetone, the cells were brought back to room temperature in 100% acetone and infiltrated with SPURRS. They were then placed in Beem capsules and the resin was allowed to polymerize at 60°C overnight. Thin sections were cut with a glass knife, collected on copper grids, and stained for 7 minutes with uranyl acetate in ethanol followed by 5 minutes in lead citrate. They were examined and photographed with a Phillips-201 transmission electron microscope.

2.4.2 Scanning electron microscopy

The aggregates were plated onto coverslips, fixed, and stained *in situ*. Fixation was in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3 at room temperature for 30 min. The cells were washed in sodium cacodylate buffer and postfixed in 1% osmium tetroxide on ice in the same buffer. They were dehydrated in ethanol stepwise from 5% to 100%. After critical point drying, they were gold coated and examined in a AMR 1000A model scanning electron microscope.

2.5 PREPARATION OF ANTISERA

The antisera to vimetin, glial fibrillar protein (GFAP), and tubulin were gifts from Dr. V.I. Kalnins (Department of Anatomy, University of Toronto). Electrophoretically pure vimentin (MW 57,000), prepared from a cytoskeletal preparation of 3T3 cells according to the method of Franke et al (1979a), was used for the immunization of rabbits. Glial filaments were isolated from calf brain (Jorgensen et al, 1976) by a slight modification of previously described methods (Yen et al, 1976) and the filament protiens separated by PAGE. The 54,000 MW band was eluted from the gel, and the electrophoretically purified protein was used for the immunization of rabbits.

The preparation of the antiserum to tubulin has been previously described (Connelly et al, 1978). Antiserum to keratin was raised in rabbits against keratin purified from hu-

man stratum corneum (Sun and Green, 1978). This was a gift from Drs. E. Fuchs and H. Green (Department of Biology, Massachusetts Institute of Technology). Antiserum to neurofilaments (Liem et al, 1978), raised in rabbits against the 160,000 MW component of bovine brain neurofilaments, was a gift from Dr. R. Liem (Department of Pharmacology, New York School of Medicine).

2.6 IMMUNOFLUORESCENCE ASSAYS

2.6.1 Filament antigens

Aggregates were plated directly onto coverslips, and fixation and staining was carried out *in situ*. The cells were rinsed once in PBS, pH 7.0, fixed for 4 min in 100% methanol, and for 2 min in 100% acetone, both at -20°C. After washing with PBS, they were treated with one of the antisera at a dilution of 1:30 (antitubulin, antineurofilament, antiglial fibrillar protein, antivimentin) or 1:50 (antikeratin). This was followed by washing three times in PBS and treatment with fluorescein-conjugated goat IgG raised against rabbit IgG (Hyland Diagnostics Div., Travenol Laboratories, Costa Mesa, CA), diluted 1:5. After a further three washes in PBS, the coverslips were mounted in 50% glycerol and examined with either a Leitz or a Zeiss Photomicroscope 2 (Carl Zeiss, Inc., New York) equipped with epifluorescent optics.

2.6.2 Tetanus toxin

The assays for tetanus toxin were carried out in collaboration with M. Rudnicki. After washing in alpha medium (buffered with PBS 1:1), the cultures were incubated at room temperature for 30 min with 50 ul of tetanus toxin (which had been dialyzed against PBS), diluted 1:20 (Connaught Research Laboratories, Willowdale, Ontario) (Mirsky et al, 1978). After washing, the coverslips were treated with horse anti tetanus toxin (Connaught) at a 1:50 dilution for 30 min, washed, and exposed to rhodamine conjugated goat anti horse IgG (Cappel Laboratories, Cochranville, PA) at a 1:50 dilution for 30 min. The coverslips were then washed and fixed in 5% acetic acid in methanol for 15 min at -20°C. Subsequent staining with anti GFP was as described above.

2.7 EMBRYONAL CARCINOMA ANTIGEN ASSAY

2.7.1 Immunofluorescence of disaggregated cells

The aggregates were dissociated in 1 mM EDTA in PBS. The cells were allowed to settle onto coverslips which had been previously coated with poly-L-lysine (1 mg/ml in H20). After washing in PBS, they were treated on ice with a 1:25 dilution of ascites fluid from hybridoma AEC3A1-9 (J.F. Harris et al, in preparation) for 30 min. The coverslips were washed and treated with fluorescein conjugated rabbit IgG raised against mouse IgM (Cedarlane Laboratories, Hornby, Ontario) diluted 1:5. The cells were fixed in 100% methanol

at -20°C, then stained with ethidium bromide (1 ug/ml in PBS) and scored immediately. Cells were scored as positive even if they had only one patch of fluorescence associated with their cell surface.

2.7.2 Immunoabsorption

Cells for the quantitative absorption analysis were fixed at 2 X10^6 /ml in 0.1% glutaraldehyde in PBS for 15 min at room temperature. Bovine serum albumin was added to give a final concentration of 1% (w/v). The cells were washed 3 times in PBS, resuspended in PBS and frozen until analysis which was carried out by Dr. J.F. Harris. The cells were diluted sequentially by factors of 2 in RPMI 1640 medium with 5% FCS and 0.02% azide and absorbed with a 10^{-4} dilution of AEC3A1-9 ascites fluid for 16h at 4°C in a humidity controlled shaking chamber. After centrifugation at 600g for 10 min, the supernatant was tested for residual activity in a two step binding assay, using fixed F9 cells and ^{125}I-F(ab)'2 rabbit anti mouse Fab (^{125}I-RAM). The cell concentration required to reduce the AEC3A1-9 activity by 50% (D50) was derived from the cell titration data and normalized to the D50 value for a control P19S1801A1 culture in order to calculate the relative amount of AEC3A1-9 antigen present on different cell populations. This method assumes a linear relationship between the relative D50 and the amount of antigen per cell.

2.8 ESTIMATION OF MEDIAN CELL VOLUME

I estimated the median cell volume from the size distributions obtained from a Coulter Counter Channalyser (Coulter Electronics Inc., Hialeah, Florida). The channalyser was calibrated with spheres of known diameter and each channel was calculated to equal 24.23 u^3. My estimation of the median cell volume was the channel midway between the two channels containing 50% of the peak number of cells. Since the distribution was somewhat skewed, this estimated value was larger than the peak.

2.9 ENZYME ASSAYS

The cells were removed from the tissue culture dishes by scraping with a rubber policeman or in some cases by trypsinization. The samples were then washed 2 times in PBS and stored at -80°C. Before assay, all samples were resuspended in an equal volume of water and sonicated. Protein concentrations were determined using Hartree's (1972) modified Lowry procedure.

Choline acetyltransferase (CAT) was assayed by M. Rudnicki using a radiochemical method described by Fonnum (1975). Eserine, an esterase inhibitor, was added to each reaction mixture to prevent degradation of acetylcholine. Acetylcholinesterase was added to a duplicate reaction mixture to determine the activity specifically attributable to the formation of acetylcholine.

The spectrophotometric method of Ellman et al (1961) was used to assay acetylcholinesterase (AchE). The activity specifically attributable to AchE was determined by adding a specific inhibitor of AchE, BW 284C51 (Sigma Chemicals, St. Louis, Mo.). In some experiments, ethopropazine which specifically inhibits pseudoesterases was added to a duplicate reaction mixture and the values thus obtained were averaged. Both inhibitors were kept in a stock solution at 10^{-2} M at 5°C and used at a final concentration of 10^{-5} M.

2.10 ISOLATION OF NONRESPONSIVE MUTANTS

Mutant cells which did not differentiate in the presence of RA were isolated by a two step procedure from the P18S18 cell line by Dr. M. W. McBurney. Initially, P19S18 cells were cultured in medium supplemented with 10^{-7} M RA. The cells were subcultured and maintained at subconfluent densities for two weeks. Undifferentiated cells were selected because of their relatively rapid growth rate. After plating at low density for a further 10 days, colonies of morphologically undifferentiated cells were obtained, one of which was expanded into a cell line and called P19S18RAC6. P18S18RAC6 was subjected to a second selection step in the presence of 10^{-5} M RA for 3 weeks. These cells were plated at low density for an additional week in the presence of the drug and one of the colonies, P19S18RAC65, was grown up for further study.

Chapter III

CHARACTERIZATION OF THE CULTURE SYSTEM

3.1 RESULTS

Pluripotent EC cells can often be induced to differentiate into various cell types if they are aggregated and cultured in suspension for several days before plating onto tissue culture grade plastic surfaces (Martin and Evans, 1975a and b). For all of my experiments, the aggregates were cultured for 5d in suspension. They were then plated and examined 2 to 3 days later when differentiated cells had migrated out of the aggregates.

For my initial experiments, I used a pluripotent cell line C145A12 (McBurney and Strutt, 1979). When 10^{-7} M RA was continuously present in the culture medium, unusually abundant numbers of neuron-like cells appeared within 48 h of plating the aggregates. It had been reported that RA has no effect on the tissue distribution which arises during the differentiation of pluripotent EC cells (Jetten et al, 1979b). My observation on C145A12 cells may, therefore, have been peculiar to that cell line. Thus I examined the response of several other EC cell lines to RA.

TABLE 3.1

RESPONSE OF DIFFERENT CELL LINES TO RETINOIC ACID (RA)

CELL LINE	REFERENCE	%AGGREGATES CONTAINING NEURONS[1]	
		IN 10^{-7} M RA	WITHOUT RA
C145A12	McBurney and Strutt, 1979	100	0[2,3]
P19	McBurney and Rogers, 1982	94	0[3]
OC15S1	McBurney, 1976	94	54
P10	McBurney and Strutt, 1980	79	0[2,3]

1. Three days after plating, aggregates were examined for the presence of neuronal-like cells using phase contrast optics. 50 aggregates were scored per measurement and a positive was scored if the aggregate contained cells with long processes (see figures 3.1c and d).

2. Although C125A12 and P10 aggregated cultures did not contain neurons 3 days after plating, neurons were routinely present 5 to 7 days after plating.

3. Some aggregates were surrounded by extra-embryonic endoderm-like cells.

The drug had a similar effect on the other three cell lines tested (Table 3.1). Virtually all RA treated aggregates contained some cells with neuronal morphology. Since aggregates from some cell lines formed no neuron-like cells in the absence of the drug, it seemed likely that the RA was inducing the formation of these neuron-like cells, rather than inhibiting the development of other tissue types.

In subsequent experiments I used P19 (fig 3.1a), an EC cell line with a normal male karyotype isolated from C3H/He mice (McBurney and Rogers, 1982). The RA effect could be easily evaluated with P19 cells because no neuron-like cells were formed in the absence of the drug. Figure 3.2 is an electron micrograph of a P19 cell showing the characteristic morphology of embryonal carcinoma cells: the nucleus is relatively large with prominent nucleoli, and the cytoplasm contains few organelles. When aggregates of P19 cells were plated in tissue culture dishes in the absence of RA, I observed only undifferentiated cells surrounded by a small amount of tissue resembling extraembryonic endoderm (fig 3.1b). Continued incubation of these cultures resulted in the proliferation of both cell types, with the appearance of no other differentiated cell types. When P19 cells were cultured as aggregates in the presence of RA, the cell types present 2 d after plating were markedly different. Within 24 h of plating, a flat layer of fibroblast-like cells migrated out from the periphery of the aggregate. These fi-

broblast-like cells did not resemble either EC cells or the endoderm-like cells seen in untreated cultures. Between 24 and 48 h after plating, neuron-like cells appeared whose processes grew rapidly from the aggregate over the fibroblast-like cell layer. Phase contrast micrographs of these cell types are shown in figs 3.1c and d. The scanning electron micrographs in figs 3.1e and f show this morphology in more detail. The processes from these neuron-like cells were frequently arranged in bundles. They had multiple branches with tips located on the fibroblast-like cells.

3.1.1 Dose-response characteristics

The differentiation of EC cells into neuron-like cells was dependent on the concentration of RA present in the culture medium. Fig 3.3 shows the response of aggregated P19 cells to various RA concentrations. At concentrations greater than 5×10^{-8} M, essentially all of the aggregates contained cells with neuron-like processes by 72 h after plating. Undifferentiated EC cells could not be identified by phase contrast microscopy in cultures containing neurons. Cells capable of forming colonies of undifferentiated cells, under conditions in which the plating efficiency of the P19 EC cells was about 50%, disappeared from these RA treated cultures. Experiments using an EC cell antigen confirmed this observation (see section 4.1.4. below). Thus the drug-induced appearance of neuron-like cells was accompanied by the

disappearance of EC cells. Between 10^{-8} and 5×10^{-8} M, many aggregates which did not contain neuron-like cells did contain fibroblast-like cells. In some experiments, a small proportion of aggregates contained small areas of beating muscle at RA concentrations of 10^{-9} to 10^{-8} M. Ms. M.K.S. Edwards has documented the appearance of both cardiac and skeletal muscle with the P19S1801A1 EC cell line (Edwards, and McBurney, 1983). At 10^{-9} M, the cultures resembled untreated controls and contained only EC cells and small amounts of extra-embryonic endoderm-like cells. In subsequent experiments discussed in this thesis, I used a dose of 5×10^{-7} M, a concentration of RA with which all aggregates contained neuron-like cells and few, if any, EC cells.

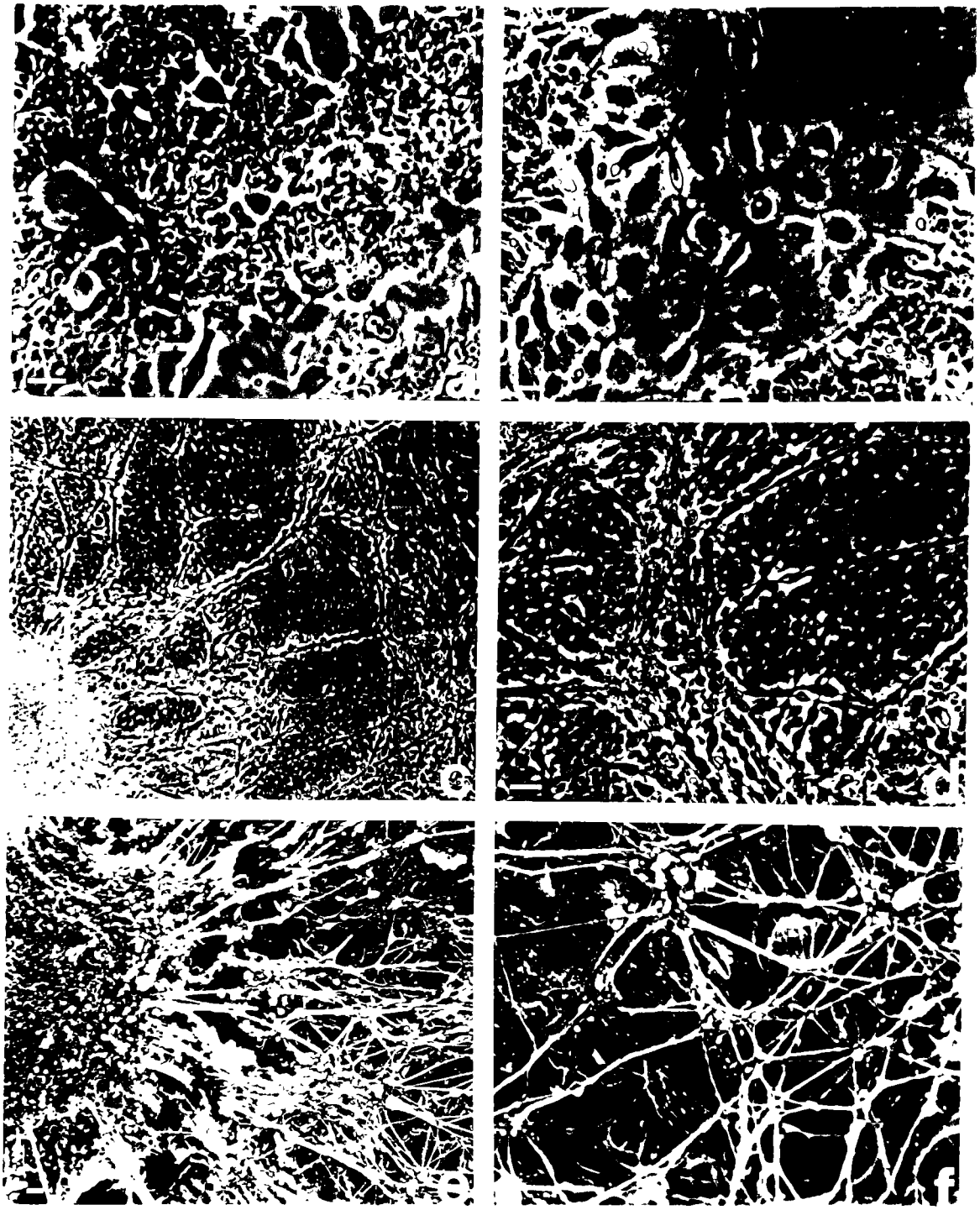


Figure 3.1. Morphologies of the P19 cells following various treatments. The undifferentiated EC cells (a) grow attached to the surface of the tissue culture dish. When they are allowed to aggregate for 5 d and the aggregates are then plated into tissue culture dishes, a small number of cells differentiate into the extra-endoderm-like cell type indicated by the arrow in b. If RA is present in the aggregated cultures, neuron-like and fibroblast- like cells appear within 2 d of plating the aggregates (c and d). Scanning electron micrographs of such RA treated cultures (e and f) show networks of processes extending over a monolayer of fibroblast-like cells. Bars: (a-d) 3.6 um; (e and f) 11.1 um.

