

# Retinoic Acid Induces Embryonal Carcinoma Cells to Differentiate into Neurons and Glial Cells

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**ABSTRACT** Murine embryonal carcinoma cells can differentiate into a varied spectrum of cell types. We observed the abundant and precocious development of neuronlike cells when embryonal carcinoma cells of various pluripotent lines were aggregated and cultured in the presence of nontoxic concentrations of retinoic acid. Neuronlike cells were also formed in retinoic acid-treated cultures of the embryonal carcinoma line, P19, which does not differentiate into neurons in the absence of the drug. The neuronal nature of these cells was confirmed by their staining with antiserum directed against neurofilament protein in indirect immunofluorescence experiments. Retinoic acid-treated cultures also contained elevated acetylcholinesterase activity. Glial cells, identified by immunofluorescence analysis of their intermediate filaments, and a population of fibroblastlike cells were also present in retinoic acid-treated cultures of P19 cells. We did not observe embryonal carcinoma, muscle, or epithelial cells in these cultures.

Neurons and glial cells appeared in cultures exposed to retinoic acid for as little as 48 h. We found no evidence for retinoic acid toxicity, suggesting 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.

Very little is known about the determination events that commit unspecialized cells to differentiate into the more specialized cell types that appear later during embryonic development. Murine embryonal carcinoma cells, the pluripotent stem cells of malignant teratocarcinomas, may provide a culture system with which to study these events (16). Lines of embryonal carcinoma cells can be isolated from teratocarcinomas and maintained in an undifferentiated state when kept in exponential growth phase in tissue culture (11, 23, 41). If left undisturbed at high density, they differentiate *in vitro* into a variety of cell types including epithelium, neurons, muscle, and cartilage (29, 30, 36).

The value of the embryonal carcinoma cells in studying determination has been limited by the complexity of their differentiation patterns; any one determination event is obscured from study by others that occur simultaneously. We attempted to limit the spectrum of cell types arising during embryonal carcinoma differentiation by identifying drugs that either blocked certain avenues of differentiation or induced embryonal carcinoma cells to differentiate into specific cell types. This paper describes the effects of retinoic acid. This

drug induces differentiation of embryonal carcinoma cells into a restricted spectrum of tissue types, namely neurons, glial, and fibroblastlike cells. This retinoic acid-mediated effect was observed using embryonal carcinoma cells from several different cell lines and was examined in detail using P19, a euploid embryonal carcinoma cell line that differentiates only into small amounts of extraembryonic endodermlike cells in the absence of retinoic acid.

Previous reports have indicated that retinoic acid alone (45) or in combination with other drugs (46) or treatments (20) can induce the differentiation of F9 embryonal carcinoma cells to form various extraembryonic endodermal tissues. Apparently different embryonal carcinoma lines respond differently after exposure to retinoic acid.

## MATERIALS AND METHODS

### *Cell Lines and Culture Techniques*

The cell lines used in these experiments are described in Table I. The cultures were grown in alpha medium (44) supplemented with 2.5% fetal calf serum and 7.5% calf serum (Flow Laboratories, Mississauga, Ontario). All cultures were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere.

TABLE I  
Response of Different Cell Lines to Retinoic Acid (RA)

Cell line	Reference	% Aggregates containing neurons*	
		In $10^{-7}$ M RA	Without RA
C145A12	(32)	100	0†§
P19	(34)	94	0§
OC15S1	(31)	94	54
P10	(33)	79	0†§

\* 3 d after plating, aggregates were examined for the presence of neuronlike cells using phase contrast optics. 50 aggregates were scored per measurement and a positive was recorded if the aggregate contained several cells with long processes (see Fig. 1 c and d).

† Although C145A12 and P10 aggregated cultures did not contain neurons 3 d after plating, neurons were routinely present 5–7 d after plating.

§ Some aggregates were surrounded by extraembryonic endodermlike cells.

Differentiation of all cell lines was carried out as follows: cells in exponential growth were treated with  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free phosphate-buffered saline (PBS) containing 0.25% trypsin and 1 mM EDTA, to remove them from the tissue culture surface. They were plated at a density of  $10^6$  cells per ml into a bacteriological grade petri dish (29) where they aggregated spontaneously. The medium was replaced after three d. The aggregates were plated into tissue culture dishes at 5 d. 2–3 d later the plated aggregates were scored and/or examined biochemically.

### Retinoic Acid Preparation

Retinoic acid was prepared as a stock solution at  $10^{-2}$  M in 95% ethanol. The stock solution was diluted directly into the culture medium to obtain the desired concentration, usually  $5 \times 10^{-7}$  M. In experiments where retinoic acid was removed from the cultures, the aggregates were washed three times with serum-free medium before resuspension in serum-containing medium.

### Preparation of Antisera

Electrophoretically pure vimentin (mol wt 57,000) prepared from cytoskeletal preparations of 3T3 cells according to the method of Franke et al. (13) was used for the immunization of rabbits. Glial filaments were isolated from calf brain (22) by a slight modification of previously described methods (49) and the filament proteins were separated by PAGE. The 54,000 mol wt band was eluted from the gel, and the electrophoretically purified protein was used for the immunization of rabbits.

The preparation of antiserum to tubulin has been previously described (6). Antiserum to keratin was raised in rabbits against keratin purified from human stratum corneum (47). This was a gift from Drs. E. Fuchs and H. Green (Department of Biology, Massachusetts Institute of Technology). Antiserum to neurofilaments (28), raised in rabbits against the 160,000-mol wt component of bovine brain neurofilaments was a gift from Dr. R. Liem (Department of Pharmacology, New York University School of Medicine).

