Chemically Induced Bidirectional Differentiation of Embryonal Carcinoma Cells *in Vitro*

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N,N-dimethylacetamide, hexamethylene bisacetamide, and Polybrene induced rapid and extensive differentiation *in vitro* in an otherwise slowly differentiating subline of embryonal carcinoma cells. The type of differentiated cell induced was dependent on the spatial organization of the stem cells during drug treatment. In monolayer culture "epithelial" cells were produced exclusively. However, treatment of aggregated suspension cultures yielded predominantly "fibroblast-like" cells. The undifferentiated embryonal carcinoma cells and the two differentiated cell types were morphologically distinct when examined by light microscopy, scanning electron microscopy, and transmission electron microscopy; and they had differences in cell surface antigens. Both differentiated cell types produced large amounts of fibronectin, whereas the embryonal carcinoma cells produced only minimal amounts. This system provides a convenient way to induce relatively synchronous differentiation of embryonal carcinoma cells into specific differentiated cell types. (Am J Pathol 97:563–584, 1979)

THE MURINE TERATOCARCINOMA is an important model for studying cellular differentiation in relation to embryogenesis and neoplasia. The malignant stem cells, called embryonal carcinoma (EC) cells, can differentiate spontaneously *in vivo*, producing a variety of cell types that, because of their limited mitotic potential, are benign.[1] Indeed, blastocyst transplants have shown that malignant EC cells in an appropriate environment can respond to developmental signals and participate in embryogenesis in an apparently normal fashion.[2–5] If one could control the differentiation process, one could possibly induce differentiation to benignancy of spontaneous neoplasms to a clinically significant degree.

Lines of murine EC stem cells have been adapted to growth *in vitro* where they may spontaneously differentiate into nearly all the cell types found within the original solid tumors.[6–16] Several investigators have found that physical aggregation of the stem cells markedly enhances differentiation.[6,9,11–16] These differentiated cultures are generally quite complex. To simplify experimental analysis EC stem cells should remain totally undifferentiated until an inductive signal causes a complete,

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synchronous change into a single differentiated cell type. One would then hope to identify the biochemical events essential for the differentiation process. The Friend erythroleukemia is the prototype of such a system.[17,18] Induction of differentiation of EC cells has been described with bromodeoxyuridine,[19] and more recently with retinoic acid[20] and hexamethylene bisacetamide (HMBA).[21]

We have studied the induction of differentiation of EC cells using N,N-dimethylacetamide (DMA) and Polybrene (PB) as well as HMBA. In monolayer culture all three agents induced an epithelial (EPI) cell type like that obtained with retinoic acid.[20] However, the same agents used at the same concentrations induced EC cells grown in suspension as cellular aggregates to develop into "fibroblast-like" (F-L) cells similar to those obtained spontaneously from aggregated cultures[12] and resembling glial cells differentiated from embryoid bodies.[22]

In this report, we describe the conditions used to induce EC cell differentiation in monolayer and suspension cultures as well as morphologic and immunohistochemical characteristics of the cell types.

Materials and Methods

Cell Lines

The EC cell line used for induction experiments was a subclone of the azaguanine resistant line PCC4.azal,[10,12] kindly provided by Dr. Robert Oshima of the University of California, San Diego. Cultures were passaged *in vitro* for approximately 6 months prior to the beginning of these studies but retained their pluripotentiality. Another cloned EC line designated 247 DES C12 and a parietal yolk sac line designated PYS2[8] were kindly provided by Dr. John Lehman of the University of Colorado Medical Center, Denver.

Culture Conditions

All cells were grown in Auto Pow minimal essential medium (MEM) (Flow Laboratories, Rockville, Md) supplemented with 10% fetal calf serum (FCS) (Flow), 1 mM sodium pyruvate, 1X nonessential amino acids, 0.14% sodium bicarbonate, 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. Monolayer cultures were grown in plastic flasks and dishes (Falcon Plastics Co., Oxnard, Calif) at 37 C in a humidified 5% CO_2 in air atmosphere. Suspension cultures were grown in glass Ehrlenmeyer flasks with airtight rubber stoppers at 37 C on a G10 Gyrotory Shaker (New Brunswick Scientific Co., New Brunswick, NJ) rotating at 80 to 90 rpm. Stock monolayer cultures were subcultured two or three times per week with trypsin–ethylenediaminotetraacetate (EDTA) (Flow), splitting at approximately 1 to 10. Experimental monolayer cultures were initiated with 3×10^5 single EC cells per 60-mm culture dish. Suspension cultures were initiated with 5×10^6 single EC cells in 50 ml MEM in 250-ml Ehrlenmeyer flasks.

Inducing Agents

HMBA was a gift from Drs. Roberta Reuben and Paul Marks (College of Physicians and Surgeons of Columbia University, NY). Stock solution of 20 mM HMBA, 100 mM DMA (Aldrich Chemical Co., Milwaukee, Wisconsin) and 400 μg/ml PB (Aldrich) were made in MEM and filter-sterilized. HMBA was used immediately, whereas DMA and PB were

stored in glass bottles for no more than 10 days, after which there seemed to be some loss of potential. Inducing agents were added at the outset and maintained in the MEM for the duration of the experiment for monolayers. Cells treated in suspension were subsequently plated in untreated MEM.