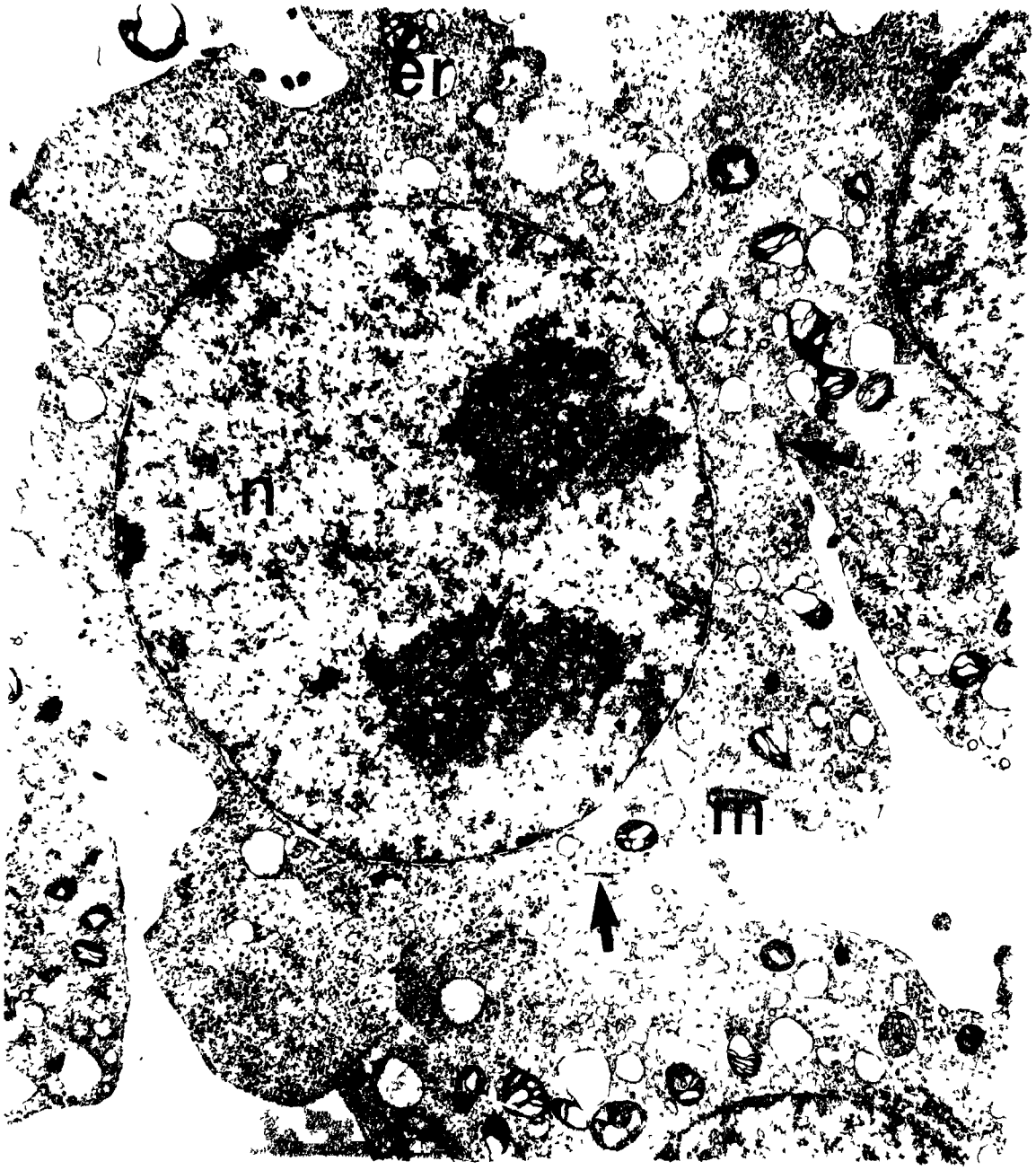

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Figure 3.2. Transmission electron micrographs of P19 cells show that they have a morphology which is typical of EC cells. The nuclear to cytoplasmic ratio is relatively large. A few mitochondria (m), small amounts of swollen endoplamic reticulum (er), and numerous scattered ribosomes characterize the cytoplasm. There appear to be some junctions between the cells (arrows). Magnification is X9420.

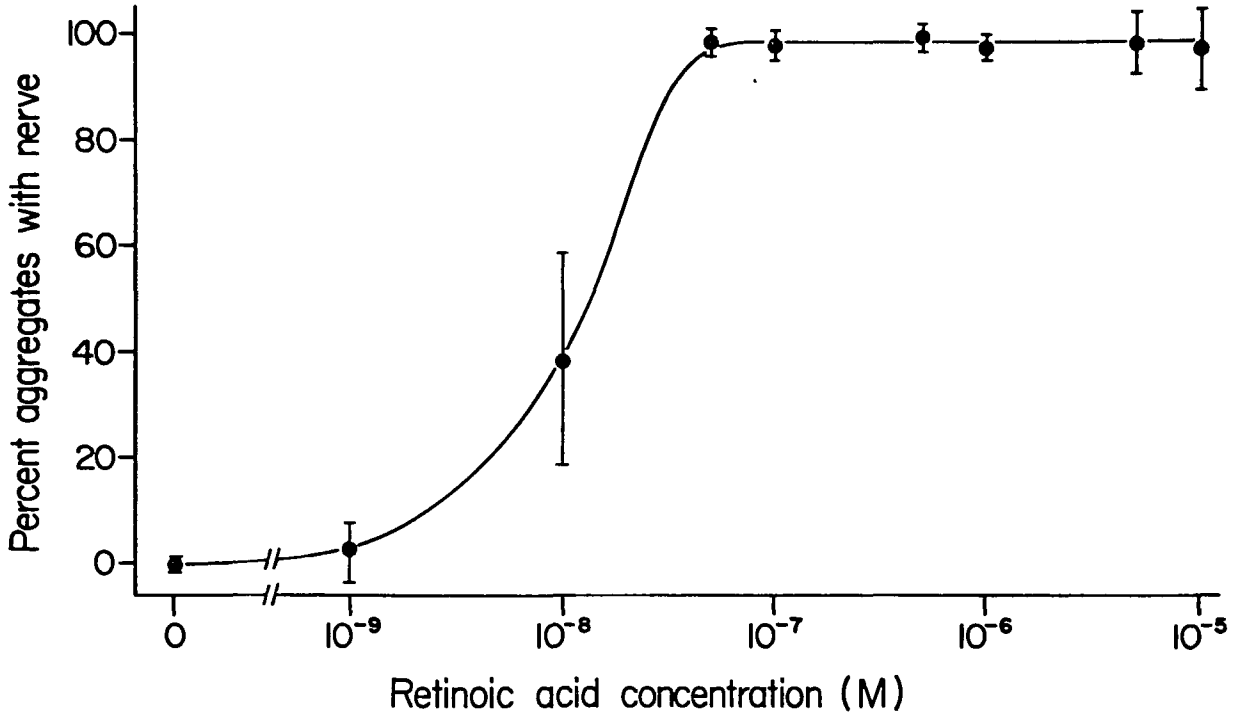
Percent aggregates with nerve
100
80
60
40
20
0
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Retinoic acid concentration (M)

Figure 3.3. Relationship between RA concentration and differentiation of neuron-like cells. The aggregates of P19 cells, continuously cultured in the presence of the drug, were plated after 5 d in suspension and scored 2 to 3 d later. Normally, 50 aggregates were scored for each drug concentration in each experiment. The points indicate the mean obtained from 6 to 11 separate experiments. The sample standard deviation is represented by the vertical bars.

3.1.2 Properties of the neuron-like cells

The experiments in this section and the next were undertaken to identify the cell types present in RA treated cultures.

Microtubules form one of the major cytoskeletal systems in cells. In neurons, the microtubules are arranged in bundles running down the axon parallel to its long axis. Such microtubule bundles were visualized in cells in RA treated cultures using indirect immunofluorescence techniques with antibodies against purified tubulin (Connolly et al, 1978). Figures 3.4a and b show that both the cell bodies and the processes of the neuron-like cells were intensely stained, in a pattern similar to that given by antitubulin staining of neurons in culture (Kalnins and Connolly, 1981). The antitubulin staining revealed varicosities on some of the processes as indicated by the arrow in fig 3.4b. It was also possible to visualize the complex patterns of neurite branching and interconnections in these cultures. The fibroblast-like cells in these cultures also showed staining of microtubules in a pattern similar to that of other fibroblasts (not shown).

Another cytoskeletal system, the 10-nm intermediate filaments, is comprised of proteins specific for various tissue types (Lazarides, 1980, 1982). Neurofilaments are the intermediate filaments specific for neurons and consist of peptide subunits of 210,000, 160,000, and 65,000 MW (Hoffman

and Losek, 1975; Schlaepfer, 1977; Liem et al, 1978). Antibodies prepared against the 160,000 MW neurofilament polypeptide were used with indirect immunofluorescence procedures to examine RA treated cultures (fig 3.4c and d). Staining was localized along the processes and cell bodies of the neuron-like cells. In contrast, the nonneuronal cells were unstained except for nucleoli which stained nonspecifically. Neither the undifferentiated nor the extraembryonic endoderm-like cells in the untreated cultures were stained by this antiserum, indicating the absence of neurofilaments from these cell types.

Cell surface tetanus toxin receptors are another marker for neurons (Bizzinni, 1979). Tetanus toxin binds to neurons in the central nervous system explant cultures via specific cells surface gangliosides (Dimpfel, 1977). Nonneuronal cells from the same cultures do not bind tetanus toxin (Mirsky et al, 1978). The binding of tetanus toxin to neurons in RA treated cultures was visualized using an indirect immunofluorescence assay. Fig 3.5a is a phase contrast micrograph of a portion of a RA treated aggregate. Both the neuronal cell bodies and their processes bound tetanus toxin (fig 3.5b). No other cell type in these cultures or in untreated cultures bound tetanus toxin.

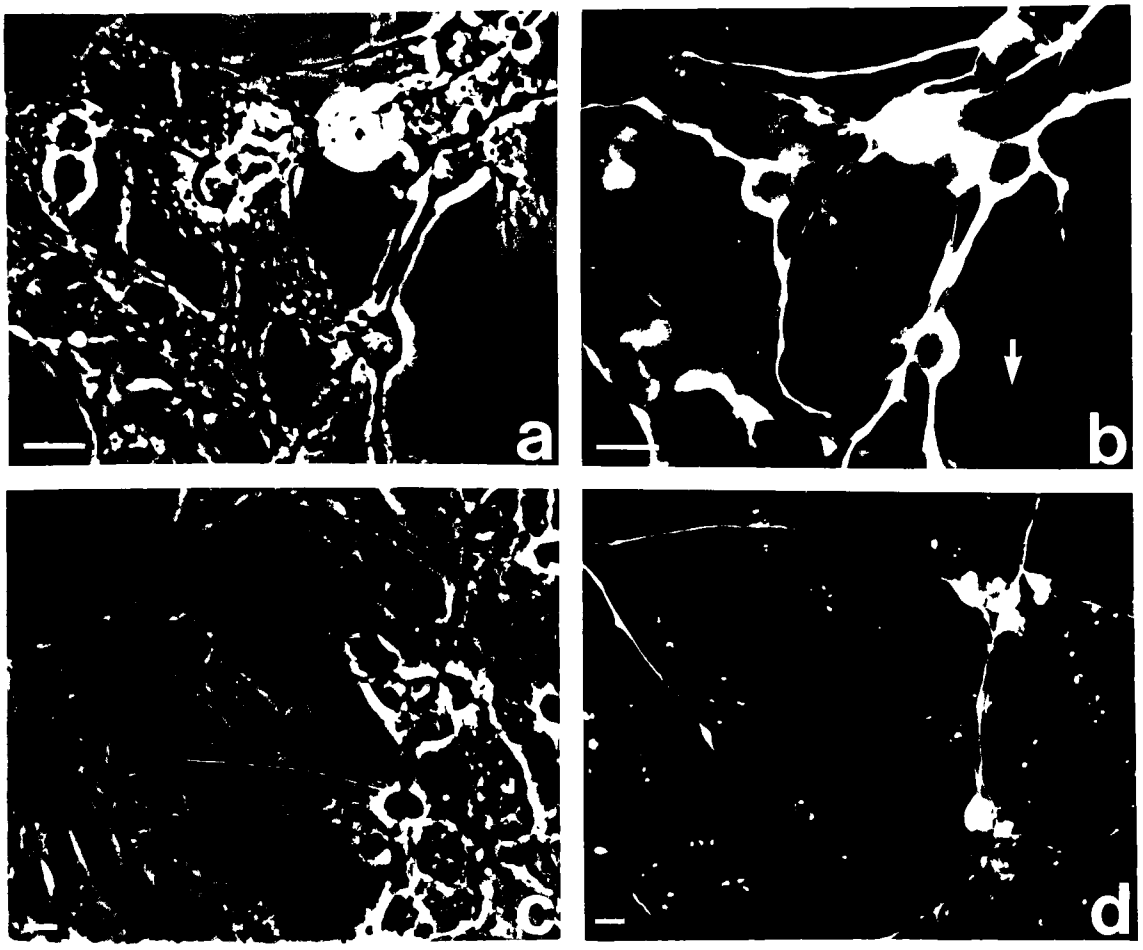
a
b
c
d

Figure 3.4. Visualization of microtubules and neurofilaments in cells from RA treated cultures. Immunofluorescence staining was with antisera to tubulin (b) and neurofilament protein (d). The antitubulin antiserum stained the neuron-like cells, showing their branching patterns. The fibroblast-like cells were lightly stained by this antiserum. The antiserum to neurofilaments stained the neuron-like cells and their processes but did not stain the cytoplasm of the underlying fibroblast-like cells (d). The phase contrast micrographs of the same cells are shown in panels a and c, respectively. Bar, 10 um.