### Immunofluorescence Assays

Aggregates were plated directly onto cover slips, and fixation and staining was carried out *in situ*. The cells were rinsed once in PBS, pH 7.0, fixed for 4 min in methanol, and for 2 min in acetone, both at  $-20^\circ\text{C}$ . Cells were treated with one of the antisera at a dilution of 1:30 (antitubulin, antineurofilament, antigial fibrillar protein, antivimentin) or 1:50 (antikeratin). This was followed by treatment with fluorescein-conjugated goat IgG raised against rabbit IgG (Hyland Diagnostics Div., Travenol Laboratories, Costa Mesa, CA), diluted 1:5. Cells were examined with a Zeiss Photomicroscope 2 (Carl Zeiss, Inc., New York) equipped with epifluorescence optics.

### Scanning Electron Microscopy

The aggregates were plated onto cover slips, fixed, and dehydrated *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.

### Acetylcholinesterase Assays

These assays were carried out using the spectrophotometric procedure described by Ellman et al. (9). The activity specifically attributable to acetylcholinesterase was determined using the specific inhibitor B.W.284C51 (Sigma Chemical Co., St. Louis, MO). The cells were removed from the tissue culture dishes by the addition of PBS containing trypsin and EDTA. After sonication, the lysates were stored at  $-80^\circ\text{C}$  until assay. Protein concentrations were determined according to a modified Lowry procedure (17).

### RESULTS

Pluripotent embryonal carcinoma cells may be induced to differentiate if they are aggregated and cultured in suspension for several days before plating onto tissue culture grade plastic surfaces (29, 30). For all of our experiments, the aggregates were cultured for 5 d in suspension. They were then plated and examined 2–3 d later when differentiated cells had migrated out of the aggregates.

For our initial experiments, we used a pluripotent cell line C145A12 (32). When  $10^{-7}$  M retinoic acid was continuously present in the culture medium, unusually abundant numbers of neuronlike cells appeared within 48 h of plating the aggregates. It has been reported that retinoic acid has no effect on the tissue distribution which arises during the differentiation of pluripotent embryonal carcinoma cells (21). Our observation on C145A12 cells may, therefore, have been peculiar to that cell line. Thus we examined the response of several other embryonal carcinoma cell lines to retinoic acid. The drug had a similar effect on the other three cell lines tested (Table I). Virtually all retinoic acid-treated aggregates contained some cells with neuronal morphology. Since aggregates from some cell lines formed no neuronlike cells in the absence of the drug, it seemed likely that the retinoic acid was inducing the formation of these neuronlike cells, rather than inhibiting the development of other tissue types.

In subsequent experiments we used P19 (Fig. 1 a), an embryonal carcinoma cell line with a normal male karyotype isolated from C3H/He mice (34). The retinoic acid effect could be easily evaluated with P19 cells because no neuronlike cells were formed in the absence of the drug. When aggregates of P19 cells were plated into tissue culture dishes in the absence of retinoic acid, we observed only undifferentiated cells surrounded by a small amount of tissue resembling extraembryonic endoderm (Fig. 1 b). Continued incubation of these cultures resulted in the proliferation of both cell types, with the appearance of no other differentiated cell type. When P19 cells were cultured as aggregates in the presence of retinoic acid, the cell types present 2 d after plating were markedly different. Within 24 h of plating, a flat layer of fibroblastlike cells migrated out from the periphery of the aggregate. These fibroblastlike cells did not resemble either embryonal carcinoma or the endodermlike cells seen in untreated cultures. Between 24 and 48 h after plating, neuronlike cells appeared whose processes grew rapidly from the aggregate over the fibroblastlike cell layer. Phase-contrast micrographs of these cell types are shown in Fig. 1 c and d. The scanning electron micrographs in Fig. 1 e and f show this morphology in more detail. The processes from these neuronlike cells were frequently arranged in bundles. They had multiple branches with tips located on the fibroblastlike cells.

### Dose-response Characteristics

The differentiation of embryonal carcinoma cells into neuronlike cells was dependent on the concentration of retinoic