The concentrations of agents employed for induction were determined empirically, based on cytotoxicity (as evidenced by floating cell debris and slow growth) and on the ability to induce a morphologic change. Both showed an obvious dose response, and the optimal final concentrations were PB, 8 μg/ml; HMBA, 2 mM; DMA, 5 or 10 mM. Early passage EC cells that responded very well to 5 mM DMA later required 10 mM DMA for the same rate and degree of differentiation.

Analysis of Differentiation

In monolayers, morphologic changes were monitored by phase contrast microscopy of living cultures and by staining of cells grown on glass coverslips. We employed May–Greenwald Giemsa stain after fixation with 1% glutaraldehyde and postfixation with 100% methanol. Aggregates that formed in suspension cultures were plated and stained after attachment and outgrowth, usually in 4 days.

Differentiation of monolayer cultures was quantitated by a point counting method.[23] Stained coverslips were examined by light microscopy with a reticle grid in one ocular lens. The slide was positioned at random, and each of 121 points of intersection was scored for the underlying cell type. Replicate counts of 200 or 300 points with a 25× or 16× objective generally had a standard deviation of less than 10%. The frequency of "hits" is directly proportional to the area covered by each cell type. The relative areas covered by EC and EPI cells was estimated by photographing confluent areas of each at the same magnification and counting the nuclei in these pictures. The average for several determinations was 5.14 EC cells/EPI cell. The relative proportion of EC cells in a mixed EC/EPI culture was computed as follows:

$$\frac{\text{EC hits} \times 5.14 \times 100}{(\text{EC hits} \times 5.14 + (\text{EPI hits})} = \% \text{ EC cells}$$

This method was not used for plated aggregates since the colony centers were often piled up, making area estimates inaccurate. Therefore, differentiation was estimated by plating aggregates sparsely and examining fixed, Giemsa-stained colonies for F-L differentiation. Prefixation with 10% formalin prior to 100% methanol fixation prevented the colonies from washing off and minimized artifactual cell shrinkage, which makes identification difficult.

Antiserum Production

New Zealand white rabbits were used to produce all antiserums. Antiserums to EC and PYS2 cells were produced by three biweekly intravenous injections of 5×10^7 undifferentiated PCC4.azal cells, 247 DES C12 cells, or PYS2 cells in phosphate-buffered saline (PBS), and monthly boosters. Immune serums were drawn 7 and 10 days after the third and subsequent injections and heat-inactivated at 56 C for 1 hour. Antiserum to differentiated F-L cells was raised against PCC4.azal aggregate cells treated for 12 to 14 days with 8 μg/ml PB. Preparations used for injection showed extensive F-L differentiation. Approximately 5×10^7 cells in PBS were emulsified with an equal volume of complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (subsequent injections), and rabbits were injected subcutaneously three times biweekly and monthly thereafter. Serum was harvested as above. None of the injected cells were treated with trypsin or EDTA, and all were washed extensively. Bovine plasma fibronectin was purified by affinity column

chromatography with gelatin bound to Sepharose 4B (Pharmacia Chemicals, Piscataway, NJ) [24] and antiserum produced by subcutaneous injection as above.

Antiserum Absorptions

EC antiserums were absorbed three times with pooled liver, spleen, and kidney cells from Strain 129 mice, twice with PYS2 cells, and once with FCS bound to Sepharose 4B. PYS2 antiserums were absorbed twice with undifferentiated PCC4.azal cells and once with FCS-Sepharose 4B. F-L antiserums were absorbed three times with a 1% Triton-×100 soluble extract of undifferentiated PCC4.azal cells bound to Sepharose 4B, twice with intact, undifferentiated PCC4.azal cells, and once with FCS–Sepharose 4B. Absorptions with cells were performed in suspension with a 1:1 ratio of packed cells to neat serum on a rocking platform for 2 hours at room temperature or overnight at 4 C. Absorptions with FCS–Sepharose 4B were performed by batch technique as above with the equivalent 10 mg protein/ml neat serum. Each absorption with EC-cell extracts bound to Sepharose 4B was performed by column chromatography with 20 mg cellular protein/ml neat serum.

Fibronectin antiserum was absorbed by passage through a gelatin–Sepharose 4B column and then a column of Sepharose 4B to which fibronectin-depleted plasma had been coupled.[24] The resulting antiserum produced a single precipitation arc against whole bovine plasma by immunoelectrophoresis. All serums were stored frozed at −20 C except for small fractions that were refrigerated at 4 C and preserved with 0.1% sodium azide.

Immunofluorescence

Viable cells were suspended by pipetting, washed, and incubated for 20 minutes at room temperature with absorbed antiserum. After washing, the cells were again incubated for 20 minutes at room temperature with rhodamine-conjugated goat antirabbit IgG (Cappel Laboratories, Cochranville, Pa), washed, and mounted with Tris (0.01 M, pH 9.4) buffered glycerol.

Cells grown on glass coverslips were completely air-dried and fixed with 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7, for 30 minutes. For localization of fibronectin, fixation preceded both antiserum incubations. For the other antiserums, fixation was performed after the first antiserum incubation. Appropriate controls were used routinely, and preimmune serums were negative.