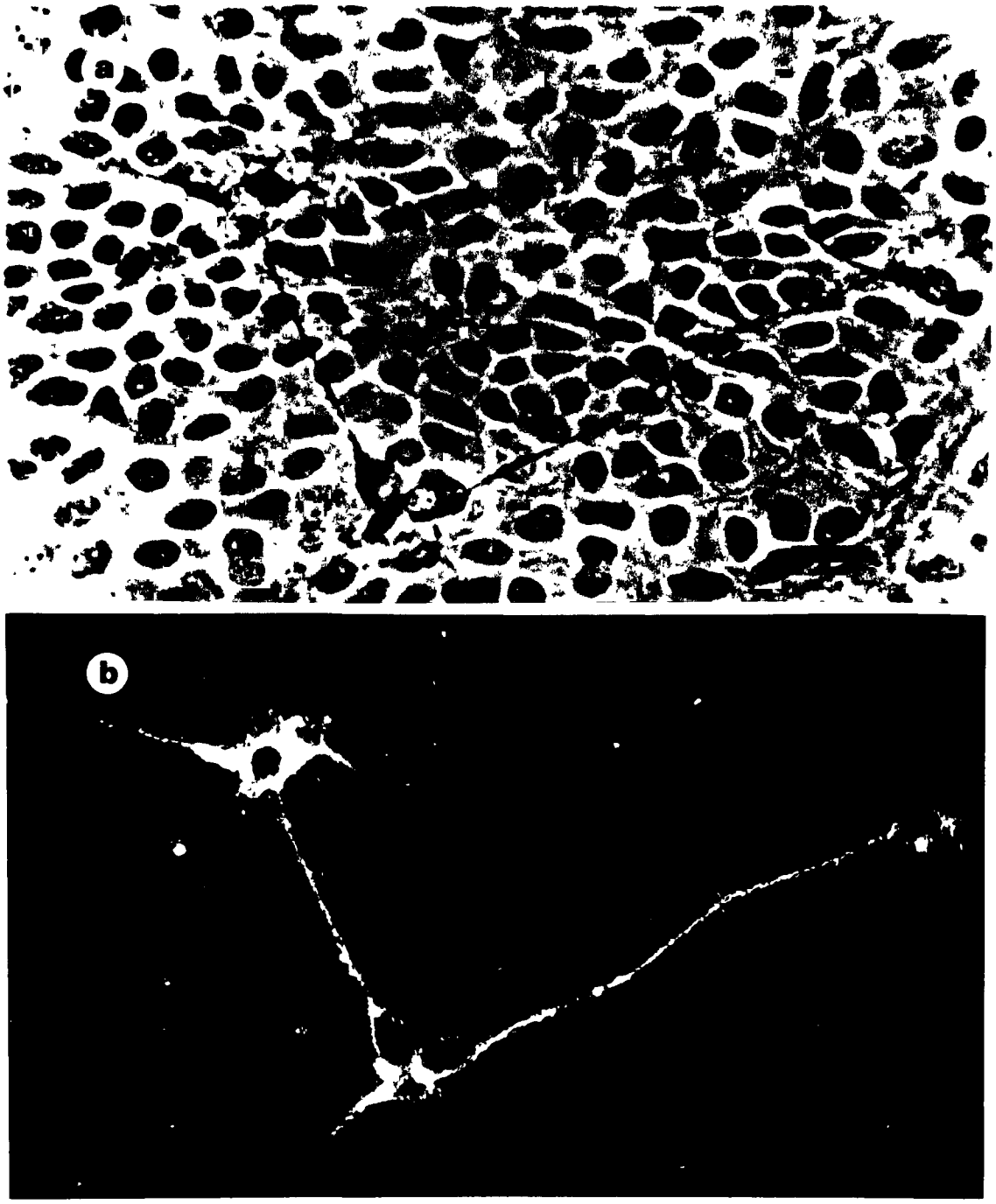
a
b

Figure 3.5. Tetanus toxin labels the neuronal cells in retinoic acid treated aggregates. Panel a shows a phase contrast micrograph of a retinoic acid-treated aggregate with neurons lying on a monolayer of flat cells. The surface of the neuronal cell body and processes are clearly labelled in a patchy fashion characteristic of tetanus toxin labelling (b). (There is some randomly scattered fluorescence associated with the underlying monlayer). Bar 10 um.

Acetylcholinesterase has been frequently used as a marker for neuronal differentiation although this enzyme is present in some nonneuronal tissues (Wilson et al, 1972; Levine et al, 1974; Adamson et al, 1977). Fig 3.6 shows the result of one of four experiments in which the AchE activity was measured in aggregated cultures of both treated and untreated cells. Although the absolute values of AchE activity varied from one experiment to another, the pattern was consistent and the results shown in fig 3.6 are representative. Untreated cultures contained little activity. However, in RA treated cultures, AchE activity peaked at a time when neurons were most numerous.

Choline acetyltransferase (CAT), the enzyme responsible for the synthesis of the neurotransmitter, acetylcholine, has also been used as a neuronal cell marker (Pfieffer et al, 1981) and was assayed in these cultures by M. Rudnicki. This activity was absent from the untreated cultures but did appear in RA treated cultures coordinately with AchE activity (fig 3.7). The decline in specific activity of these enzymes at 10 days is probably a consequence of the proliferation of nonneuronal cells in these cultures.

The above information on the biochemical, immunofluorescent, and anatomical aspects is consistent and indicates the presence of neurons in RA treated cultures.

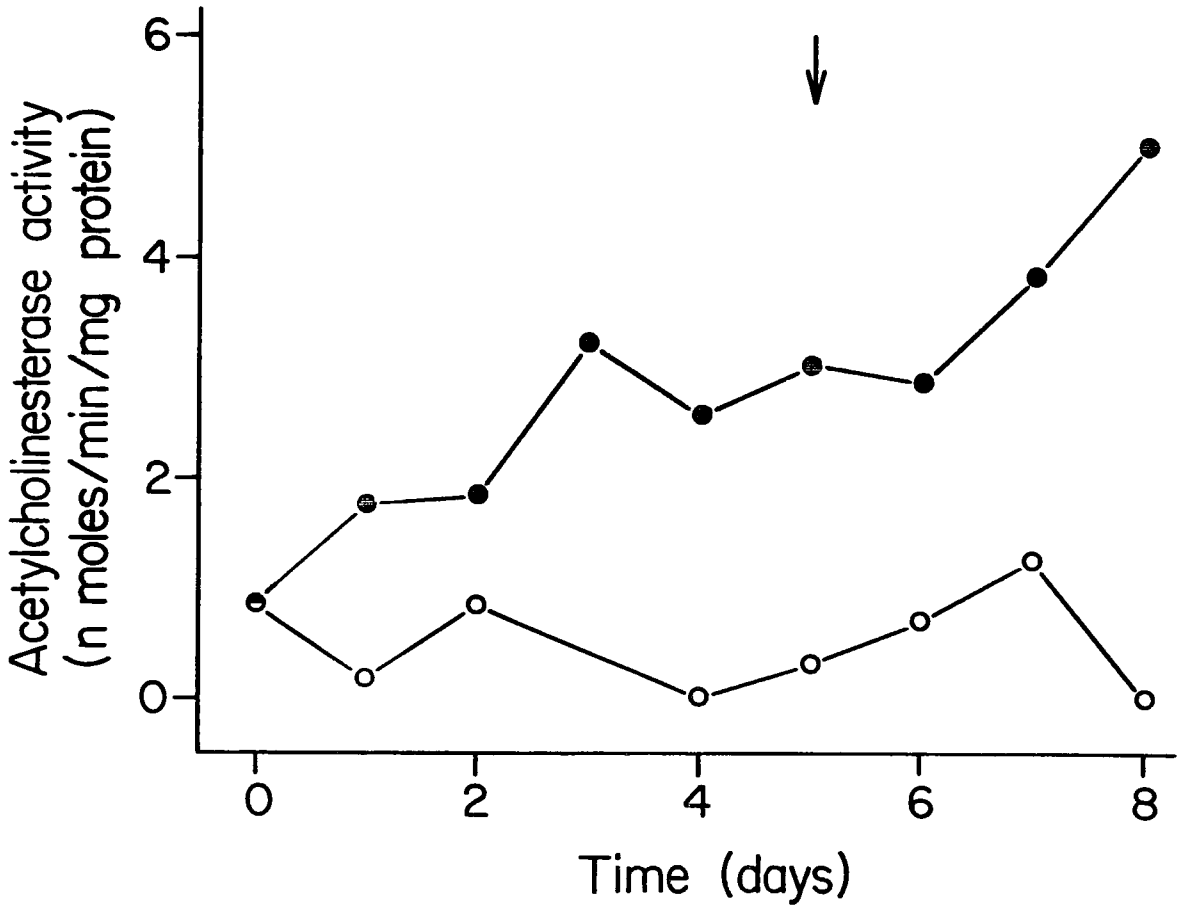
Acetylcholinesterase activity
(n moles/min/mg protein)
6
4
2
0
0
2
4
6
8
Time (days)

Figure 3.6. Acetylcholinesterase appears in RA treated but not untreated aggregate cultures. The specific activity of AchE was determined in treated (closed circles) and untreated (open circles) aggregated P19 cultures. Aggregates were plated at 5 d (arrow) and neurons became abundant by 7 d. Each point represents the activity which was specifically inhibited by BW 284C51 using acetylthiocholine as a substrate. The activity of acetylcholinesterase in adult mouse brain homogenates (strain CH3/He, the genotype of P19 cells) was 53 nmol/min/mg protein, about 10 times the maximum activity seen in RA treated cultures.

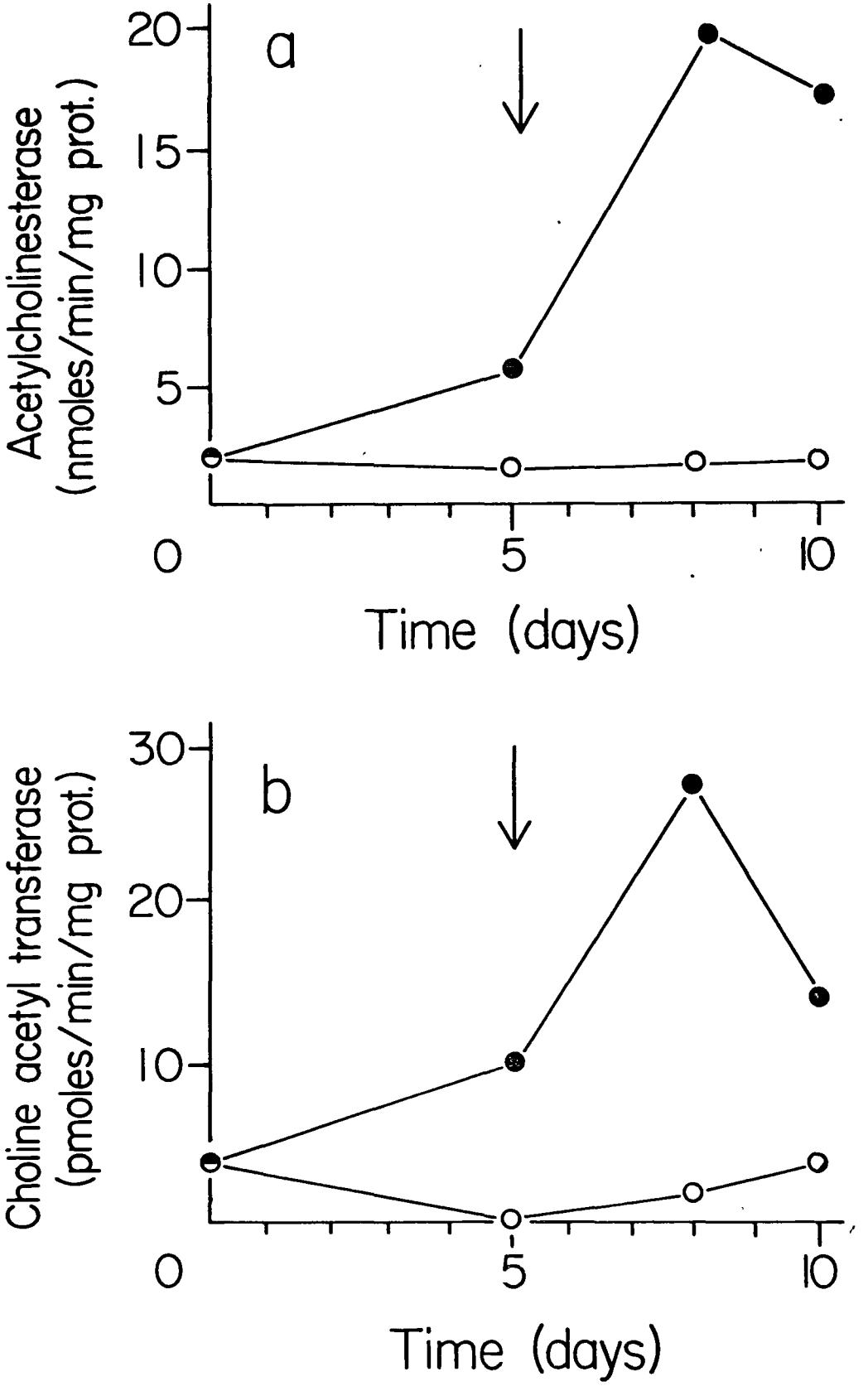
a
Acetylcholinesterase
(nmoles/min/mg prot.)
20
15
10
5
0
5
10
Time (days)
b
Choline acetyl transferase
(pmoles/min/mg prot.)
30
20
10
0
5
10
Time (days)

Figure 3.7. Choline acetyltransferase and acetylcholinesterase activities rise coordinately in RA treated cultures. The specific activities of CAT and AchE were determined in treated (filled circles) and untreated (open circles) aggregate cultures of P19 cells. Aggregates were plated at 5 days (arrow) and neurons became abundant at 7 days. (a) Each point represents an average of the specific activity of AchE which was specifically inhibited by BW 284C51 and the specific activity remaining after ethopropazine was added to the reaction mixture to inhibit pseudoesterases. Adult C3H/He mouse brain extracts contained a specific activity of 120 nmoles/min/mg protein. (b) Each point represents the specific activity of CAT obtained in the presense of eserine, an esterase inhibiter. An activity of 40 pmoles/min/mg protein was found in adult mouse brain.

3.1.3 Nonneuronal cells in retinoic acid treated cultures

The experiments described in this section were undertaken to characterize the nonneuronal cells present in RA treated aggregates of P19 cells.

As discussed above, the tissue-specific intermediate filament proteins provide a means of identifying some tissue types. Antisera directed against vimentin, keratin and glial fibrillar protein were used in immunofluorescence experiments to determine whether mesodermal-like, epithelial, and glial cells were present in these cultures.

Vimentin is an intermediate filament protein originally thought to be present only in mesodermal cells. It is, however, present in many tissue culture cells of nonmesodermal origin (Franke et al, 1978; Franke et al, 1979b). The fibroblast-like cells contain an intermediate filament network which stains with antiserum to vimentin (fig 3.8b). The staining pattern is typical of that of other vimentin-containing intermediate filament systems (Franke et al, 1979b).

The keratins are a class of proteins, ranging in MW from 41,000 to 65,000, found in the intermediate filaments of epithelial cells (Fuchs and Green, 1978). Antibodies directed against keratin did not stain intermediate filaments in any of the cells in RA treated cultures (fig 3.8d). This result suggests that RA treated cultures do not contain epithelial cells.

Glial fibrillar protein (GFP) is the major component of the intermediate filaments in glial astrocyte cells (Kalnins and Connolly, 1981). It is very similar, if not identical, to glial acidic fibrillar protein, a soluble protein isolated from glial cells which has been shown to be a major component of glial filaments (Eng et al, 1971; Bignami et al, 1972). Neither the neurons nor the fibroblast-like cells stained with antiserum specific for GFP. However, 4-5 d after plating RA treated aggregates, a population of cells containing this protein appeared at the junction of the fibroblast-like monolayer and the aggregate. Fig 3.8f shows the staining patterns obtained from this population of glial cells. Thus, RA treated cultures contained three distinct cell types based on the antigenic characteristics of their intermediate filaments (Table 3.2).