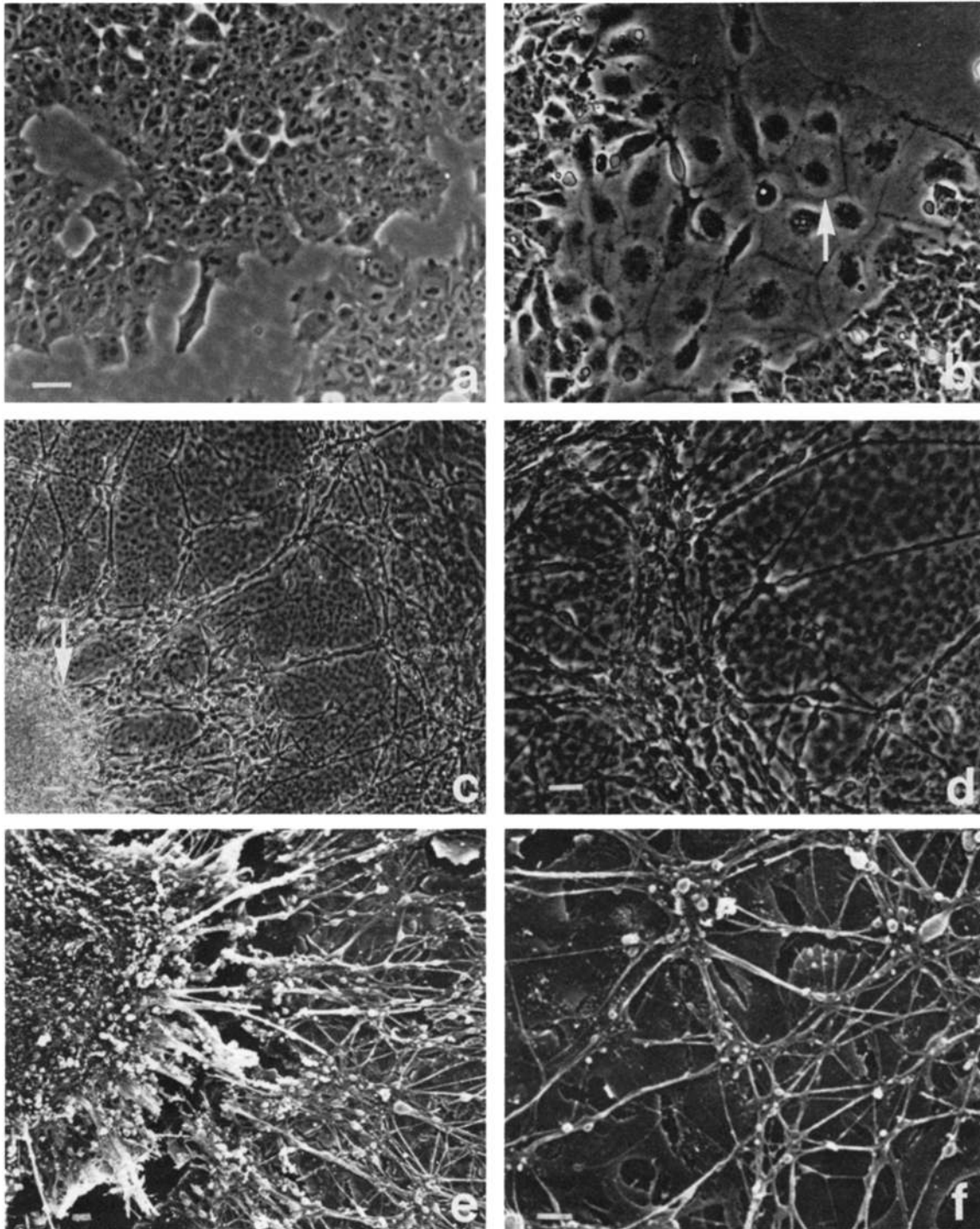


FIGURE 1 Morphologies of P19 cells following various treatments. The undifferentiated embryonic carcinoma cells (a) grow attached to the surface of the tissue culture dish. When they are allowed to aggregate for 5 d and the aggregates are then plated into tissue culture dishes, a small number of cells differentiate into the endodermlike cell type indicated by the arrow in b. If retinoic acid is present in the aggregated cultures, neuronlike and fibroblastlike cells appear within 2 d of plating the aggregates (c and d). Scanning electron micrographs of such retinoic acid-treated cultures (e and f) show networks of processes extending over a monolayer of the fibroblastlike cells. Bars: (a-d) 3.6  $\mu\text{m}$ ; (e and f) 11.1  $\mu\text{m}$ .

acid present in the culture medium. Fig. 2 shows the response of aggregated P19 cells to various retinoic acid concentrations. At concentrations  $>5 \times 10^{-8}$  M, essentially all of the aggregates contained cells with neuronlike processes by 72 h after plating. Undifferentiated embryonic carcinoma cells could not be identified by phase-contrast microscopy in cultures containing neurons. The absence of embryonic carcinoma cells from these

retinoic acid-treated cultures has been confirmed by the disappearance from these cultures of cells capable of forming colonies under conditions in which the plating efficiency of P19 embryonic carcinoma cells is about  $\sim 50\%$  (34). Thus the drug-induced appearance of neuronlike cells was accompanied by a disappearance of embryonic carcinoma cells. Between  $10^{-8}$  and  $5 \times 10^{-8}$  M, many aggregates which did not contain

neuronlike cells did contain fibroblastlike cells. Below  $10^{-8}$  M, the cultures resembled untreated controls and contained only embryonal carcinoma cells and small amounts of extraembryonic endodermlike cells. In subsequent experiments discussed in this paper, we used a dose of  $5 \times 10^{-7}$  M, a concentration of retinoic acid with which all aggregates con-

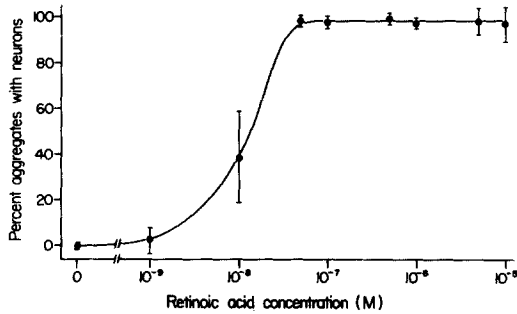


FIGURE 2 Relationship between retinoic acid concentration and differentiation of neuronlike cells. The aggregates of P19 cells, continuously cultured in the presence of the drug, were plated after 5 d in suspension and examined 2-3 d later. Normally, 50 aggregates were scored for each drug concentration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

tained neuronlike cells and few, if any, embryonal carcinoma cells.

### Properties of the Neuronlike Cells

The experiments in this section and the next were undertaken to positively identify the cell types present in retinoic acid-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 retinoic acid-treated cultures using indirect immunofluorescence techniques with antibodies prepared against purified tubulin (6) (Fig. 3 *a* and *b*). Both the cell bodies and their processes were intensely stained, in a pattern similar to that given by antitubulin staining of neurons in culture (24). This staining procedure clearly showed that some of the processes contained varicosities, an anatomical characteristic of some kinds of neuronal cell processes. It was also possible to visualize the complex patterns of neurite branching and interconnections in these cultures. The fibroblastlike 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 fila-