Histochemical Determinations

For alkaline phosphatase histochemistry, acetone-fixed cells on coverslips were stained with naphthol AS-MX phosphate (Sigma Chemicals, St. Louis, Mo) at pH 8.6.[25] For acid phosphatase histochemistry, acetone-fixed cells were stained with naphthol AS-B1 phosphoric acid (Sigma) and Fast Garnet GBC (Sigma) at pH 5.2.[26]

Electron Microscopy

Preparation of samples for transmission and scanning electron microscopy has been described.[27] The indirect labeling of cell surfaces with hemocyanin-conjugated antibodies has been previously described.[28] Areas to be thin-sectioned were carefully selected to exclude extraneous cell types in mixed populations.

Results

Light Microscopy

The schema for chemically induced differentiation of EC cells is summarized in Table 1. Each cell type was defined by distinctive morphologic characteristics seen by light microscopy.

Table 1—Schema for Induction of Differentiation of Embryonal Carcinoma (EC) Cells

EC on monolayer + no inducing agent	→ EC
EC on monolayer + inducing agent*	→ Epithelial cells
EC as aggregate + no inducing agent	→ EC (in monolayer)
EC as aggregate + inducing agent*	→ Fibroblast-like cells (in monolayer)

* N,N-dimethylacetamide, hexamethylene bisacetamide, or Polybrene.

EC Cells

EC cells in stock monolayer cultures differentiate minimally. Occasional EPI cells are seen but routinely constitute less than 1–2% of the population. By phase microscopy of living cells or by conventional light microscopy of fixed stained preparations the EC cells are seen to be small and rounded with scant cytoplasm and often indistinct cell borders, giving the appearance of a syncytium (Figure 1). They usually have a single, large, central nucleolus and little in the way of cytoplasmic structures. Mitotic figures are common, and in dense cultures the cells have a tendency to pile up.

EPI Cells

When the EC stem cells are plated sparsely ($5–20 \times 10^3/cm^2$) in monolayer cultures, about 10% differentiate spontaneously into EPI cells (Figure 1). After treatment of similar cultures with 10 mM DMA, 2 mM HMBA, or 8 μg/ml PB, more than 99% of the EC cells differentiate into EPI cells (Figure 2) morphologically identical to those seen in untreated cultures. Morphologic changes appear as early as 24 hours and become well developed in 2 to 3 days. After 7 days of drug treatment, only rare undifferentiated EC cells can be identified, but if the drug is removed, occasional EC colonies do appear within several days. In up to 14 days, only EC and EPI cells have been identified in treated or untreated monolayer EC cultures. EPI cells, when followed carefully, have never been seen to revert to EC cells nor to change into F-L cells.

The well-differentiated EPI cells (Figure 2) are polygonal, with distinct linear or refractile cell borders, when viewed by phase contrast microscopy. EPI cells are approximately five times as large as EC cells and are usually very flat. They have a relatively small nucleus with one or more small nucleoli. Juxtanuclear cytoplasmic granules are often abundant. Soon after differentiation the EPI cells divide actively, but mitosis eventually ceases.

F-L Cells

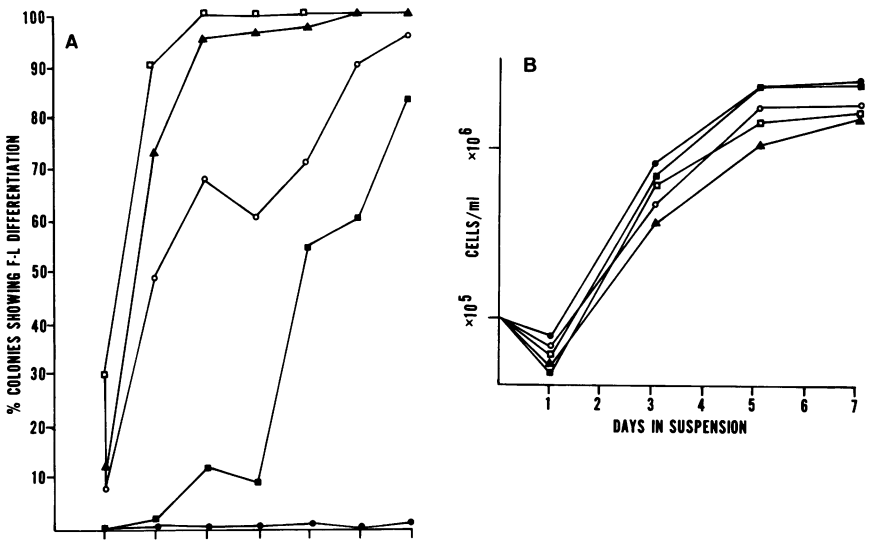
When single EC cells are seeded in suspension culture, they quickly begin to aggregate and form smooth-surfaced spherical aggregates within 24

hours. These continue to grow by accretion and cellular division and may after several days reach over 1 mm in diameter. Morphologic changes cannot be seen in the intact aggregates, but when they are plated in culture dishes, the aggregates attach and spread out as discrete colonies that are easily examined. When untreated aggregates from an early passage of EC cells were plated, a new cell type resembling fibroblasts was found. The F-L cells differentiated spontaneously after as little as 4 days of aggregation; after 7 days, 60–70% of the plated colonies showed at least some F-L cells; and the remaining colonies consisted of EC cells and/or EPI cells. With continued passage, stock EC cells lost their ability to spontaneously differentiate into F-L cells (Figure 3) even after growth in suspension for 3 weeks. However, when these EC cells were treated in suspension with DMA, HMBA, or PB, most of the plated colonies developed F-L cells (Figure 4).