RA treated aggregates which were exposed to tetanus toxin were also treated with antiserum directed against GFP (fig 3.9). Fig 3.9d shows that the monolayer of cells underlying the neurons in fig 3.9c is composed of glial cells. The neurons did not stain with GFP. Thus, neurons and glia form two distinct cell populations in RA treated cultures. The glial cells did not label uniformly with anti GFP (fig 3.9d), probably because of the asynchrony with which the mitotically active glioblasts (GFP-) terminally differentiate into mature astrocytes (GFP+).

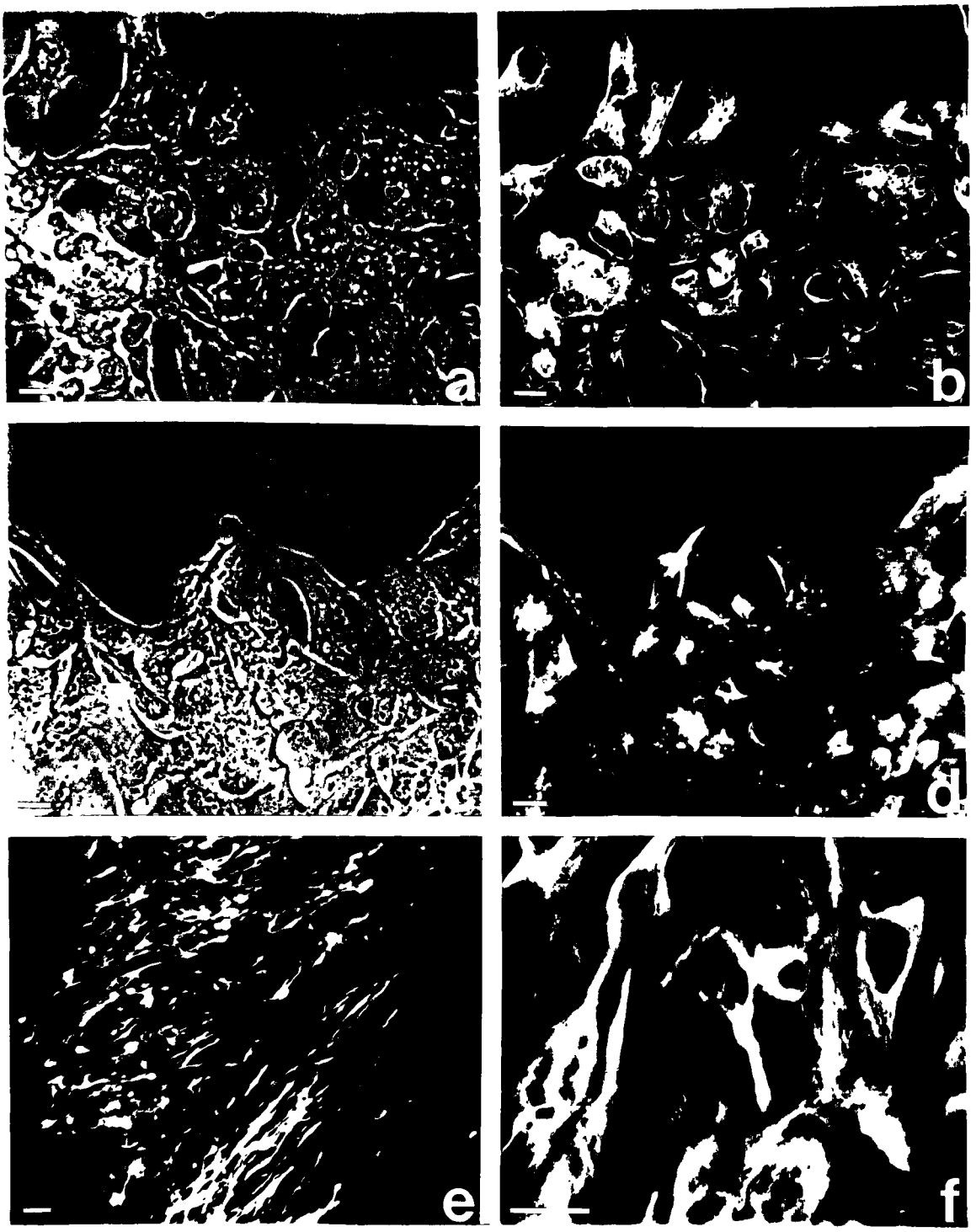
a
b
d
e
f

Figure 3.6. Immunofluorescence staining of intermediate filaments in cells from RA treated cultures. a and b show the same field of fibroblast-like cells photographed by phase contrast (a) and following immunofluorescence staining with antiserum raised against vimentin (b). The typical "basket" pattern of vimentin-containing intermediate filaments is present in virtually all cells. c and d show the same field photographed by phase contrast (c) and following immunofluorescence staining using antiserum against keratin (d). Some nonspecific perinuclear staining was observed, but no intermediate filaments stained with this procedure. Antiglial fibrillar protien antiserum was used in panel e and f. A population of glial filament-containing cells appeared 4-5 d after plating the aggregates. Bar, 20 um.

b
d
a
c

Figure 3.9. Tetanus toxin and anti GFP antiserum label two different cell populations in retinoic acid treated aggregates of P19 cells. All panels show the same field of cells after double labelling with tetanus toxin and anti GFP antiserum. Panels a and c were photographed at a higher focal plane than b and d. Panel c shows the cells photographed using rhodamine filters which allow tetanus toxin binding to be visualized. Panel a is the corresponding phase micrograph. Glial intermediate filaments are present in most of the flat cells but the neurons are clearly not stained with the anti GFAP antiserum (d). The arrows in a and b indicate the position of one of the neurons. Bar 50 um.

3.1.4 Cell types present in untreated F19 cultures

The untreated aggregated cultures of F19 cells contained undifferentiated EC cells and cells which resembled extra-embryonic endoderm. These cultures were analyzed with the antisera described above.

Neither glial fibrillar protein-containing intermediate filaments nor neurofilaments were observed in either cell type. Fig 3.10b shows that the extra-embryonic endoderm-like cells in untreated cultures contained intermediate filaments which were stained with antibody to vimentin. I also observed these filaments in undifferentiated EC cells (not shown). Paulin et al (1980) have shown that PCC3/A11 EC cells also contain vimentin. Thus, vimentin is present in many cell types in both treated and untreated cultures (Table 3.2).

The extra-embryonic endoderm-like cells in untreated cultures contained bundles of intermediate filaments that were stained with antiserum directed against keratin (figs 3.10d and f). These filaments extended from the nuclear region to the periphery of the cell, ending on desmosomes which were shared by the neighbouring cells (fig 3.10f). The presence of cytokeratin-containing filaments in the extra-embryonic endoderm-like cells in untreated aggregates indicates that these cells are different from the fibroblast-like cells present in RA treated cultures. The undifferentiated F19 cells did not stain with antiserum to keratin.

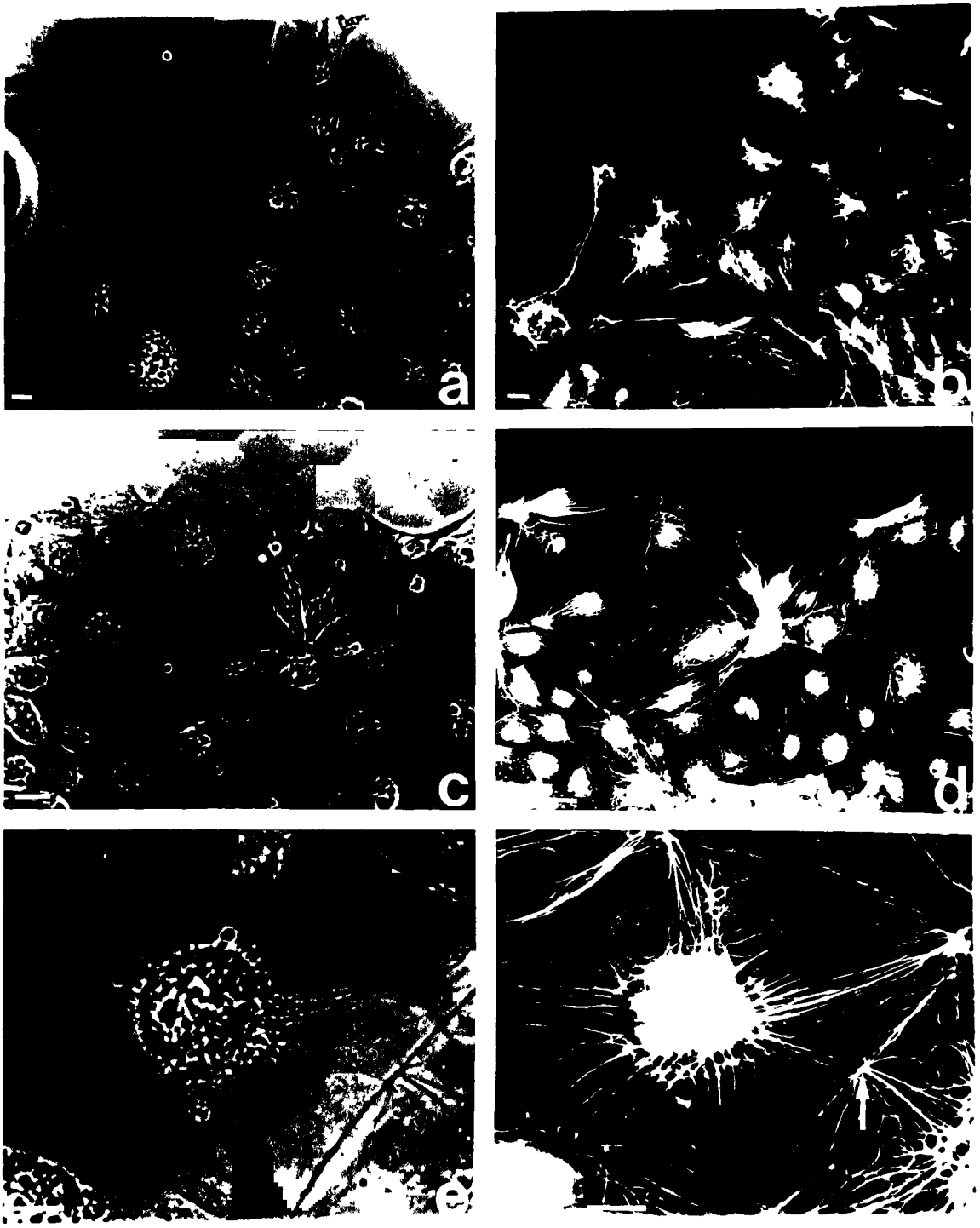
a
b
c
d
e

Figure 3.10. Immunofluorescence staining of the intermediate filaments in the extra-embryonic endoderm-like cells formed in the absence of RA. These cultures were stained with the antibody to vimentin (b) and the antibody to keratin (d and f). Phase contrast micrographs of the same cells are shown in a,c and e respectively. The vimentin intermediate filament system is similar to that seen in the fibroblast-like cells present in RA treated cultures. The endoderm-like cells also contain an intermediate filament system stained by antibodies to keratin. These keratin-containing intermediate filaments extend from the periphery of the nucleus to the cell border where they appear to end on desmosomes shared by neighbouring cells (arrow, f). Bar, 20 um.

TABLE 3.2

CELL TYPES PRESENT IN AGGREGATED P19 CULTURES

	Untreated		Retinoic Acid Treated		
Intermediate Filament Protein	embryonal carcinoma	extra-embryonic endoderm	neuron	astrocyte	fibroblast
vimentin	+	+	-	n.d.*	+
keratin	-	+	-	-	-
glial fibrillar protein	-	-	-	+	-
neurofilament protein	-	-	+	-	-

* n.d. not determined

3.2 DISCUSSION

The aim of this project was to use drugs to simplify the differentiation patterns of EC cells *in vitro* so that it would be possible to examine the important determination events leading to the differentiation of a limited number of cell types. My experiments showed that, in the presence of RA, EC cells differentiated into a limited spectrum of cell types, namely neurons, glia, and fibroblast-like cells. The experiments described in this chapter convincingly demonstrate that neurons and glial cells are present in RA treated cultures of aggregated P19 EC cells.

The neurons present in these cultures were initially identified on the basis of their distinctive morphology. As observed with both the light and scanning electron microscopes, the processes from these neurons formed a branched and interconnected network. Immunofluorescent staining of these cells with antitubulin antiserum highlighted the varicosities on some of the neuronal processes, structures which are often seen on isolated neurons using this technique (see example in Kalnins and Connolly, 1981). The neuronal nature of these cells was confirmed by the presence of neurofilaments in their cytoplasm and tetanus toxin receptors on their cell surface, two neuron-specific cell markers. Identification of neurotransmitters and associated enzymes is another way of characterizing neurons. This approach was taken by Pfeiffer et al (1981) who have isolated a line of

EC cells which appeared to be spontaneously committed to the formation of cholinergic neurons. Elevated levels of AchE and CAT were present in the differentiated cultures derived from these cells. The activities of these enzymes were elevated coordinately in RA treated cultures of P19 cells and were highest when the cultures contained the largest numbers of morphologically identifiable neurons. However, the activities increased many days before neurons became apparent. Perhaps these enzymes are expressed very soon after neuronal determination. The presence of CAT and AchE suggests that these neurons may be cholinergic.

In addition to neurons, RA treated cultures of P19 cells also contained glial astrocytes, identified by their staining with antibody to GFP. In the normal embryo both neurons and glia are derived from the ectodermal germ layer. Both neural cell types resemble the embryonic rather than the adult because they lack markers of mature neural cells. For example, the neurons do not stain with antibody to Thy-1 glycoprotein (see review by Fields, 1979) and the glial cells do not contain S100 protein (Moore, 1968; reviewed by Zomzely-Neurath and Walker, 1980) (Dr. J. Bell, personal communication). I have not been able to fully characterize the fibroblast-like cells. The presence of vimentin-containing intermediate filaments in these cells does not imply that they are mesodermally-derived, since vimentin is present in most cells in tissue culture (Franke et al, 1978;

Franke et al, 1979b), including P19 EC cells. Since the fibroblast-like cells did not develop into muscle, adipose, or cartilagenous tissue, it seems likely that these fibroblasts are analogous to cells of similar morphology present in cell cultures derived from embryonic brain (Abney et al, 1981).

The extra-embryonic endoderm-like cells in untreated but aggregated cultures of P19 cells contained two intermediate filament networks, one of which could be visualized by antiserum to prekeratin and the other by antiserum to vimentin. Paulin et al (1982) have reported prekeratin-containing intermediate filaments in parietal extra-embryonic endoderm cells obtained from F9 cells treated with RA in monolayer and it has recently been reported that vimentin and cytokeratin-containing intermediate filament networks coexist in the parietal endoderm cells of the embryo (Lane et al, 1983). I did not observe cells with prekeratin-containing intermediate filaments in RA treated aggregated P19 cells and therefore conclude that extra-embryonic endoderm does not appear under these conditions with this cell line. The absence of endoderm from these cultures indicates that the differentiation of this tissue type is not a prerequisite first step in EC cell differentiation. More importantly, it demonstrates that P19 cells differentiate into a different set of cell types in the presence of RA than they do in its absence. This suggests that RA might affect the decision events which determine the fate of undifferentiated P19 cells.