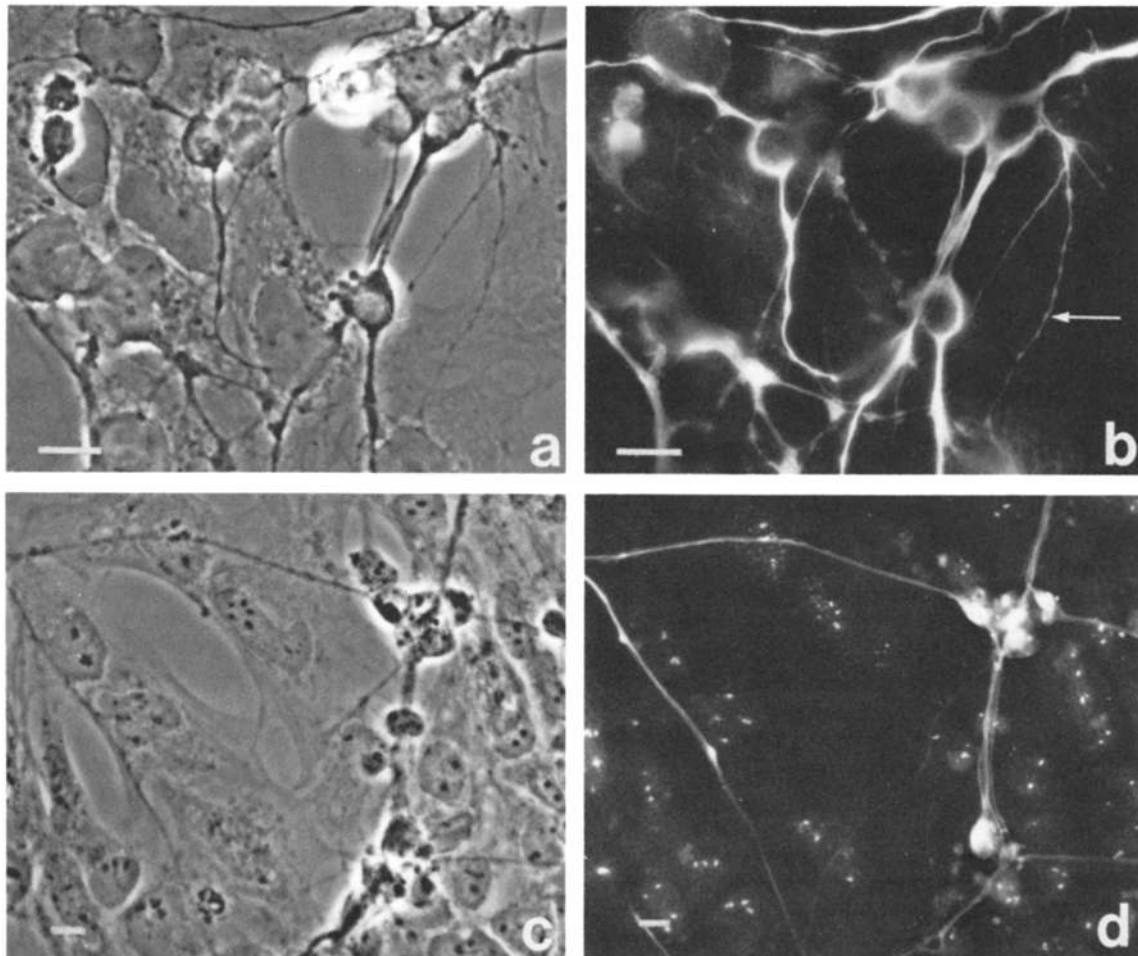


FIGURE 3 Visualization of microtubules and neurofilaments in cells from retinoic acid-treated cultures. Immunofluorescence staining was with antisera to tubulin (*b*) and neurofilament protein (*d*). The antitubulin staining revealed varicosities on some of the processes as indicated by the arrow in *b*. The fibroblastlike cells were lightly stained by this antiserum. The antiserum to neurofilaments stained the neuronlike cells and their processes but did not stain the cytoplasm of the underlying fibroblastlike cells (*d*). The phase-contrast micrographs of the same cells are shown in panels *a* and *c*, respectively. Bar, 10  $\mu$ m.

ments, is comprised of proteins specific for various tissue types (26). Neurofilaments are the intermediate filaments specific for neurons and consist of peptide subunits of 210,000, 160,000 and 68,000 mol wt (19, 28, 42). Antibodies prepared against the 160,000-mol wt MW neurofilament polypeptide were used with indirect immunofluorescence procedures to examine retinoic acid-treated cultures (Fig. 3 *c* and *d*). Staining was localized along the processes and cell bodies of every one of the neuronlike cells. In contrast, the cytoplasm of the nonneural cells were unstained except for nucleoli which stained nonspecifically. Neither the undifferentiated nor the extraembryonic endodermlike cells in the untreated cultures were stained by this antiserum, indicating the absence of neurofilaments from these cell types.

Acetylcholinesterase has been frequently used as a marker for neuronal differentiation although this enzyme is present in some nonneural tissues (2, 27, 48). Fig. 4 shows the result of one of four experiments in which the acetylcholinesterase activity was measured in aggregated cultures of both treated and untreated cells. Although the absolute values of acetylcholinesterase varied from one experiment to another, the pattern was consistent and the results shown in Fig. 4 are representative. Untreated cultures contained little activity. However, in retinoic acid-treated cultures, acetylcholinesterase activity appeared soon after aggregation and increased with time, reaching its highest value 2–3 d after plating, a time when neuronlike cells became evident in the plated aggregates. Preliminary experiments also indicate the presence of choline acetyltransferase in these retinoic acid-treated cultures. At day 8, the choline acetyltransferase activity was 28 pmol/min/mg protein in the retinoic acid-treated culture. This compares with 2.3 pmol/min/mg in untreated cultures of the same age and 50 pmol/min/mg in adult mouse brain.

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

### Nonneural Cells in Retinoic Acid-treated Cultures

The experiments described in this section were undertaken to characterize the nonneural cells present in retinoic acid-treated aggregated cultures 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, also present in many tissue culture cells of nonmesodermal origin (12, 14). The fibroblastlike cells contain an intermediate filament network which stains with antiserum to vimentin (Fig. 5 *b*). The staining pattern is typical of that of other vimentin-containing intermediate filament systems (12).