The intact control and drug-treated aggregates are initially remarkably similar in suspension. All aggregates are uniformly spherical up to 5 to 7 days, when DMA-treated and HMBA-treated aggregates become elongated or dumbbell-shaped. The surfaces of drug-treated aggregates are quite smooth; control aggregates have a slightly more irregular surface. The control and treated aggregates are approximately equal in size, as estimated by ocular micrometer, for the first 3 to 4 days, after which time the control aggregates grow slightly faster than the induced aggregates.

The F-L cells in monolayer colonies (Figure 4) range from small, slightly elongated cells near the colony center, to well-oriented spindle cells in the midzone, to medium-sized, relatively flat cells at the periphery. Transitions from one form to another are easily found. For example, an area of spindle cells will merge imperceptibly with an area of flat, peripheral cells. Spindle cells and EC cells form distinct boundaries when juxtaposed in culture. F-L cells have oval to elongated nuclei with one or two small nucleoli. Cytoplasmic granules can be seen near the nucleus. The spindle variant is the most easily identified by light microscopy. The small central cells closely resemble EC cells but can be distinguished by their slight elongation, smaller nucleoli, and their tendency to remain piled up. Likewise, the flat peripheral cells may closely resemble EPI cells but can be distinguished by their tendency to separate from one another, their more irregular outlines, and their lack of distinct linear cell borders.

The time required for the induction of F-L cells in suspension was determined by sequentially plating aliquots from aggregate cultures and scoring the resulting colonies for F-L cells. The experiment illustrated in Text-figure 1A shows the relative potency of the three agents as well as a clear dose response relationship for HMBA. F-L cells appear after only



TEXT-FIGURE 1A—Differentiation of EC cells after drug treatment in suspension. On Day 0, trypsinized EC cells were seeded in suspension culture at 10^5/ml with continuous drug treatment as follows: ●, CTL, □, 10 mM DMA, ■, 1 mM HMBA, ▲, 2 mM HMBA, ○, 8 μg/ml PB. Every day fractions were seeded sparsely in monolayers in MEM and after 4 more days were fixed, Giemsa-stained, and scored for F-L differentiation by light microscopy. Points represent an average of duplicate cultures. B—Growth of EC cells in suspension. Fractions of same suspension cultures described in A were trypsinized and counted in a hemocytometer. Points represent an average of duplicate cultures.

one day of treatment with 10 mM DMA or 2 mM HMBA, and by 3 days almost all of colonies have F-L cells. The growth curves for the same experiment (Text-figure 1B) are remarkably similar and show that drug-treated cultures increase nearly as rapidly as the nondifferentiating control cultures. F-L cells have been isolated in pure culture and have continued to divide. In several generations there has been no reversion to EC cells nor further change to EPI cells.

Electron Microscopy

The morphologic differences among the three cell types seen by light microscopy correlate with ultrastructural differences. By transmission electron microscopy the EC cells are characterized by a high nucleus-to-cytoplasm ratio with few mitochondria, scattered ribosomes, short, narrow profiles of endoplasmic reticulum, and a small Golgi body (Figure 5). The EC cells are generally loosely arranged, with short stretches of adjacent plasma membranes quite uniformly apposed. Adherens junctions [29,30] characterized by close apposition of membranes with associated condensation of cytoplasmic microfibrils are common. Occludens or nexus

types of junctional structures,[29,30] characterized by five distinct layers and no apparent intercellular space, are common between EC cells grown in suspension but are less common in monolayers. Sometimes these junctional structures are located just beneath the apical surface, thus imparting a certain degree of polarity.

EPI cells appear quite large and thin, with the surface in contact with medium being generally flat (Figure 6). The cells are distinctly polarized, as defined by a subsurface adherens junction that is always present. A few microvilli are always found near this junction that is always present. A few microvilli are always found near this junction and occasionally over the entire cell surface. Beneath the adherens junction, a zone of interdigitating processes is commonly seen and occasionally a short occludens-type junction. The cytoplasmic structures are better developed than in EC cells. The rough endoplasmic reticulum is dilated slightly and contains relatively lucent material. The Golgi apparatus is usually large, consisting of numerous rounded profiles. Microfibrils are more prominent than in EC cells; they are usually associated with junctional structures but also occur as small bundles beneath the plasma membrane. Microtubules are uncommon. Membrane-enclosed particles, morphologically consistent with "intracisternal A-type" viral particles, are commonly seen in EPI cells. C, B, and budding viral forms have not been identified. The nuclei of the EPI cells are more irregular in shape than those of EC cells, and the nucleolar condensations are more often attached to the nuclear membrane. Fine fibrillar extracellular material is occasionally seen between cells, but not cross-banded collagen nor basement membrane material.

By transmission electron microscopy monolayer F-L cells are characterized by well-developed cytoplasmic structures and a relative paucity of junctional structures (Figure 7). The rough endoplasmic reticulum is prominent and often widely dilated. The Golgi body occurs as stacked narrow profiles; and numerous microfilaments occur in thick, long bundles. There is little in the way of surface polarization; adherens junctions are sparse, randomly distributed, and associated with the microfilament bundles. Intracisternal "A-type" particles are even more common than in EPI cells. The extracellular space focally contains fine fibrillar material as well as larger fibrils, occasionally with discrete periodic bands, interpreted as collagen. Extracellular material has not been seen on the surface in contact with the culture medium.