RA has been previously shown to induce the differentiation of EC cells. The cell type(s) formed by RA-exposed EC cells seems variable depending on the particular cell line used and on whether the cells are exposed to the drug as aggregates or in monolayer cultures. The EC cell line F9 (Berstine et al, 1973), has been most extensively studied. Strickland and Mahdavi (1978) first showed that RA treated monolayer cultures of F9 cells differentiate into cells resembling extra-embryonic endoderm cells. If subsequently treated with dibutryl cAMP, these cells further change into parietal endoderm (Strickland et al, 1980). Hogan et al (1981) have shown that some cells in aggregates of RA treated F9 cells develop into visceral endoderm. Kuff and Fewell (1980) have observed 'neuron-like' cells in RA treated cultures of F9 cells but the neuronal nature of these cells has not been unequivocally established and these cells may be in fact have been the parietal endoderm cells described by Strickland et al (1980). I found that some F9 cells differentiated into neurons when they were aggregated and treated with high concentrations of RA (greater than 10^{-6} M). However, only small numbers of neurons were formed, many of the aggregates contained no neurons, and some of the nonneuronal cells did contain prekeratin intermediate filaments. Thus, extra-embryonic endoderm was formed by F9 cells in conditions in which none is made by P19 cells, and F9 cell only inefficiently developed into neurons in our hands.

Aggregation was first used by Martin and Evans (1975a and b) to induce differentiation of EC cells in culture. Speers et al (1979) have shown that hexamethylene bisacetamide treatment of an EC cell line results in the formation of differentiated cells with epithelial or fibroblast morphologies depending on whether or not cells are aggregated during drug treatment. The effects of RA reported in this thesis and those on F9 cells reported by Hogan et al (1981) are also dependent on cell aggregation. The effects of aggregation may result from inside-outside interactions similar to those hypothesized in other mammalian developmental processes (Herbert and Graham, 1974).

I have observed that the differentiated cell types appeared in a reproducible sequence after plating RA treated aggregates. Fibroblast-like cells appear initially (1 d), followed by neurons (2 d), and glial cells (4-5 d). The morphology of the cells and the sequence of their appearance is identical to that seen in explants of brain from 10 d-old rat embryos (Abney et al, 1981; Raju et al, 1981). This sequence probably reflects the rates of neuronal and glial maturation and suggests that RA causes a rapid commitment to embryonic ectoderm and neuroepithelium which is followed by differentiation of neural cells. This is discussed further in chapter 5. One could speculate that the aggregate supplies the necessary three dimensional environment found <u>in vivo</u> in the neural tube.

Chapter IV

MECHANISM OF ACTION OF RETINOIC ACID

4.1 RESULTS

4.1.1 Induction versus selection

There are at least two different models which could be used to explain the effects of RA on P19 cells. The first proposes that RA acts by directly inducing P19 cells to differentiate along the developmental pathway leading to neurons and glial cells. Alternatively, RA could act by selecting for cells capable of differentiating into these cell types. The observation that neurons and glial cells do not appear in P19 cultures, which have not been exposed to RA, suggests that RA might act by induction. The following experiments provide further support for the induction model.

RA did not kill EC cells. Fig 4.1a shows that the number of colonies formed by P19 cells in the presence of RA at concentrations up to 10^{-5} M was similar to the number obtained in the absence of the drug. The colonies formed in RA concentrations above 5×10^{-8} M were composed of the fibroblast-like cells, whereas colonies formed at lower RA concentrations were composed of cells with EC morphology.

The growth rate of P19 cells in medium containing RA was measured during a 48 h drug exposure. 48 hours of exposure

to RA is sufficient for neurons and glial cells to differentiate in all aggregates exposed to RA. As can be seen in fig 4.1b, RA had little effect on the growth rate during this time. In other experiments, I found that aggregates grown in the presence of 5 $X10^{-7}$ M RA for 9 d in suspension culture contained 80% of the number of cells found in untreated aggregates grown for the same length of time. Thus, it seems unlikely that the effects of RA can be explained by it simply killing cells destined to develop into other cell types such as muscle.

The cells of the P19 cultures may be heterogeneous with respect to their response to RA. Although undifferentiated cells disappeared from RA treated cultures, RA might act by selecting for the overgrowth of a subpopulation of cells precommitted to form neurons and glial cells (see section 4.1.4 below). To test this possibility, 25 P19 cells were individually picked and plated into separate culture dishes. 19 formed colonies, and 17 were sucessfully expanded into clonal cell lines. 15 of these 17 cell lines responded to RA in a manner similar to that for the parental culture (Table 4.1). The exceptions were P19S8, a tetraploid clone, which gave rise to a few neurons even in the absence of RA, and P19S11, which also gave very small numbers of neurons in a minor fraction of untreated aggregates. Thus, each cell within the P19 cultures appears to be capable of responding to RA.

TABLE 4.1

RESPONSE OF SEVERAL SUBCLONES OF P19 TO RETINOIC ACID (RA)

SUBCLONE	NUMBER OF AGGREGATES CONTAINING NEURONS	
	WITH RETINOIC ACID[1]	WITHOUT RETINOIC ACID
S1	79/80[2]	0/50
S2	60/60	0/50
S3	56/60	0/50
S4	39/40	0/44
S5	37/40	0/50
S6	49/50	0/76
S7	40/40	ND[3]
S8	33/36	39/83
S9	70/70	ND
S10	60/60	0/50
S11	61/61	10/42
S12	75/75	0/50
S13	61/61	0/41
S14	55/56	0/18
S15	65/65	0/70
S16[4]	99/100	0/50
S17	60/60	0/46

1. Cultures were treated with 5 x 10^{-7} M retinoic acid.

2. Number of aggregates containing neurons 3 days after plating aggregates/ Number of aggregates examined.

3. ND = no data.

4. S16 cells are referred to as P19S18 in text of thesis.

If RA affects determination events, it might be possible to remove the drug following commitment but before cytodifferentiation. RA was therefore removed from aggregated cultures at various times after the beginning of the experiment. All cultures were plated 5 d after aggregation and scored 2-3 d later. The results, illustrated in fig 4.2, indicate that a 48 h exposure to the drug was adequate to ensure that neurons formed in virtually all aggregates. Thus, RA acts very early, 3 to 4 days before differentiated cells first become evident. There would be little time for selection to occur and as demonstrated above, there are no toxic effects during this 48 hour period.

Monolayer cultures of P19 cells could be treated for 48 h with RA before aggregation and subsequent culture in the absence of the drug. In such experiments, neurons developed abundantly from each aggregate. When monolayer cultures were treated with RA but not aggregated, virtually all the cells differentiated into fibroblast-like cells and few, if any, neurons were found. Apparently neuronal differentiation was greatly facilitated by both RA treatment and the high density achieved by aggregation. Aggregation and RA treatment are two separate conditions which may be met simultaneously or RA treatment may precede aggregation.

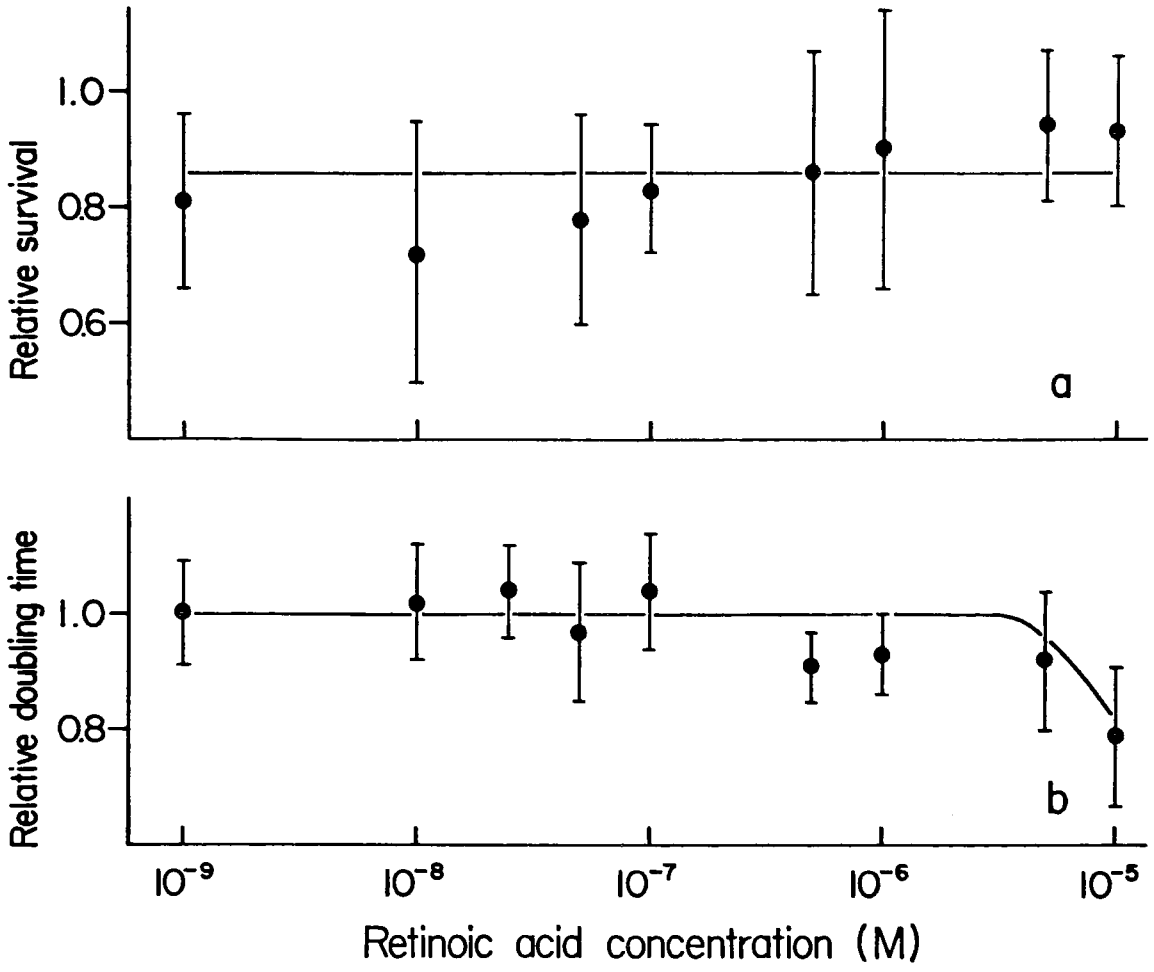
Relative survival
1.0
0.8
0.6
a
Relative doubling time
1.0
0.8
b
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Retinoic acid concentration (M)

Figure 4.1. RA is not toxic to P19 cells. The plating efficiency and growth rate of P19 cells were measured in cultures containing RA. The relative survival (a) was calculated from experiments in which P19 cells were plated at low density (°200 cells per 60 mm diameter petri dish) and the number of colonies counted after 10 d. The points represent the mean in 3-5 separate experiments. The mean plating efficiency in the absence of RA was 41%. The doubling time of P19 cells was calculated after growing the P19 cells (seeded at 10^5 cells / ml) for 48 h in the presence of RA. The ratio of the doubling time of the treated cultures to the doubling time of the untreated cultures is plotted versus the RA concentration in b. The points represent the mean of 12 or 24 separate determinations obtained in 3 or 6 separate experiments. The mean doubling time of the controls was 16 h. Vertical bars represent the sample standard deviation.

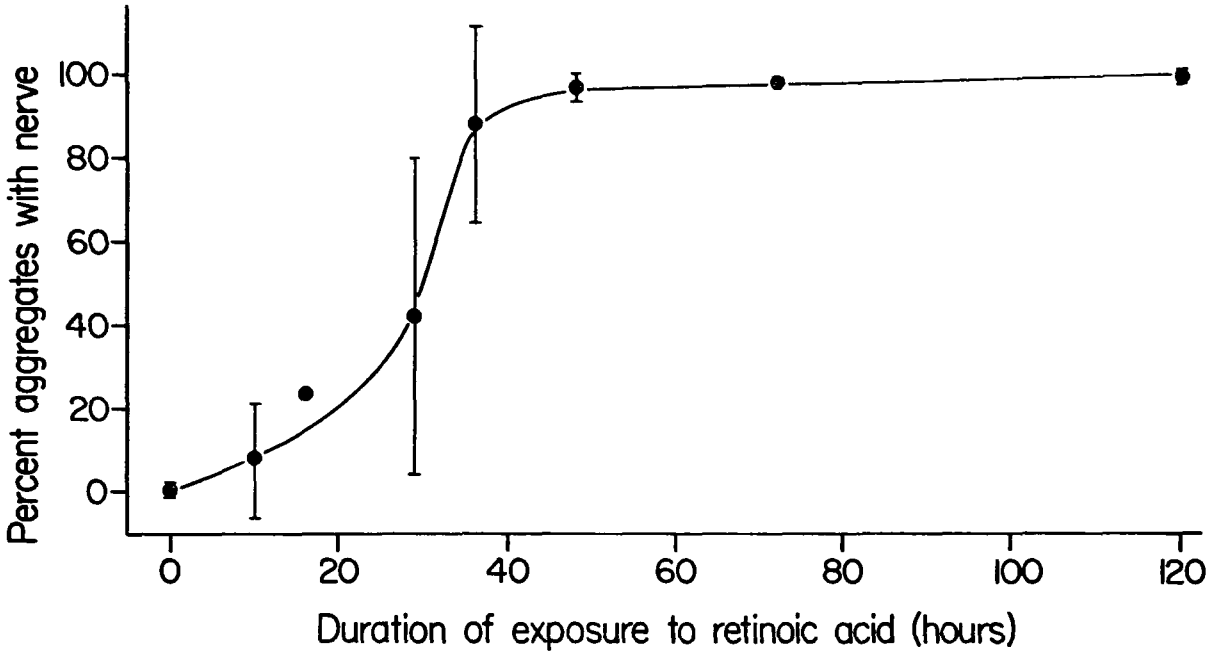
Percent aggregates with nerve
100
80
60
40
20
0
0
20
40
60
80
100
120
Duration of exposure to retinoic acid (hours)

Figure 4.2. RA need not be continuously present in aggregated cultures. RA (5×10^{-7} M) was added at the initiation of aggregation and removed at various times by washing 3 times with normal medium. Aggregates were plated at 5 d and scored for the presence of neurons 2-3 d after plating. Each point represents the mean of 2-6 separate experiments. The sample standard deviation was calculated for those concentrations which were tested in at least 3 experiments.

4.1.2 Retinoic acid analogues

RA has diverse biological effects on many different cell types. The intracellular targets of RA which are important in the differentiation of P19 cells, may be similar to those in other RA-sensitive biological systems (although the consequences of RA action are very different). Structural analogues of RA have been used to investigate which parts of the RA molecule are important for biological activity (Lotan, 1980). A number of retinoids were tested for their ability to induce P19 cells to differentiate into neurons. Table 4.2 shows that retinoids with major modifications to the C15 carboxylic acid group of RA required much higher concentrations to attain the same efficiency as RA. The relative efficencies of the analogues are similar to those obtained in some other biological systems (Jetter and Jetter, 1979; Lotan, 1980; Lotan et al, 1981) and suggest that the intracellular mechanisms of retinoid action in P19 cells are similar to those in other systems and may involve binding to the cRABP.