The keratins are a class of proteins, ranging in mol wt from 41,000–65,000, found in the intermediate filaments of epithelial cells (15). Antibodies directed against keratin did not stain intermediate filaments in any of the cells in retinoic acid-treated cultures (Fig. 5 *c* and *d*). This result suggests that retinoic acid-treated cultures do not contain epithelial cells.

Glial fibrillar acidic protein is a major component of the intermediate filaments in glial astrocyte cells (5, 10). Neither

the neurons nor the fibroblastlike cells stained with antiserum specific for this protein. However, 4–5 d after plating retinoic acid-treated aggregates, a population of cells containing this protein initially appeared at the junction of the fibroblastlike monolayer and the aggregate. Fig. 5 *e* and *f* show the staining patterns obtained from this population of glial cells. Thus, retinoic acid-treated cultures contained three distinct cell types based on the antigenic characteristics of their intermediate filaments (Table II).

### Cell Types Present in Untreated P19 Cultures

The untreated aggregated cultures of P19 contained undifferentiated embryonal carcinoma cells and cells which resembled extraembryonic endoderm. These cultures were analyzed with the antisera described in the previous section.

Neither glial fibrillar protein-containing intermediate filaments nor neurofilaments were observed in either cell type. Fig. 6 *a* and *b* show that the extraembryonic endodermlike cells in untreated cultures contained intermediate filaments which were stained with antibody to vimentin. We also observed these filaments in undifferentiated P19 cells (not shown). Paulin et al. (37) have shown that PCC3/A11 embryonal carcinoma cells also contain vimentin. Thus, vimentin is present in many cell types in both treated and untreated cultures (Table II).

The extraembryonic endodermlike cells in untreated cultures contained bundles of intermediate filaments that were stained using antiserum directed against keratin (Fig. 6 *c* and *d*). These filaments extended from the nuclear region to the periphery of the cell, ending on desmosomes which were shared with the neighboring cells (Fig. 6 *e* and *f*). The presence of cytokeratin-

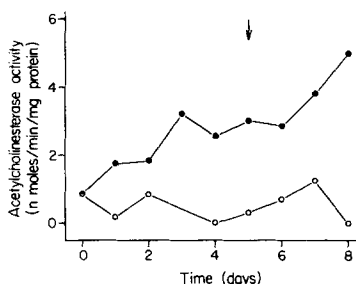


FIGURE 4 Acetylcholinesterase appears in retinoic acid-treated but not untreated aggregate cultures. The specific activity of acetylcholinesterase was determined in treated (●) and untreated (○) aggregated P19 cultures. Aggregates were plated at 5 d

(arrow) and neurons became abundant by 7 d. Each point represents the activity that was specifically inhibited by B.W. 284C51 using acetylthiocholine as a substrate. The activity of acetylcholinesterase in adult mouse brain homogenates (strain C3H/He, the genotype of P19 cells) was 53 nmol/min/mg protein, ~10 times the maximum activity seen in retinoic acid-treated cultures.

TABLE II  
Cell Types Present in Aggregated P19 Cultures

Intermediate filament protein	Untreated		Retinoic acid treated		
	Embryonal carcinoma	Extraembryonic endoderm	Neuron	Astrocyte	Fibroblast
Vimentin	+	+	–	nd*	+
Keratin	–	+	–	–	–
Glial fibrillar protein	–	–	–	+	–
Neurofilament protein	–	–	+	–	–

\* nd not determined.