The cellular morphologic characteristics within aggregates was also examined directly by transmission electron microscopy and with stained 1-μ thick sections. The earliest and most obvious difference between control and 10-mM DMA-treated aggregates occurs within 24 hours and remains

present for at least 4 days. The treated aggregates have a greater degree of cellular compaction, as evidenced by the paucity of intercellular space and smoother surfaces. Although not strictly quantitated, treated aggregate cells appear to have more frequent occludens junctions during the first 2 days, but at Days 3 and 4 fewer occludens and adherens structures are seen than in control aggregates. By Days 3 and 4 the treated aggregate cells have accumulated more cytoplasm with more microtubules, the nuclei are more irregular in contour, and focal fine fibrillar extracellular material is common. Bundles of microfilaments and "A-type" particles similar to those found in plated F-L cells have not been identified in treated aggregates. The treated aggregates lack a surface layer of yolk-sac epithelium and a basement membrane and therefore do *not* resemble embryoid bodies.

The three cell types are quite distinctive by scanning electron microscopy. EC cells (Figures 8 and 9) are small and rounded, with a few irregularly spaced microvilli. Occasionally, monolayer EC cells appear more polygonal, with the cell borders partially outlined by uniform microvilli. EPI cells (Figures 10 and 11) are seen to be larger and flatter than EC cells and F-L cells; all borders of adjacent EPI cells are well demarcated by uniform microvilli, but longer cell processes are absent. F-L cells (Figures 10 and 11) have generally smooth surfaces except for prominent long processes. Microvilli are less common and more randomly arranged on F-L cells than on EC and EPI cells.

Immunohistochemical Observations

Heterologous antiserums were raised against EC cells, PYS2 cells, and F-L cells and extensively cross-absorbed. Our purpose was first to establish that each cell type had differences in cell surface antigens and then to closely monitor the differentiation process in terms of antigenic changes. PYS2 antiserum was used because these cells morphologically resembled EPI cells, and they could be grown in large numbers.

EC antiserum stains viable EC cells in monolayers or suspension with a fine granular pattern. In monolayers staining at cell borders outlines the individual cells (Figures 12 and 13). Drug-treated EC cells gradually lose their reactivity with EC antiserums, and neither well-differentiated EPI cells (Figure 12) nor F-L cells (Figure 13) are stained. This loss of reactivity with EC antiserum begins after 1 day of treatment with 10 mM DMA in monolayer or in suspension and with EPI differentiation clearly parallels the morphologic transformation.

Fully absorbed PYS2 antiserum lost all indirect immunofluorescence ac-

tivity against cell surface antigens of PYS2 cells as well as EC and EPI cells and stained only extracellular material. Similarly, fully absorbed F-L antiserum showed no cell surface activity against F-L cells by indirect immunofluorescence but stained extracellular material brightly.

Because antiserums to PYS2 and F-L cells are relatively crude preparations, we used affinity-purified fibronectin antiserum to more carefully examine the production of extracellular matrix. This serum gives minimal staining of EC cells by indirect immunofluorescence, whereas both EPI and F-L cells have linear strands and coarse granules (Figure 14). The linear pattern is seen with both viable and fixed differentiated cells and is presumably extracellular. Coarse granular staining is seen in fixed preparations only and is interpreted as representing internal fibronectin.

Monolayers produce internal and external fibronectin after only two days' treatment with 10 mM DMA. It is clearly associated with EPI cells only. By 7 days an extensive network of long interwoven strands is produced. DMA-treated aggregates also produce fibronectin after only 2 days in suspension, and after plating it can be clearly identified associated only with F-L cells.

To localize more carefully the antigenic determinants defined by these antiserums, we examined cells by scanning electron microscopy, using a hemocyanin conjugate as the second antibody layer. EC antiserum selectively stains EC cell surfaces as seen by indirect immunofluorescence. PYS2 antiserum has no apparent selectivity for any cell type, and extracellular material cannot be identified on the cell surfaces in contact with the culture medium. Fibronectin antiserum does not stain any cell surfaces but does deposit heavily on extracellular material exposed from beneath F-L cells.

Scanning immunoelectron microscopy with F-L antiserum gives results quite different from those of immunofluorescence. The F-L cell surfaces stain heavily, including the cellular processes (Figure 11). All variants of F-L cells, small central cells, spindle cells, and flat peripheral cells, stain approximately equally. EC and EPI cells are negative or, rarely, trace-positive. In some coverslip preparations EC, EPI, and F-L were all present, the former cell types serving as a convenient internal control for demonstrating the specificity of F-L antiserum. Several samples of F-L antiserum were absorbed independently, giving identical results, ie, strongly positive surface stain by scanning electron microscopy, but no cell surface staining by immunofluorescence. Possibly the glutaraldehyde prefixation used for scanning electron microscopy preserves an otherwise highly labile surface determinant recognized by the F-L antiserum, and the fixa-

tion procedures used for immunofluorescence do not. Glutaraldehyde was not used as a fixative for immunofluorescence due to high background.

In addition to these immunologic stains, histochemical staining was used to distinguish the various cell types. EC cells have very high levels of alkaline phosphatase, well-differentiated EPI cells are negative, and F-L cells range from negative near the colony periphery to intermediate in the more central areas. All three cell types have detectable acid phosphatase, the F-L cells having perhaps more activity; the differences are not great. Thus, the various cell types can be discriminated immunologically and histochemically as summarized in Table 2.