TABLE 4.2

EFFICIENCIES OF SOME RETINOIDS ON INDUCTION OF NEURONAL DEVELOPMENT

RETINOID	HALF-EFFECTIVE DOSE[1] ($\times 10^{-8}$ M)	EFFICIENCY RELATIVE TO ALL TRANS RA
All trans RA	2.8	1
13-cis RA	3.8	.72
retinal	50	.06
retinol	280	.01
retinyl acetate	430	.007
TMMP[2] analogue of ethyl retinoate	450	.006
TMMP analogue of N-ethyl retinamide	>1000	-

1. Dose at which 50% of plated aggregates contained neurons 3 days after plating the aggregates.

2. TMMP = trimethylmethoxyphenol.

4.1.3 Polyamines

RA is an anti tumour promotor. This activity may result from RA mediated suppression of ornithine decarboxylase, a key enzyme in polyamine biosynthesis (Verma et al, 1978). Since polyamines may play a role in some other differentiation systems (Fish et al, 1981; Scott et al, 1982), it seemed possible that the RA effect on P19 could be mediated by a decrease in intracellular polyamine levels. Cultures of aggregated P19 cells were exposed to the following drugs, both in the presence and absence of RA: 3×10^{-6} M spermidine, a polyamine, 3×10^{-4} M alpha-methylornithine and 10^{-7} M methylglyoxal-bis-(guanyl-hydrazone), two inhibitors of polyamine biosynthesis, a tumour promotor, 10^{-5} M phorbol myristate acetate and 3×10^{-6} M dexamethasone, an anti-tumour promotor. These drug concentrations were non-toxic in 48 h growth tests. None of these drugs had any effect on the P19 cultures, suggesting that changes in polyamine metabolism do not mediate the developmental effects induced by RA and that tumour promotors are ineffective in altering the response of P19 cells.

4.1.4 Mutant cell lines

Since the important biological consequences of RA treatment were not obvious, I used a genetic approach to attempt to determine which events are crucial to the differentiation process. This section describes the stepwise isolation of

P19 derived cell lines which do not differentiate into neurons in the presence of RA. P19S18 cells, a subclone of P19 (see section above), were cultured in the continuous presence of 10^{-7} M RA, and a partially nonresponsive clone P19S18RAC6 was isolated (fig 4.3). P19S18RAC65 (RAC65) is a subclone of P19S18RAC6 which was isolated in the presence of 10^{-5} M RA. RAC65 cells do not differentiate into neurons at concentrations of RA as high as 10^{-6} M (fig 4.3). RAC65 cells are similar in morphology to P19 EC cells and have 42 chromosomes.

No cells with EC morphology were apparent in RA treated P19 aggregates by 7-8 days after the beginning of the experiment. In contrast, 7-8 day old identically treated cultures of RAC65 cells consisted entirely of EC-like cells. In order to document this observation, I used a monoclonal antibody, AEC3A1-9, which detects an antigen found on undifferentiated EC cells but which is not present on differentiated cells (J.F. Harris et al, in preparation). The AEC3A1-9 antigen is closely related to the antigen detected by SSEA-1 (Solter and Knowles, 1978). As can be seen in fig 4.4b, the number of cells carrying the antigen in RA treated P19 aggregates, as detected by indirect immunofluorescence, decreased after 2 days and reached a plateau of 25% by 6-8 days. Cells from DMSO treated aggregates showed a similar pattern. The fluorescence on the cells after 6 days was very weak and quantitative immunoabsorption (Dr. J.F. Har-

ris) experiments indicated that the cells in these RA treated cultures contained only about 1% of the antigen concentration present on untreated cells (fig 4.4a). The amount of antigen on the control untreated cells decreased, but to a much lesser extent. This decrease can be attributed to the formation of extraembryonic endoderm. Fig. 4.4c shows that the amount of antigen on RA treated RAC65 cells did not decrease during the experiment confirming the observation that these cultures consisted of EC cells. Experiments on the growth rate of RAC65 cells in monolayer cultures provided further support for this idea. The RAC65 cells grew continuously and rapidly in both the presence and absence of RA (fig 4.5b), whereas the parental P19 cells did not grow continuously in RA treated cultures (fig 4.5a). After 2 d in RA, proliferation ceased and the P19 cells changed into cells with a fibroblast morphology. No such change occured in RA-treated RAC65 cultures.

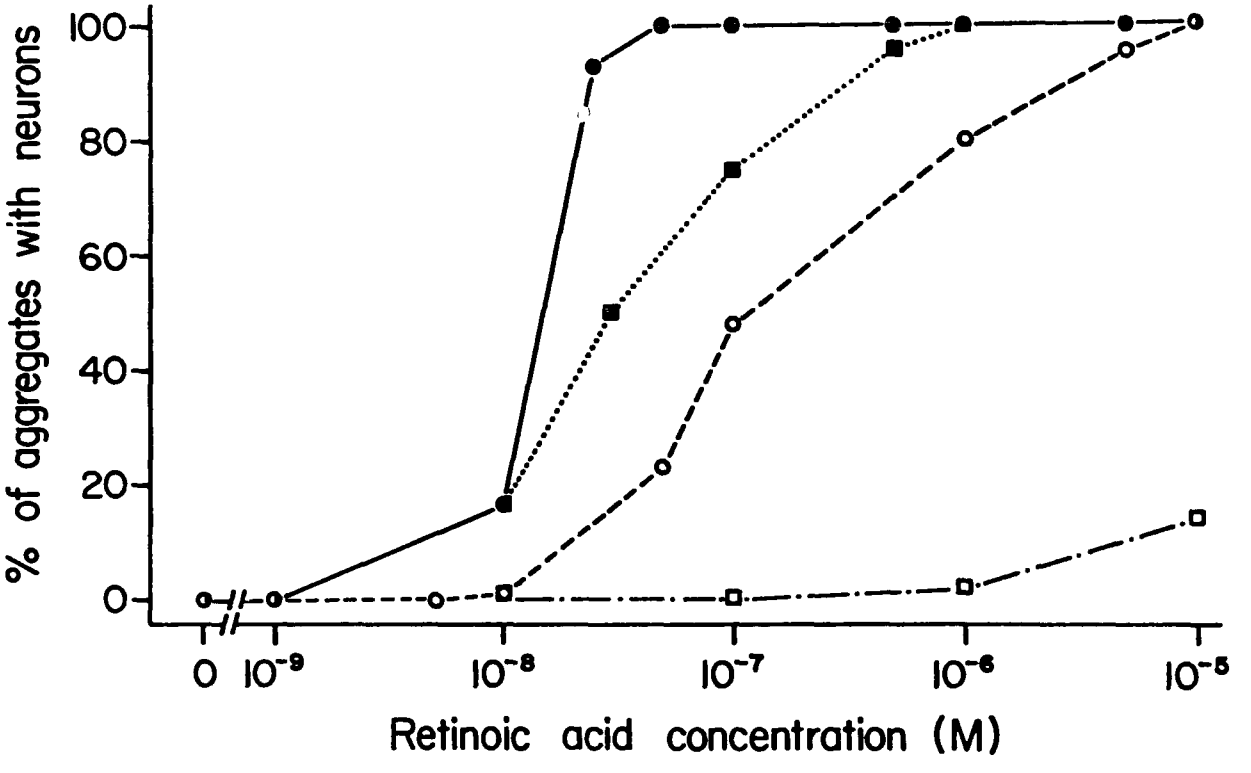
% of aggregates with neurons
100
80
60
40
20
0
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Retinoic acid concentration (M)

Figure 4.3. RAC65 cells do not differentiate into neurons in the presence of RA. Aggregates of cells were cultured for 5 days in the presence of drug, plated and scored 2-3 days later. Normally, 50 aggregates were scored for each drug concentration in each experiment. The points indicate the mean obtained from several experiments. P19S18 (filled circles), P19S18RAC6 (open circles), RAC65 (open squares), and HY-1 (filled squares).

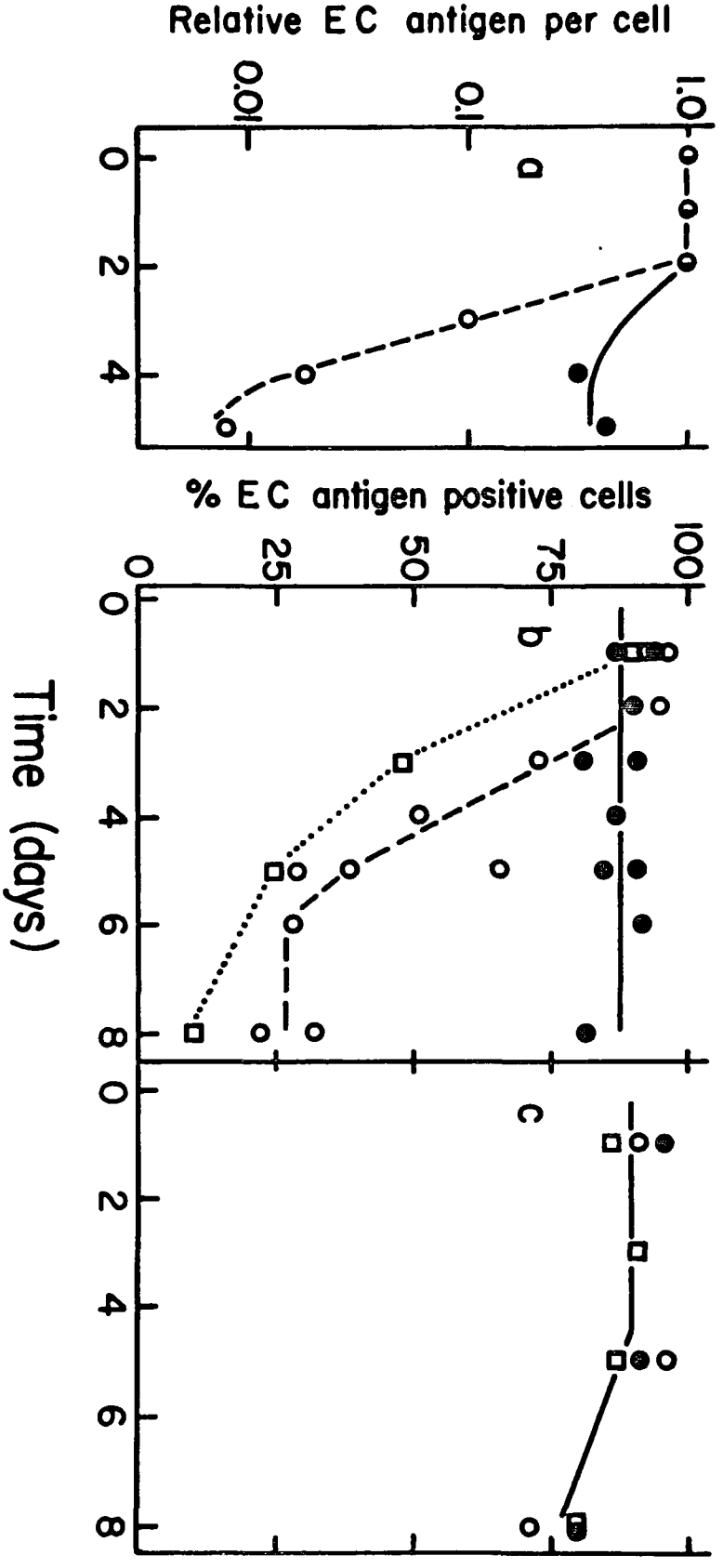
Relative EC antigen per cell
0.01
0.1
1.0
a
% EC antigen positive cells
0
25
50
75
100
b
c
Time (days)
0
2
4
6
8

Figure 4.4. AEC3A1-9 embryonal carcinoma cell associated antigen disappears from RA and DMSO treated aggregate cultures of P19 cells but not from similarily treated RAC65 cells. (a) The amount of antigen per P19 cell was determined by an absorption procedure after gluteraldehyde fixation of the cells. The percent of antigen positive cells in P19 (b) and RAC65 (c) cultures was measured using an indirect immunofluorescent procedure. Each point represents the mean of 2-3 experiments. 500 cells were scored for each point in each experiment of panels b and c. Untreated cells (filled circles), 5X 10^{-7} M RA treated (open circles), and 1% DMSO treated (open squares).

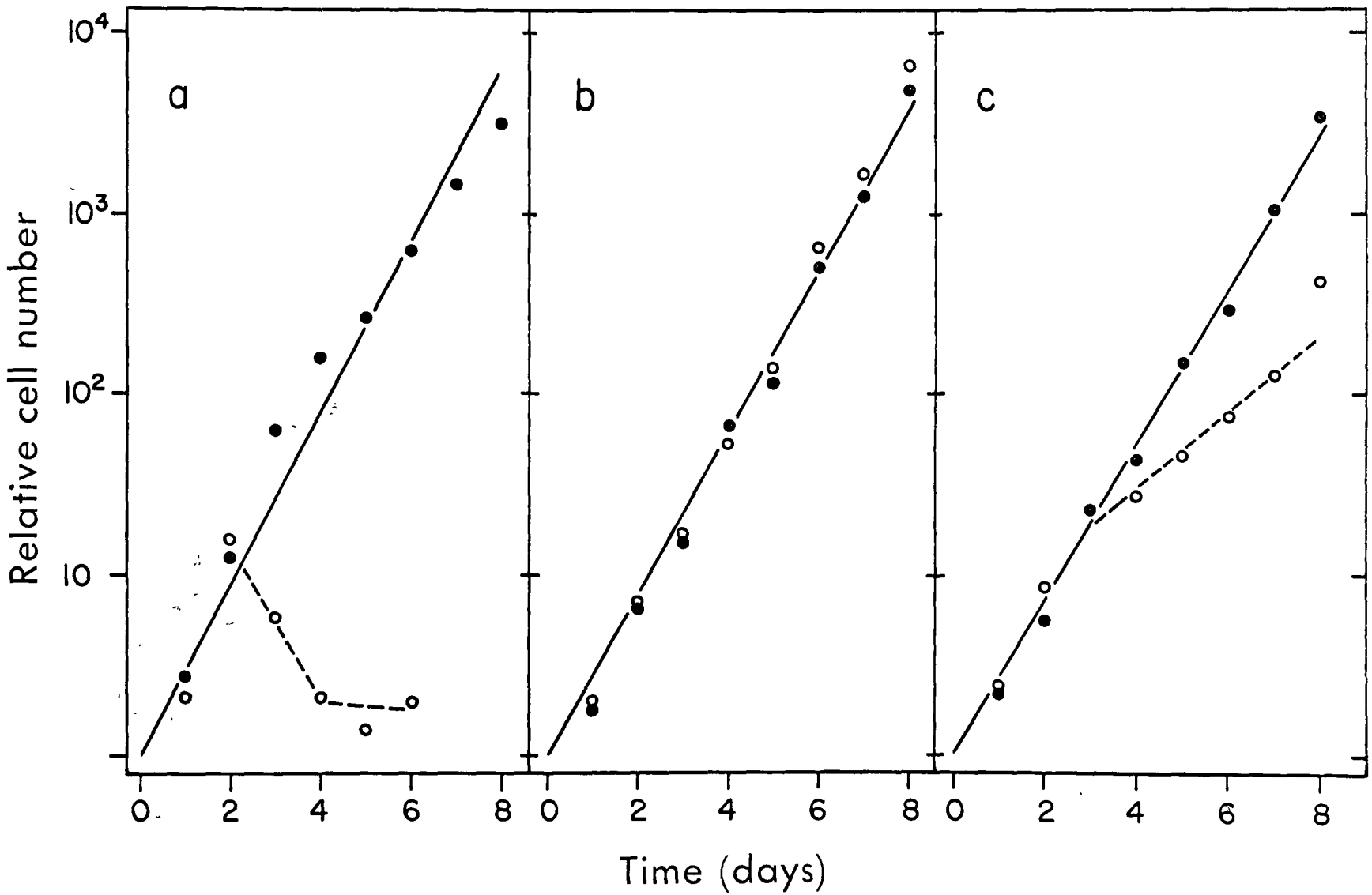
a
b
c
Relative cell number
10^4
10^3
10^2
10
0
2
4
6
8
Time (days)

Figure 4.5. The growth rate of RAC65 cells is not changed in the presence of 5×10^{-7} M RA. The growth rate of cells in monolayer cultures was determined by plating cells at a concentration of 10^5 cells/ml into duplicate tissue culture dishes. The cells from the two dishes were counted after 24 and 48 hours respectively. Two new dishes were seeded from the cells in the 48 hour dish and the process was continued for 8 days. The points represent the mean of 2 experiments. P19 cells (a), RAC65 cells (b), and HY-1 cells (c) were grown in the continuous presence (open circles) or absence (filled circles) of retinoic acid. The doubling times were as follows: P19; 14.7h in untreated cultures, RAC65; 16.3h in treated and untreated cultures, and HY-1; 16.7h in treated and untreated cultures for 3 days at which time it increased to 20.1h in treated cultures.