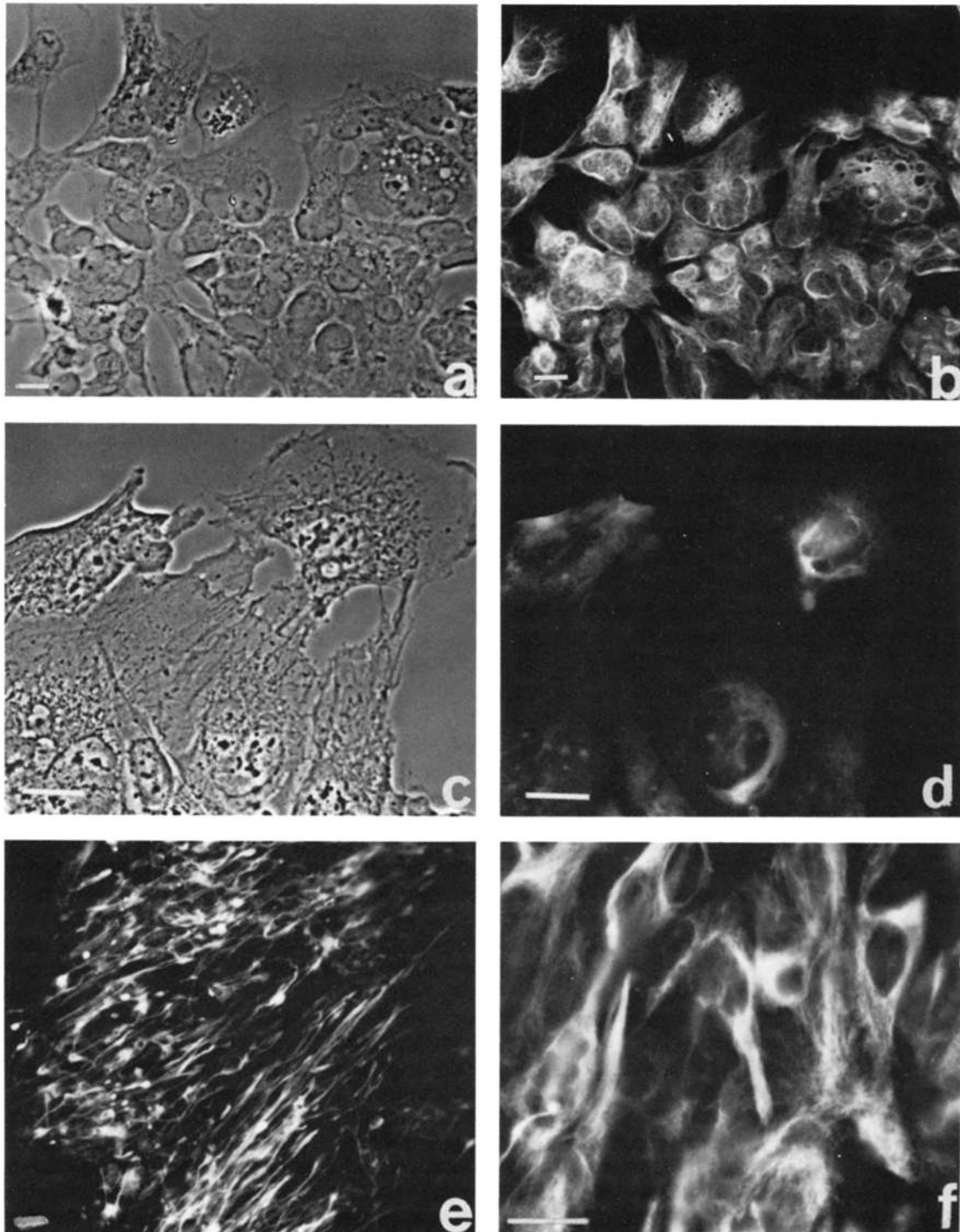


FIGURE 5 Immunofluorescence staining of intermediate filaments in cells from retinoic acid-treated cultures. *a* and *b* show the same field of fibroblastlike cells photographed by phase contrast (*a*) and following immunofluorescence staining with antiserum raised against vimentin (*b*). The typical "basket" pattern of vimentin-containing intermediate filaments is present in virtually all cells. *c* and *d* show the same field photographed by phase-contrast microscopy (*c*) and following immunofluorescence staining using antiserum against keratin (panel *d*). Some nonspecific perinuclear staining was observed, but no intermediate filaments stained with this procedure. Antigial fibrillar protein antiserum was used in panels *e* and *f*. A population of glial filament-containing cells appeared 4-5 d after plating the aggregates. Bar, 20  $\mu$ m.

containing filaments in the extraembryonic endoderm cells in untreated aggregates indicates that these cells are different from the fibroblastlike cells present in retinoic acid-treated cultures. The undifferentiated P19 cells did not stain with antibodies to keratin.

#### *The Mechanism of Retinoic Acid Action*

The following experiments were conducted to determine whether the retinoic acid affects the decision events which determine the fate of the undifferentiated embryonal carci-