Discussion

DMA, HMBA, and PB induce rapid, extensive differentiation in a subline of PCC4.azal EC cells. The type of differentiated cell induced depends on the spatial relationships of the EC cells during the period of drug treatment: EPI cells are produced in monolayer culture, and F-L cells are produced in suspension culture. The three cell types differ morphologically, ultrastructurally, antigenically, and biochemically.

The induced cell types are "differentiated" by the criteria of irreversibility, production of a luxury molecule (fibronectin), and increased cytoplasmic complexity. The induced cell types are presumably "normal" derivatives of EC cells, since essentially identical cells have been found in spontaneously differentiating, untreated cultures. The cells are capable of mitosis, but EPI cells appear to be end cells, since older confluent cultures cease division even in the presence of untreated medium.

The differentiated cells have not been definitively classified. If one assumes that EC cells are analogous to inner-cell-mass cells of the mouse embryo as has been suggested,[31] the EPI cells induced in monolayers

Table 2—Immunologic and Histochemical Characteristics of Cell Types

Staining reaction	Cell type		
	EC	EPI	F-L
Anti-EC*	+++	–	–
Anti-F-L†	– to tr	– to tr	+++
Anti-fibronectin*	–	+++	+++
Alkaline phosphatase‡	+++	– to tr	– to ++

EC = ebryonal carcinoma; EPI = epithelial; F-L = fibroblast-like.
* By indirect immunofluorescence.
† By scanning electron microscopy.
‡ By histochemical localization.
– = no staining; tr = minimal, focal staining; + = minor staining; ++ = moderate staining; +++ = heavy staining.

might be yolk-sac endoderm. Indeed, the EPI cells do resemble PYS2 cells when examined by phase contrast and scanning electron microscopy, especially when the latter are plated sparsely. However, EPI cells do not have the dilated cisternae of rough endoplasmic reticulum seen in PYS2 cells, nor do they produce morphologically identifiable basement membrane material.[8,31] Cells apparently similar to EPI cells induced with retinoic acid [20] and hexamethylene bisacetamide [21] were classified as endoderm, the classification based on biochemical criteria, namely, the production of plasminogen activator, a marker enzyme of parietal endoderm.[32]

The F-L cells could represent a mesodermal or a neuroectodermal derivative again by analogy to early differentiation of the inner cell mass. In other situations, when induced aggregate cultures composed predominantly of F-L cells are left in monolayer for several weeks, numerous foci of adipose cells form, suggesting mesodermal origin. Other mesodermal derivatives have not yet been identified. On the other hand, some of the F-L cells could represent glial differentiation. Glial tissue is a common component of well-differentiated murine teratocarcinomas *in vivo*,[1] and the F-L cels do resemble differentiating glial tissue, as described in embryoid body differentiation *in vitro*.[22] Focal neuronal differentiation has been occasionally found associated with F-L cells, further suggesting this possibility. We cannot completely rule out the possibility that the F-L cells are a mixed population of morphologically similar cells representing more than one germ layer. We have recently succeeded in eliminating contaminating EC cells from a mixed population by complement-mediated EC-antiserum–directed killing. There is no apparent toxic effect on the F-L cells, and they continue to divide. Additional studies with these cells should permit more definitive classification.

The immunologic reagents used in this work were relatively crude but clearly distinguished the cell types from one another. It seems probable that each cell type has a specific "fingerprint" of cell surface components, which when characterized biochemically should allow absolute identification. The changes evident with immunohistochemical staining followed or accompanied the morphologic changes of differentiation and so were of little help in identifying early or transition stages in differentiation.

The production of fibronectin is a convenient marker for cellular differentiation in this system, although it does not distinguish between the EPI and F-L cell types. Wartiovaara et al [33] also reported that cells differentiated from EC stem cells produce fibronectin. It was associated with presumptive endodermal cells in monolayer culture and in the basal lamina of embryoid bodies. In contrast, Zetter and Martin [34] describe both multi-

potent and nullipotent EC cell lines that produce fibronectin in the undifferentiated state, but also mention fibronectin-negative EC cell lines. In the developing mouse embryo,[34] fibronectin is first associated with differentiation of the inner cell mass into endoderm and ectoderm. Trophectoderm and cleavage-stage embryos are negative. Therefore, fibronectin-negative EC cells may represent a slightly earlier stage of development than fibronectin-positive lines. Fibronectin may represent a developmentally significant second message for continued differentiation or for structural organization,[35] and further studies are needed.

It is not surprising that the spatial relationships of the EC cells during induction are important in determining the pathway of differentiation. Internal–external differences are apparently determinative for trophoblast [40] and endoderm [41,42] differentiation in the mouse embryo. In spontaneously formed embryoid bodies *in vitro*,[31] it also appears that only the surface cells differentiate into yolk-sac epithelium. The mechanism for this differentiation is obscure. It seems clear that the yolk-sac epithelium is not itself an inductive agent, since aggregate differentiation in our system proceeds in the absence of embryoid body formation and in many individual aggregates with no epithelial component at all.