In order to determine whether the RAC65 phenotype was dominant or recessive, cell-cell hybridization experiments were carried out in which RAC65 cells were fused to P19S18O1A1, a 6-thioguanine and ouabain-resistent clone of P19 cells (McBurney et al, 1982). Clones of hybrid cells were selected in HAT (Littlefield, 1964) medium supplemented by 1.5 mM ouabain. Two clones, HY-1 and HY-2 (isolated by Dr. M.W. McBurney), were examined in detail. The basic observation was that the mutant phenotype was recessive because both hybrid lines differentiated into neurons when aggregated in the presence of RA (fig 4.3), although a few undifferentiated cells remained in the aggregates. Hybrids between RAC65 and O1A1D3, which I isolated, also differentiated into neurons (data not shown). O1A1D3 cells are responsive to RA but non-responsive to DMSO (Edwards, MSc. thesis, 1983). In monolayer growth experiments, the results were not as clear-cut. HY-1 and HY-2 cells grew more slowly after 3 days in RA but did not abruptly cease proliferation as did P19 (fig 4.5c). Some of the hybrid cells had a chromosome number less than the combined parental number of 82. Thus, the intermediate phenotype of the hybrids may be a consequence of the heterogeneity of the chromosome numbers in the hybrids and the apparent segregation of recessive alleles.

4.1.5 Cell volume changes during differentiation

The cells in P19 aggregates became smaller in RA treated cultures as compared to those from untreated cultures. Fig 4.6 shows a cell size distribution obtained 4 days after aggregation. I estimated the median cell volume from these and other cultures (see Materials and Methods) and these are plotted in fig 4.7. The 30% decrease in volume of untreated cells probably reflects accumulation of cells in the G1 phase of the cell cycle. RA treated P19 cultures showed a much more dramatic 75% decrease in cell volume, but only if the cells were aggregated. Monolayer cultures of P19 cells treated with RA did not show changes in cell volume. In contrast, RAC65 cells treated with RA did not show this extensive volume decrease and behaved like untreated P19 cells.

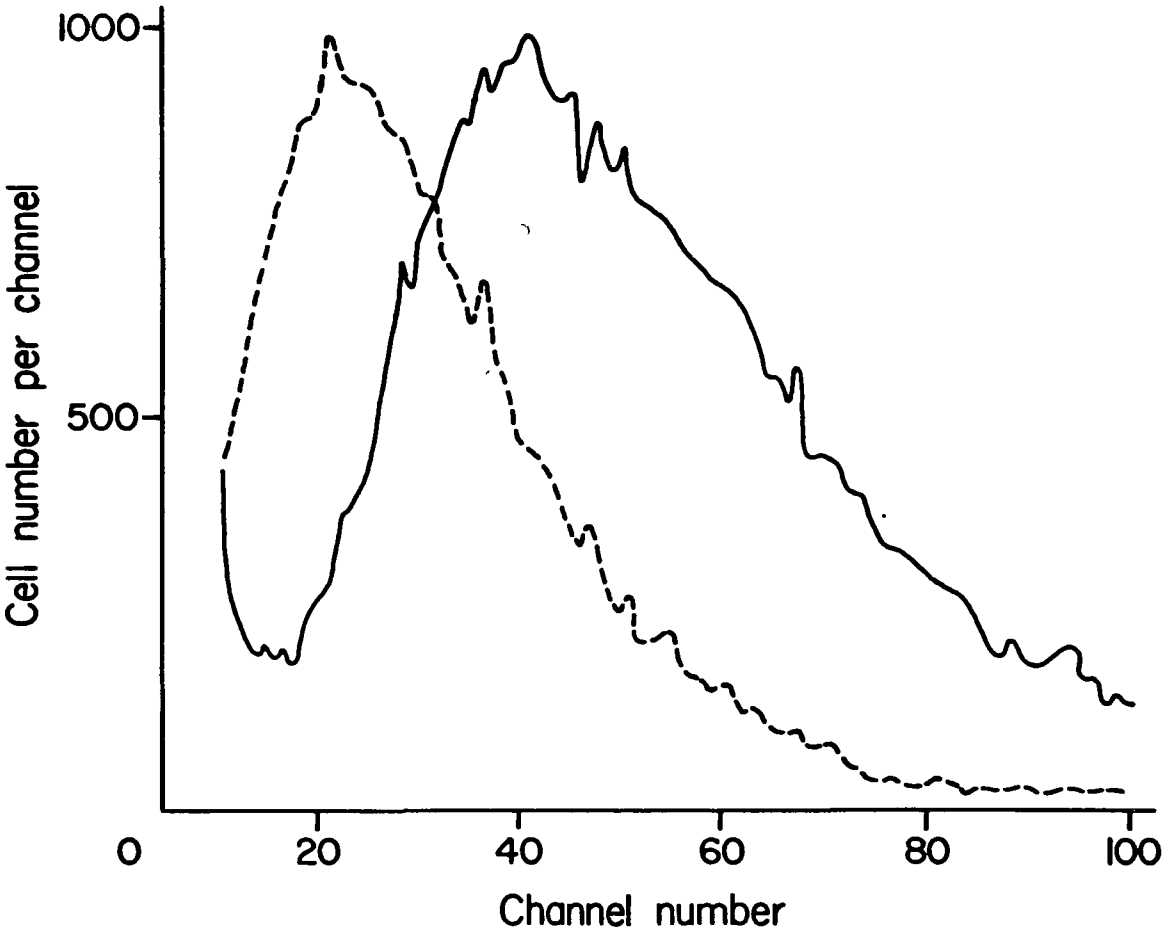
1000
500
Cell number per channel
0
20
40
60
80
100
Channel number

Figure 4.6. The size distribution of cells from 4 day old RA treated (dotted line) and untreated (solid line) P19 aggregates. The aggregates were disassociated and the size distibution of the cells obtained using a Coulter Counter Channalyser. The peak channel of the distribution obtained from unaggregated P19 cells was 60.

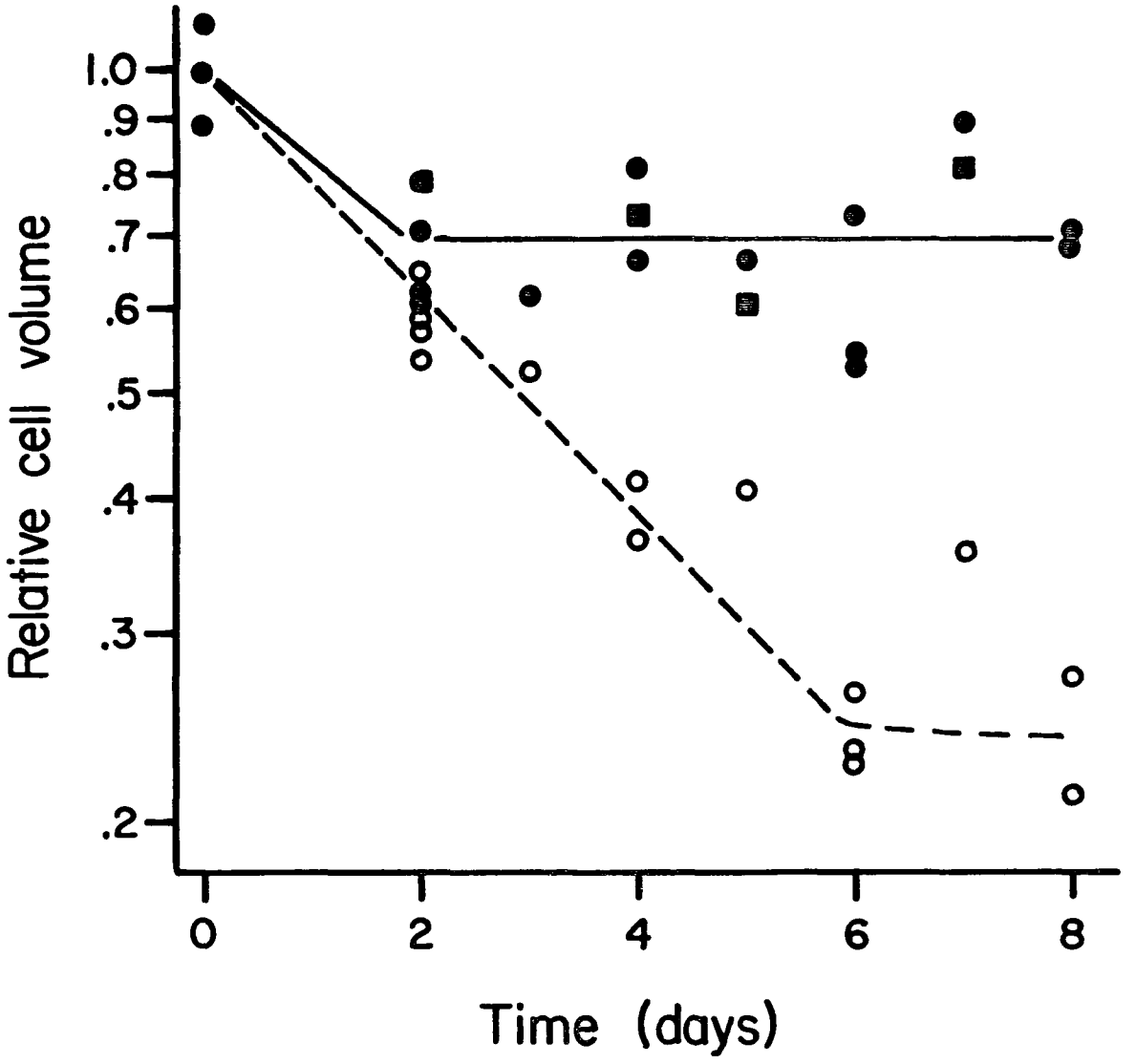
Relative cell volume
1.0
.9
.8
.7
.6
.5
.4
.3
.2
0
2
4
6
8
Time (days)

Figure 4.7. The volume of cells from RA treated aggregates decreases. Aggregated cells were dissociated at daily intervals. Each point represents the relative median cell volume calculated from a size distribution obtained with a Coulter Counter Channalyser. Relative cell volume is defined as the median volume at time t divided by the median volume of untreated cells. Untreated P19 cells (filled circles), 5 $X10^{-7}$ M RA treated P19 cells (open circles), and 5 $X10^{-7}$ M RA treated RAC65 cells (filled squares).

4.2 DISCUSSION

My results argue against models which attempt to explain the action of RA by cell selection. RA was not toxic to P19 cells during the first 48 hours when RA must be present. RA also allowed unreduced plating efficiencies of EC cells plated into RA-containing medium. The population of EC cells was found to be homogeneous with respect to its response to RA treatment. It could be argued that the P19 population as a whole is committed to making neurons, glia, and fibroblast-like cells and that RA acts to enhance the differentiation process but has no role in directing the differentiation of these cells. However, since P19 cells can differentiate into extra-embryonic endoderm-like cells and muscle (McBurney et al, 1982), it is more probable that they comprise a pluripotent EC cell population. This is supported by experiments in which P19 cells were injected into mouse blastocysts (Rossant and McBurney, 1982). The P19 cells contributed to most of the tissues in the consequent developing mouse embryo. In addition, other pluripotent EC cell lines also respond to RA, indicating that the effect is not peculiar to P19. Most consistent with the observations is the model which proposes that RA acts at the level of determination to induce a set of events which do not occur in the absence of the drug. Thus, the intracellular consequences of RA treatment may be similar to the normal developmental signals which determine neuronal and glial tissues.

It remains unclear how RA triggers the neural developmental pathway. RA has a number of diverse biological effects (Lotan, 1980). The relative efficiencies of RA analogues are similar in many *in vitro* systems (Lotan, 1980; Lotan et al, 1980). This heirarchy of efficiencies is correlated with the affinity of the retinoids for the cellular retinoic acid binding protein (cRABP) (Jetten and Jetten, 1979; Lotan, 1980). The effectiveness of the various retinoids that I have tested in the P19 system suggests that the reaction of RA with the cRABP may be a first step in inducing P19 cells to differentiate into neurons. cRABP complexed RA might then be transported to the nucleus where it would act directly upon the DNA. Liau et al (1981) have, in fact, demonstrated that retinol, bound to its cellular binding protein, is transported to the nucleus of rat liver cells where it binds specifically to sites located on the chromatin. The observations of Schindler et al (1981) are consistent with this type of model for the mechanism of action of RA in EC cells. They have isolated RA non-responsive PCC4 EC cells and have demonstrated that they lack cRABP activity. In some systems RA acts by inhibiting the induction of ornithine decarboxylase, a key enzyme in polyamine biosynthesis (Verma et al, 1976; Lotan, 1981; Fish et al, 1981; Scott et al, 1982). However, our data suggest that polyamine biosynthesis is not involved in the triggering of the neural developmental pathway.

I could find no evidence the RAC65 cells differentiated in the presence of RA. RAC65 cells have maintained their nonresponsive phenotype for many generations in tissue culture suggesting that a stable genetic change has occurred in these cells. It seems unlikely that this change is the result of a single point mutation since two selection steps were needed for its isolation. I have been unsuccessful in several attempts to isolate a nonresponsive mutant with only one selection step. Since the inability to respond to RA seems to behave as a recessive character in the cell hybrids, the two-step isolation requirements probably reflects the presence of two wild-type alleles in the diploid P19 cells. These non responsive mutants should be useful as a basis of comparison to the responsive P19 cells with respect to specific biochemical properties. Their effect on P19 cells in mixing experiment might provide information on the importance of intercellular interactions during determination.

The volume of cells in RA treated P19 aggregates began to decrease relative to the control by 48 h after the initiation of the experiment. The decrease in cell volume is an early event in the neural development of P19 cultures but it is not necessary for all forms of EC differentiation because no drop in cell volume is seen in DMSO treated aggregates of P19 cells destined to develop into nonneural tissues, including muscle (Edwards, MSc. thesis, 1983). This observa-

tion suggests that changes in ion flow might be important in neural differentiation. Decreases in cell volume have been shown to be an early event in the commitment of Friend erythroleukemic cells (Loritz et al, 1977), and the spontaneous differentiation of an EC cell line called 1009 (Pfeiffer et al, 1981). The effects noted on RA treated P19 aggregates are more dramatic because the cells do not simply accumulate in the G1 phase of the cell cycle, but shrink to 25% of their original volume. RAC65 cells from RA treated aggregates behaved like P19 cells from untreated aggregates, showing a small decrease in volume which stabilized after 3 days. This observation is consistent with the previous observations that RAC65 cells are non-responsive to RA.

Different concentrations of RA are effective in inducing the development of different cell types (Edwards and McBurney, 1983) and structurally unrelated drugs such as DMSO, butyrate, and 6-thioguanine have similar differentiation-inducing properties to those of low concentrations of RA (Edwards, MSc. thesis, 1983, Appendix A). The fact that RAC65 cells fail to respond to any of these drugs suggests that all of these drugs may have a common intracellular pathway of action and that the defect(s) in RAC65 cells affects some component of this shared pathway.