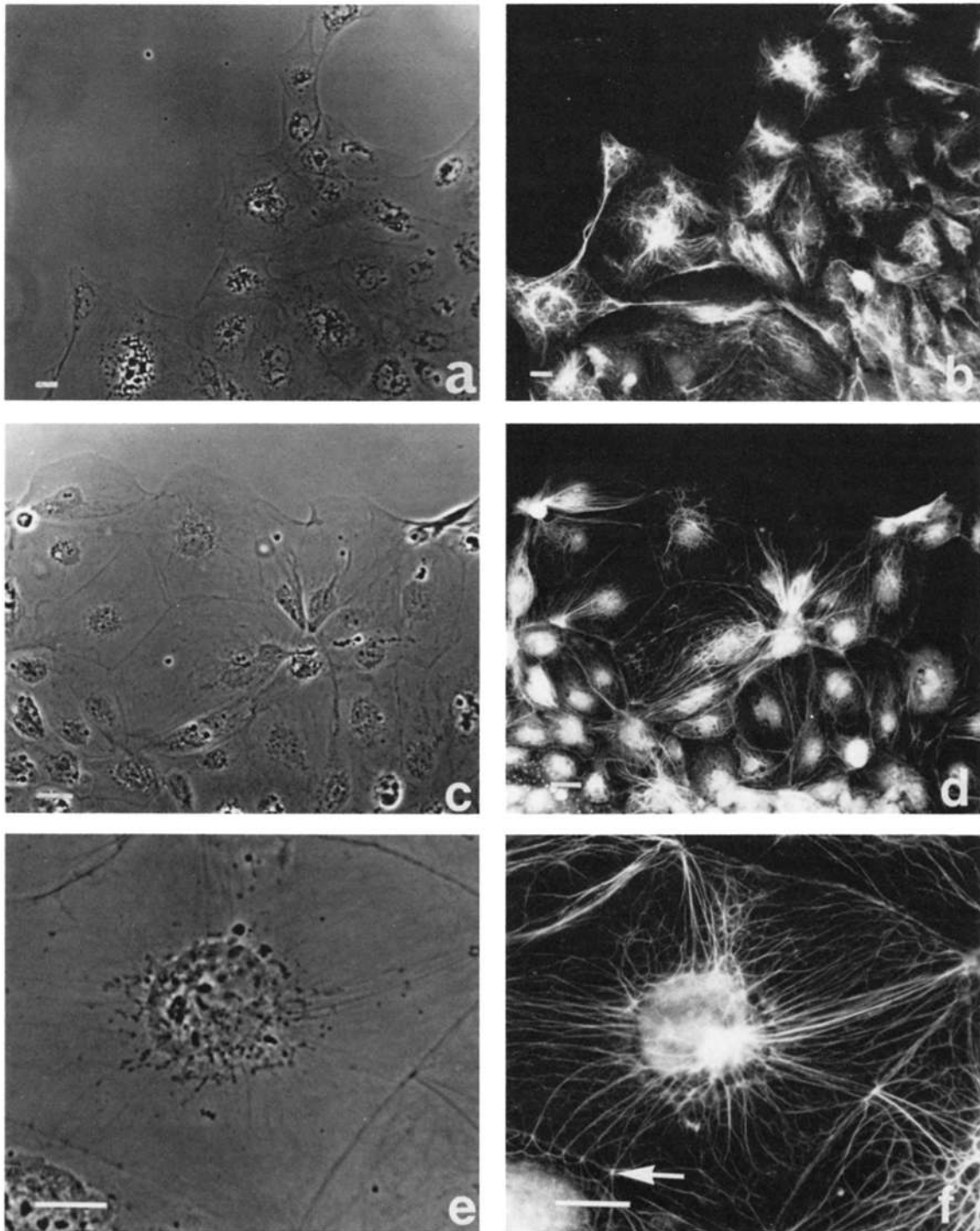


FIGURE 6 Immunofluorescence staining of the intermediate filaments in the extraembryonic endodermlike cells formed in the absence of retinoic acid. These cultures were stained with the antibody to vimentin (*b*) and the antibody to keratin (*d* and *f*). Phase-contrast micrographs of the same cells are shown in *a* and *c* and *e*, respectively. The vimentin intermediate filament system is similar to that seen in the fibroblastlike cells present in the retinoic acid-treated cultures. The endodermlike 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 neighboring cells (arrow, *f*). Bar, 20  $\mu\text{m}$ .

noma cells, or whether its effects are mediated by selection.

The retinoic acid did not kill embryonal carcinoma cells. Fig. 7 *a* shows that the number of colonies formed by P19 cells in the presence of retinoic acid at concentrations up to  $10^{-5}$  M was similar to the number obtained in the absence of the drug. The colonies formed in retinoic acid concentrations above  $5$

$\times 10^{-8}$  M were composed of the fibroblastlike cells, whereas colonies formed at lower retinoic acid concentrations were composed of cells with embryonal carcinoma morphology.

The growth rate of P19 cells in medium containing retinoic acid was measured during a 48-h drug exposure. As can be seen in Fig. 7 *b*, retinoic acid had little effect on the doubling

time within this period. In other experiments, we found that aggregates grown in the presence of  $5 \times 10^{-7}$  M retinoic acid 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 retinoic acid can be explained by its effects on cell viability or growth rate.

The cells of the P19 cultures may be heterogeneous with respect to their development potentials. To test this possibility, 25 P19 cells were individually picked and plated into separate culture dishes. 19 formed colonies, and 17 of these were successfully expanded into clonal cell lines. 15 of these 17 cell lines responded to retinoic acid in a manner similar to that for the parental culture (Table III). The exceptions were P19S8, a tetraploid clone, which gave rise to a few neurons even in the

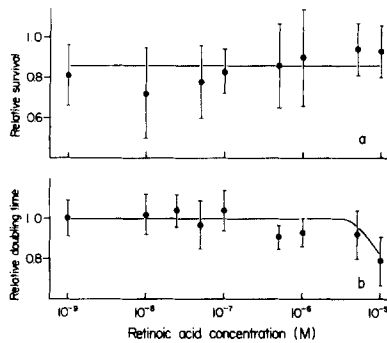


FIGURE 7 Retinoic acid is not toxic to P19 cells. The plating efficiency and growth rate of P19 cells were measured in cultures containing retinoic acid. The relative survival (a) was calculated from experiments in which P19 cells were plated at low density (~200 cells per 60 mm diameter petri dish) 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 retinoic acid was 41%. The doubling time of P19 cells was calculated after growing P19 cells (seeded at  $10^5$  cells/ml) for 48 h in the presence of retinoic acid. The ratio of the doubling time of treated cultures to the doubling time of untreated cultures is plotted vs. retinoic acid concentration in b. The points represent the mean of 12-24 separate determinations. The mean doubling time of the controls was 16 h. Vertical bars represent sample standard deviation.

TABLE III  
Response of Several Subclones of P19 to Retinoic Acid

Subclone	Number of aggregates containing neurons	
	With retinoic acid*	Without retinoic acid
S1	79/80‡	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§
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	99/100	0/50
S17	60/60	0/46

\* Cultures were treated with  $5 \times 10^{-7}$  M retinoic acid.

‡ No. of aggregates containing neurons 3 d after plating aggregates/no. of aggregates examined.

§ ND, no data.

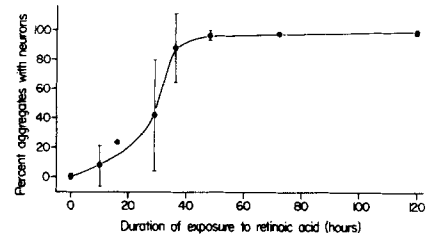


FIGURE 8 Retinoic acid need not be continuously present in aggregated cultures. Retinoic acid ( $5 \times 10^{-7}$  M) was added at the initiation of aggregation and removed at various times by washing three times with normal medium. Aggregates were plated at 5 d and scored for the presence of neurons 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 three experiments.

absence of retinoic acid, and P19S11, which also gave very small numbers of neurons in a minor fraction of untreated aggregates. Thus, the P19 population appears to be homogeneous with respect to its response to retinoic acid.

If retinoic acid affects determination events, it might be possible to remove the drug following commitment but before cytodifferentiation. Retinoic acid 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. 8, indicate that a 48-h exposure to the drug was adequate to ensure that neurons formed in virtually all aggregates.

Monolayer cultures of P19 cells could be treated for 48 h with retinoic acid 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 retinoic acid but not aggregated, virtually all cells differentiated into fibroblastlike cells and few, if any, neurons were found. Apparently neuron differentiation was greatly facilitated by both retinoic acid treatment and the high cell density achieved by aggregation. These two conditions may be met simultaneously or, retinoic acid treatment may precede aggregation.

## DISCUSSION

By adding drugs to the culture medium during differentiation of embryonal carcinoma cells, we hoped to reduce the spectrum of tissue types formed by interfering with some of the events involved in the commitment of undifferentiated cells to particular differentiation pathways. Our experiments showed that, in the presence of retinoic acid, embryonal carcinoma cells differentiated into a limited spectrum of tissue types, namely neurons, glial, and fibroblastlike cells. Our results suggest that retinoic acid acts by inducing all the embryonal carcinoma cells in the aggregated cultures to differentiate and that these cells are committed to form a limited variety of cell types.