The mechanism of induction is unknown. One relevant observation is that DMA-treated aggregates become more compact than untreated controls within 24 hours, and compaction precedes fibronection production and extensive F-L cell differentiation by 24–48 hours. DMA and HMBA also induce differentiation in erythroleukemia cells by unknown mechanisms.[36,37] Activity in such divergent systems suggests that the drugs may be acting nonspecifically. These agents may merely sensitize the cells to normal signals already present within the environment, perhaps by increasing cell–cell cohesiveness. PB, for example, is known to cause neuraminidase-sensitive agglutination of erythrocytes.[38] Disruption of cellular compaction by heterologous anti-EC Fab fragments clearly inhibits differentiation of early mouse embryos *in vitro*.[39] On the other hand, it is more difficult to invoke cellular compaction as a facilitative influence in monolayers where these compounds also have an inductive effect. Whatever the mechanism, it is clear that the chemical agents are inducing differentiation in two distinct directions. The fact that this induction is relatively synchronous and exclusive should permit informative comparisons of the processes of differentiation.

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Acknowledgments

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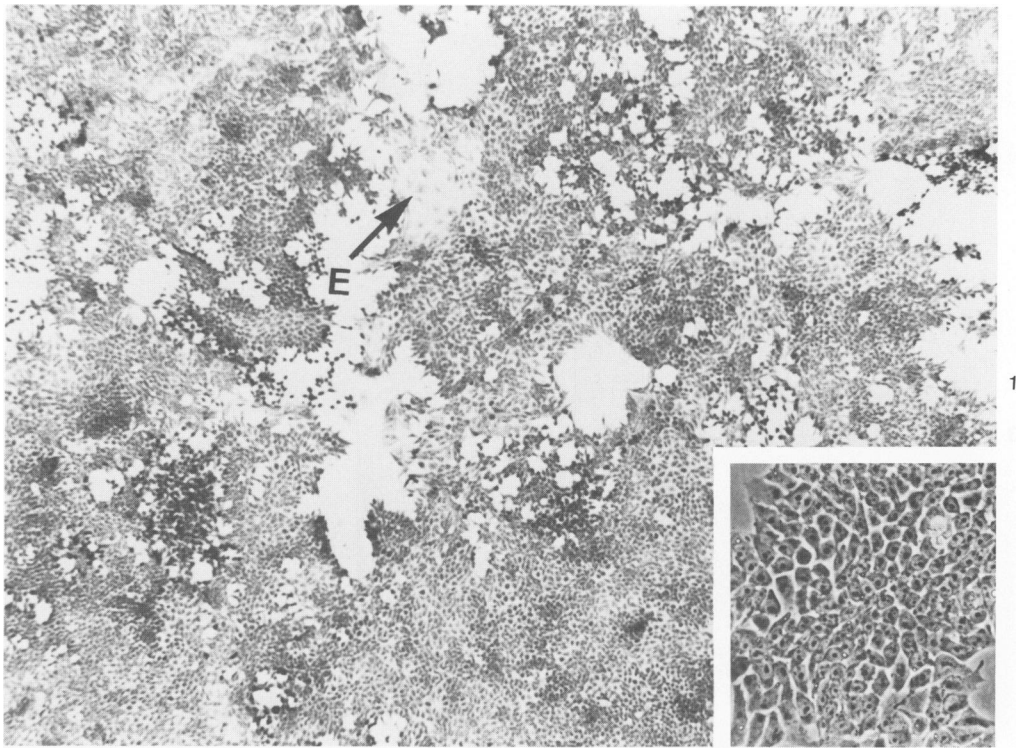
[*Illustrations follow*]



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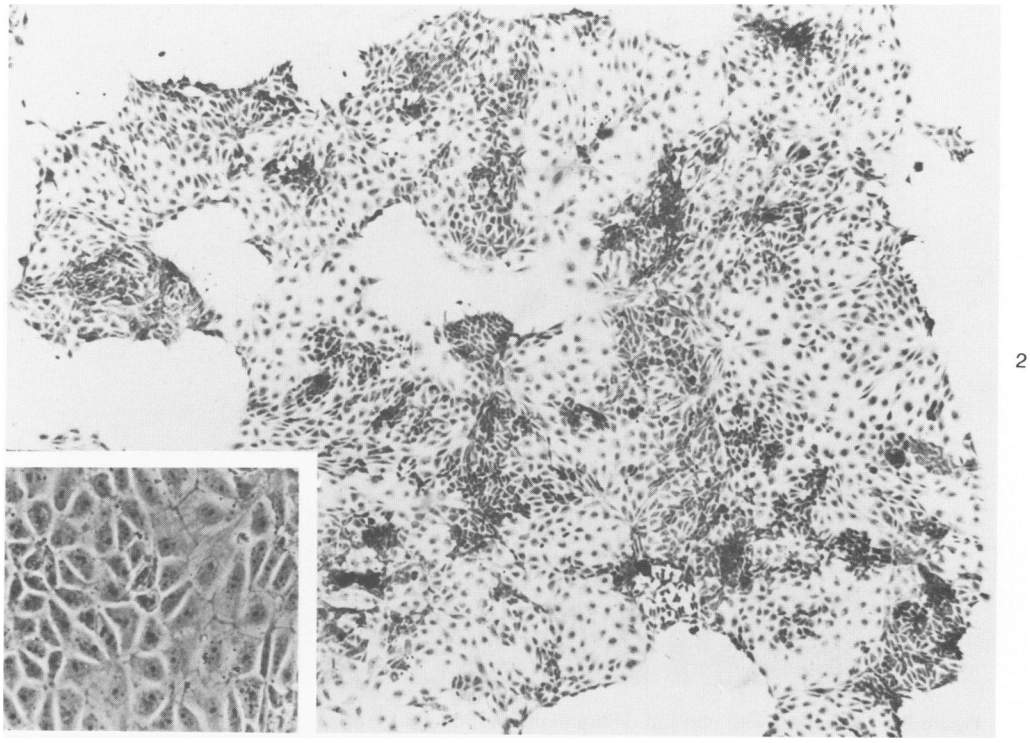