The data in this chapter can be used to formulate an interim working model for the mechanism of action of RA in this system. In the presence of RA, P19 cells become com-

mitted to differentiating into a neuroepithelial-like cell type and subsequently the cell types of the neural developmental pathway. This may be accomplished via the intracellular binding of RA to its binding protein. This step of the process does not require aggregation and but RA must be present for 48 hours for maximum effect. After this time, the determined P19 cells must be aggregated in order to express the differentiation program which leads to neurons and glial cells. Some aspects of this model will be discussed further in the next chapter.

Chapter V

CONCLUSIONS

The primary objective of this project was to find a way of manipulating the differentiation pattern of EC cells as a first step in studying the events involved in committing pluripotent cells to particular developmental pathways. I have observed that P19 EC cells differentiate entirely into neurons, glial astrocyte cells, and fibroblast-like cells in the presence of non-toxic concentrations of RA greater than 10^{-7} M. The fact that oligodendrocytes were not observed in RA treated P19 cultures may be a simple consequence of the presence of serum in the medium. Oligodendrocytes, while sharing a common precursor with some astrocytes, differentiate only in cultures with low concentrations of serum (Raff et al, 1983). Neural cell types were not observed in untreated P19 cultures. The simplest conclusion is that RA induces P19 cells to differentiate along an neuroectodermal developmental pathway. This system should, therefore, be useful for studying commitment to neuroectodermal differentiation.

Several pieces of information would be useful in constructing a model of the events which occur when RA is added to a culture of P19 cells. The first involves ascertaining

the embryonic cell type which is equivelent to P19 cells. This would enable us to establish the minimum number of cell type changes undergone by the P19 cells as they differentiate into neurons. The fact that P19 cells differentiate into extra-embryonic-like cells *in vitro* and can contribute to yolk sac *in vivo* (Rossant and McBurney, 1982) suggests that they are equivalent to ICM cells. Injecting single ICM cells into genetically distinct blastocysts has shown that the ICM is partitioned into primitive endoderm and primitive ectoderm by 4 1/2 days and that the latter cell type can not give rise to the former after this time (Gardner, 1981). However, some *in vitro* studies (Pederson et al, 1977: Dziadek, 1979) suggest that primitive ectoderm does retain the capacity to differentiate into primitive endoderm for a short period. Therefore it is possible that P19 cells may be equivalent to an early primitive ectodermal cell in spite of their ability to give rise to small amounts of extra-embryonic ectoderm-like cells. The commitment of P19 cells to differentiate into neural cells requires 48 hours, during which time the cells would be able to divide three times. If cell division is necessary for commitment to occur and P19 cells do indeed represent ICM cells, then there would be very little time for a P19 cell to develop from an ICM-like to an ectodermal-like and finally into a cell committed to neuroepithelial differentiation. This time frame may argue that P19 cells are, in fact, ectodermal equivalents.

The correlation observed between the ability of retinoid analogues to induce the differentiation of P19 cells and their reported ability to bind to the cRABP indirectly suggests that a possible mechanism for the biochemical action of RA might involve, as its first step, binding to the cRABP. A defect in cRABP activity could explain the recessivity of the RAC65 mutation, the non-responsiveness of RAC65 cells to RA, and the apparent requirement for two hit kinetics in obtaining the RAC65 mutants. However, RAC65 cells do not respond to DMSO (Edwards, MSc. thesis 1983). Since it is difficult to understand how DMSO could bind or affect cRABP, this suggests that the RAC65 block is at event(s) after binding to cRABP. RAC65 cell should be tested for cRABP activity. The DMSO non-responsive cell line, 01A1D3 (Edwards MSc.thesis, 1983) which does differentiate into neurons in the presence of RA, should be useful in investigating whether RA and DMSO act independently in the P19 cells.

Another possible mechanism of action of RA, which I did not investigate in this thesis, would involve changes in cell surface glycoproteins. In this type of model, RA would enter P19 cells and be converted to a retinyl phosphate. It has been suggested that a metabolite of RA can form retinyl phosphate which can then act as a carrier for mannosyl residues, transporting them across the plasma membrane (De Luca et al, 1979). In this manner, the cell surface glycopro-

teins of the P19 cell could be modified. These changes would presumably cause surrounding cells to act differently and trigger the changes leading to neural differentiation.

After the initial 48 hours of RA treatment, the P19 cells must be aggregated in order to differentiate into neurons. RA is no longer required. Non-aggregated cells develop into fibroblast-like cells. Presumably, the three dimensional arrangement of the differentiating cells is important just as it is in vivo. I have no information on the spatial organization of the differentiating neural cell types within the aggregate, but it is tempting to compare it to the neural tube with neurons and glial cells generated in an inner layer and migrating outwards after they begin to differentiate. This system offers exciting possibilities for studying neural precursor cell types and for examing the cytodifferentiation of neurons and glial cells. In particular, it is still not known if neurons and glial cells share a common precursor. With this system, it should be possible to identify this cell type, if it does exist, by examining the progeny of suitably labelled cells.

Edwards and McBurney (1983) have presented data which show that RA, at concentrations of 10^{-8} M and lower can induce P19 cells to differentiate into cardiac and skeletal muscle. Thus, RA has different effects at different concentrations. Maden et al (1982) and Tickle et al (1982) have shown that RA can affect pattern formation in developing and

regenerating limbs, a developmental process which seems to involve gradients of morphogens. One can speculate that the different concentrations of RA effective in the P19 system might represent a gradient effect *in vivo*.

In conclusion, the effect of RA on P19 cells appears to be an effect on the determination events which commit pluripotent cells to ectodermal pathways of differentiation. This system can be used to study the events involved in this determination process and also can be exploited for studying neural development.

Appendix A

RESPONSE OF P19 CELLS TO DMSO

This appendix contains the preliminary report of the effect of DMSO on the differentiation of P19 cells.

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

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Pluripotent murine embryonal carcinoma cells can differentiate in culture into many tissue types similar to those normally found in early embryos[1] and may be useful in investigating some developmental events[1,2]. Central to our understanding of embryonic development are explanations of cellular determination, that is, the commitment of early embryonic cells to form divergent cell types. Of relevance is recent work with the F9 line of embryonal carcinoma cells which suggests that certain extra-embryonic cell types are specifically formed following treatment of undifferentiated cells with drugs[3,4] and the manipulation of culture conditions[5]. We report here that the P19 line of embryonic carcinoma cells[6] may provide an analogous system in which drugs can be used to manipulate the formation of tissues which normally comprise the fetus. In the presence of dimethyl sulphoxide (DMSO) aggregates of P19 cells differentiate rapidly to form large amounts of cardiac and skeletal muscle but no neurones or glia. We have previously shown that in the presence of high concentrations of retinoic acid ($>5\times10^{-7}$ M), aggregates of these same cells develop into neuronal and glial tissues but not muscle[7]. Thus, drugs can be used to generate two quite different spectra of embryonic tissue types from the same population of embryonal carcinoma cells.

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

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

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

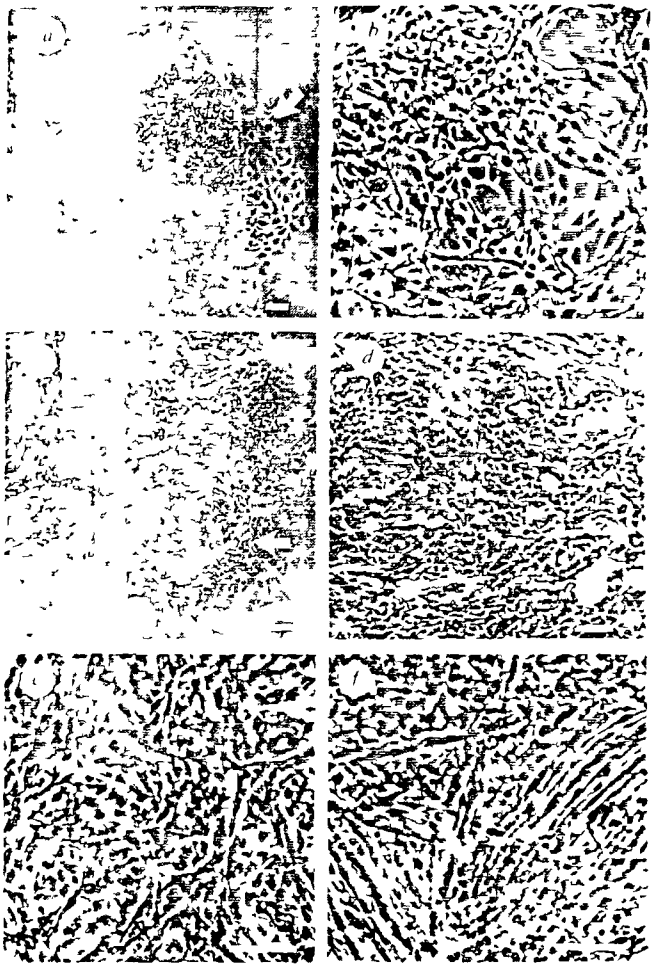

Fig. 1 Phase contrast photomicrographs of live teratocarcinoma cells. The conditions for cell culture[7,9] and aggregation[8] have been described previously. Cell aggregates were formed from a stock culture of P19S1801A1 cells and parallel cultures were carried in *a* normal medium, *a* medium plus 10%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

The DMSO treated aggregates of P10S1801A1 cells developed muscle but neither neurones nor glia. Treatment of the same cells with retinoic acid resulted in the development of neurones (Fig. 1*b*), glial cells, fibroblast-like cells but no muscle. Cultures exposed to both retinoic acid (5×10^{-7} M) and DMSO (0.5 or 1.0%) developed as if exposed only to retinoic acid, that is, neurones and glia but no muscle were formed.

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

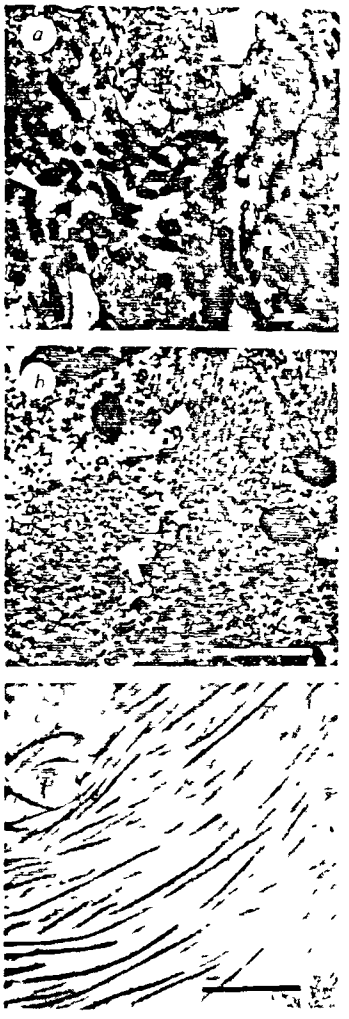

Fig 2 Electron micrographs of some of the tissues formed in DMSO treated cultures *a* A section through cardiac muscle shows bundles of thick and thin filaments in longitudinal section (arrows) glycogen granules (arrowheads) and numerous mitochondria *b* A section through a multinucleate myotube shows the thick and thin filaments in cross section (arrow) and glycogen (arrowhead) Many of the non muscle cells in these cultures secreted collagen (*c*) which was often seen forming part of an intercellular matrix Scale bars 0 5 μm

When 10 μM adrenaline was added to DMSO-treated cultures, the cardiac muscle responded by a 2-2 5-fold increase in contraction frequency and some previously quiescent areas of the culture were stimulated into rhythmic activity Therefore β-adrenergic receptors were present Such receptors are apparently acquired by cardiac muscle after the acquisition of spontaneous contractility[10]

DMSO was not demonstrably cytotoxic to the P19S1801A1 cells at concentrations effective in differentiation experiments Figure 3 shows that the efficiency of colony formation was unaffected by DMSO at concentrations up to 1 0% Virtually all colonies formed in DMSO contained only embryonal carcinoma cells In other experiments, monolayers of cells were cultured for 20 days in 1% DMSO without change in growth rate or morphology At the end of this 20-day period, the DMSO-treated cells were aggregated in the presence or absence of DMSO (0.5%) Those aggregates formed in the absence of DMSO did not differentiate while those cultured in the drug formed muscle and fibroblasts in the usual way Thus, it seems that the DMSO had no effect on the P19S1801A1 cells cultured as monolayers and that both the drug and cell aggregation are necessary for muscle differentiation DMSO could be removed after 2-3 days but cardiac muscle still developed at 6-7 days as in the continuous presence of the drug

The effects of DMSO described above were observed not

Table 1 Presence of α actin in DMSO treated cultures

Treatment	Total actin*	α actin*	% Muscle actin
Untreated day 7	1 91 ± 0 07	0 16 ± 0 02	8 4
Retinoic acid, day 7	2 31 ± 0 03	0 30 ± 0 02	13 0
DMSO day 7	2 62 ± 0 15	0 52 ± 0 01	19 8
DMSO day 11	3 05 ± 0 14	0 68 ± 0 02	22 3

Aggregated cultures were prepared as described in Fig 1 legend Two days after plating (day 7) or 6 days after plating (day 11) the cultures were collected for analysis The DMSO treated day 7 culture contained rhythmically contracting but not multinucleate muscle By day 11 both muscle types were present Protein and peptide isolation was carried out as previously described[24] except for the use of trypsin instead of chymotrypsin for peptide generation The actin contents were calculated by measuring the amounts of material which co purified during electrophoresis at *p*H 6 5 2 1 and 3 5 with tryptic peptides generated from muscle actin Total actin values were calculated from the amounts of radioactive material co-migrating with the two chemically modified peptides CmCys-Asp Ile Asp Ile Arg and CmCys Phe All known actins contain peptides which should co purify with these two α-Actin values were calculated from the amount of material co-purifying with an 18 residue CmCys containing peptide generated from the N terminal region of α actin Actins from other tissues differ from α actin in this region[9] so should not co-purify with this peptide Total actin is higher in differentiated than in undifferentiated cultures and there is more muscle specific α actin in DMSO treated cultures The 8-13% of muscle actin present in untreated and in retinoic acid treated cultures may represent a background of radioactive label derived from non α actin peptides which co purify with the legitimate peptide

* mg actin per 100 mg total protein

only on P19S1801A1 cells, but also on the parental P19 cells and on all of the subclones from this line which were tested However, DMSO had no effect on the differentiation of the embryonal carcinoma cell lines F9[11], OC15S1[12] and C86S1[12] whereas some clones of P10 cells[13] appear to form an excess of neurones in the presence of DMSO (G D Paterno and M W M , unpublished) Variation has also been observed in the response of different embryonal carcinoma lines to retinoic acid[3,7] and to aggregation in the absence of drugs[1,2]

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

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

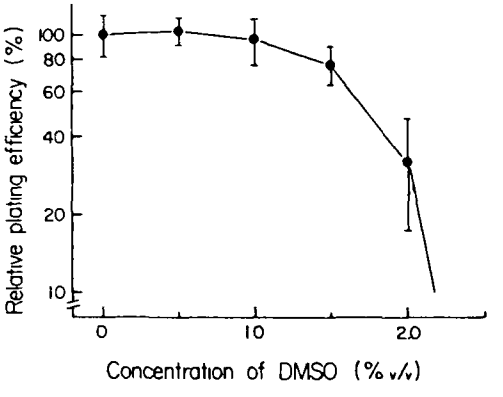

Fig. 3 The efficiency of colony formation of P19S1801A1 embryonal carcinoma cells in the presence of DMSO indicates the absence of toxicity at concentrations of less than 1% (v/v) About 200 cells were introduced into replicate 60 mm diameter disnes containing various concentrations of DMSO dissolved in α medium supplemented with 10% fetal bovine serum and 10^{-4} M β mercaptoethanol Incubation was for 8 days at 37 °C All colonies consisted of cells with embryonal carcinoma morphology Those colonies formed in 1 5% DMSO were smaller than control colonies indicating that at these concentrations the rate of cell proliferation was decreased

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

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