### Characterization of Cells

The neurons present in retinoic acid-treated cultures of P19 cells 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 revealed



varicosities on some neuronal processes, a characteristic of some kinds of neurons. The neuronal nature of these cells was confirmed by the presence of neurofilaments in their cytoplasm. These cells also display tetanus toxin surface receptors (Rudnicki, M. A., E. M. V. Jones-Villeneuve, and M. W. McBurney, manuscript in preparation), another neuron-specific property (8, 35).

Darmon, Bottenstein, and Sato (7) recently reported that a serum-free medium induced the formation of neurons from a clone of embryonal carcinoma cells. The neurons were identified by their morphology, isoenzyme pattern, and Na<sup>+</sup> channels. Identification of neurotransmitters and associated enzymes is another way of characterizing neurons. This approach was taken by Pfeiffer et al. (39) who have isolated a line of embryonal carcinoma cells which appeared to be spontaneously committed to the formation of cholinergic neurons. Elevated levels of acetylcholinesterase and choline acetyltransferase were present in differentiated cultures derived from these cells. We found that acetylcholinesterase activities were elevated in retinoic acid-treated cultures of P19 cells and were highest when the cultures contained the largest numbers of morphologically identifiable neurons. However, the activity of acetylcholinesterase increased many days before neurons became apparent. Perhaps this enzyme is expressed very soon after neuronal determination. The presence of acetylcholinesterase suggests that these neurons may be cholinergic, and this contention is supported by the presence of choline acetyltransferase in these cultures.

In addition to neurons, retinoic acid-treated P19 cultures also contained glial astrocytes, identified by their staining with antibody to glial fibrillar protein. In the normal embryo, both neurons and glial cells are derived from the ectodermal germ layer. We have not been able to fully characterize the fibroblastlike 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 (12, 14), including P19 embryonal carcinoma cells. Since the fibroblastlike cells did not develop into muscle, adipose, or cartilaginous tissue, it seems likely that these fibroblasts are analogous to cells of similar morphology present in explants of embryonic brain (1). We have observed that the differentiated cell types appear in a reproducible sequence after plating retinoic acid-treated aggregates. Fibroblastlike cells appear initially (1 d), followed by neurons (2 d), and glial cells (4–5 d). This sequence of tissue type appearance is identical to that seen in explants of brain from 10-d-old rat embryos (1). In addition, the morphologies of cells in cultures of central nervous system (1, 40) and peripheral nervous system (3) tissues are strikingly similar to those we observe with retinoic acid-treated P19 cells shown in Fig. 1.

The extraembryonic endodermlike 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. (38) have reported prekeratin-containing intermediate filaments in parietal extraembryonic endoderm cells obtained from retinoic acid-treated F9 cells (see discussion below). We did not observe cells with prekeratin-containing intermediate filaments in retinoic acid-treated P19 aggregated cultures and therefore conclude that extraembryonic 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 embryonal carcinoma cell differentiation.

## Mechanism of the Retinoic Acid Effect

Retinoic acid has been previously shown to induce the differentiation of embryonal carcinoma cells. The effect induced by retinoic acid seems variable depending on the particular cell line used and on whether or not cells are exposed to the drug as aggregates or in monolayer cultures. The embryonal carcinoma line, F9 (4), has been most extensively studied. Strickland and Mahdavi (45) first showed that retinoic acid-treated F9 monolayer cultures differentiate into extraembryonic endodermlike cells. If subsequently treated with dibutyryl cAMP, these cells further change into parietal endoderm (46). Hogan et al. (20) have recently shown that some cells in aggregates of retinoic acid-treated F9 cells develop into visceral endoderm. Kuff and Fewell (25) have observed "neuron-like" cells in retinoic acid-treated cultures of F9 but the neuronal nature of these cells was not unequivocally established and these cells may in fact have been the parietal endoderm cells described by Strickland et al. (46). We found that some F9 cells differentiated into neurons when they were aggregated and treated with high concentrations of retinoic acid ( $>10^{-6}$  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, extraembryonic endoderm was formed by F9 cells in conditions under which none is made by P19 cells, and F9 cells only inefficiently developed into neurons in our hands.

Aggregation was first used by Martin and Evans (29, 30) to induce differentiation of embryonal carcinoma cells in culture. Speers et al. (43) have shown that hexamethylene bisacetamide (HMBA) treatment of an embryonal carcinoma line results in the formation of differentiated cells with epithelial or fibroblastic morphologies depending on whether or not cells are aggregated during drug treatment. The effects of retinoic acid reported in the present communication and those on F9 cells reported by Hogan et al. (20) 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 (18).

Retinoic acid was not toxic to the P19 cells. The population of embryonal carcinoma cells was found to be homogeneous with respect to its response to retinoic acid treatment. It could be argued that the P19 population as a whole is committed to making neurons, glial, and fibroblastlike cells and that retinoic acid acts to enhance the differentiation process but has no role in determination of these cells. However, since P19 cells can differentiate into extraembryonic endodermlike cells and into muscle under certain conditions (M. W. McBurney, E. M. V. Jones-Villeneuve, M. K. S. Edwards, and P. J. Anderson, manuscript submitted for publication), it is more probable that they comprise a pluripotent embryonal carcinoma cell population. In addition, other pluripotent embryonal carcinoma cells also respond to retinoic acid, indicating that the effect is not peculiar to P19. Our results argue against models which attempt to explain the action of retinoic acid by cell selection. Most consistent with the observations is the model which proposes that retinoic acid acts as the level of determination to induce a set of differentiation events which do not occur in the absence of this drug. Thus the intracellular consequences of retinoic acid treatment may be similar to the normal developmental signals which determine neuronal and glial tissues.

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