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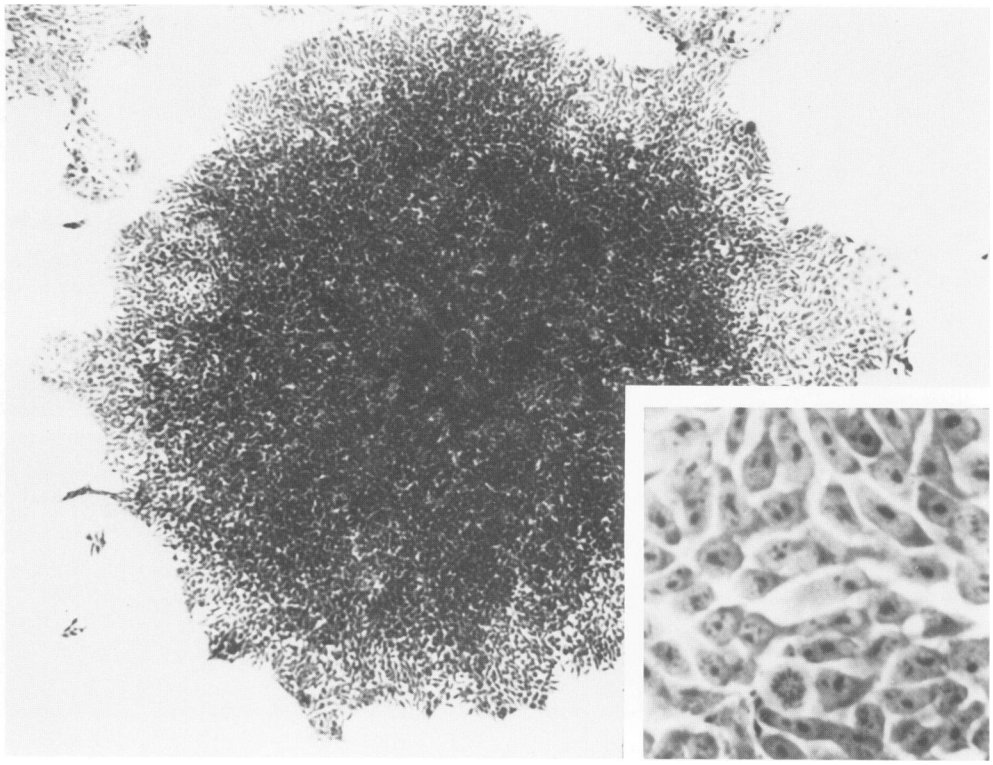
Figure 1—Untreated EC cells grown in monolayer culture for 4 days. Note focal differentiation into EPI cells (*E*) (May–Greenwald Giemsa, ×64) **Inset**—EC cells. (Phase contrast, ×160) **Figure 2**—Nearly complete differentiation into EPI induced by treating EC cells in monolayer with 10 mM DMA for 4 days. Most of the smaller cells are in fact EPI cells that have not spread out. (May–Greenwald Giemsa, ×64) **Inset**—EPI cells showing distinct linear borders. (Phase contrast, ×160)



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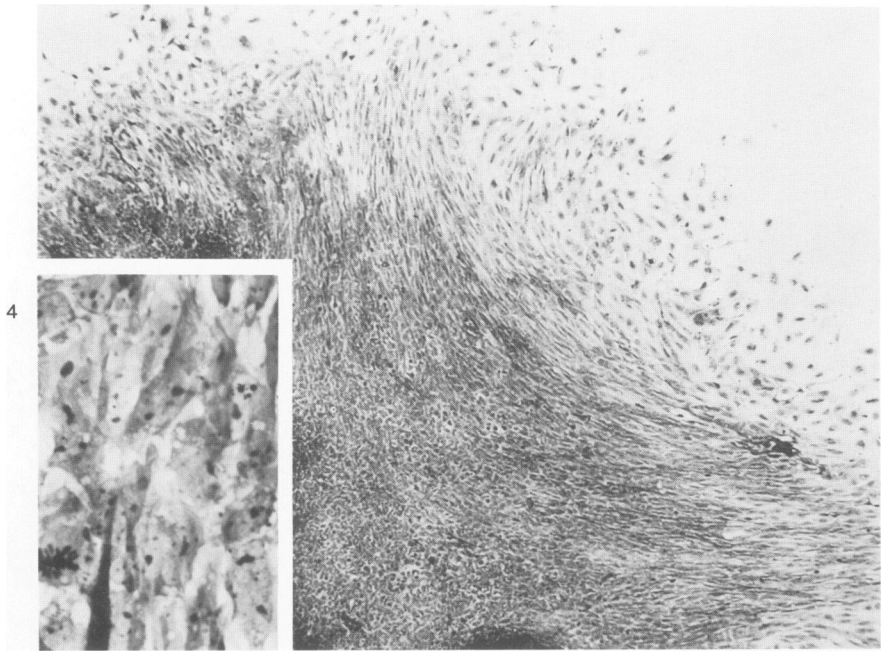

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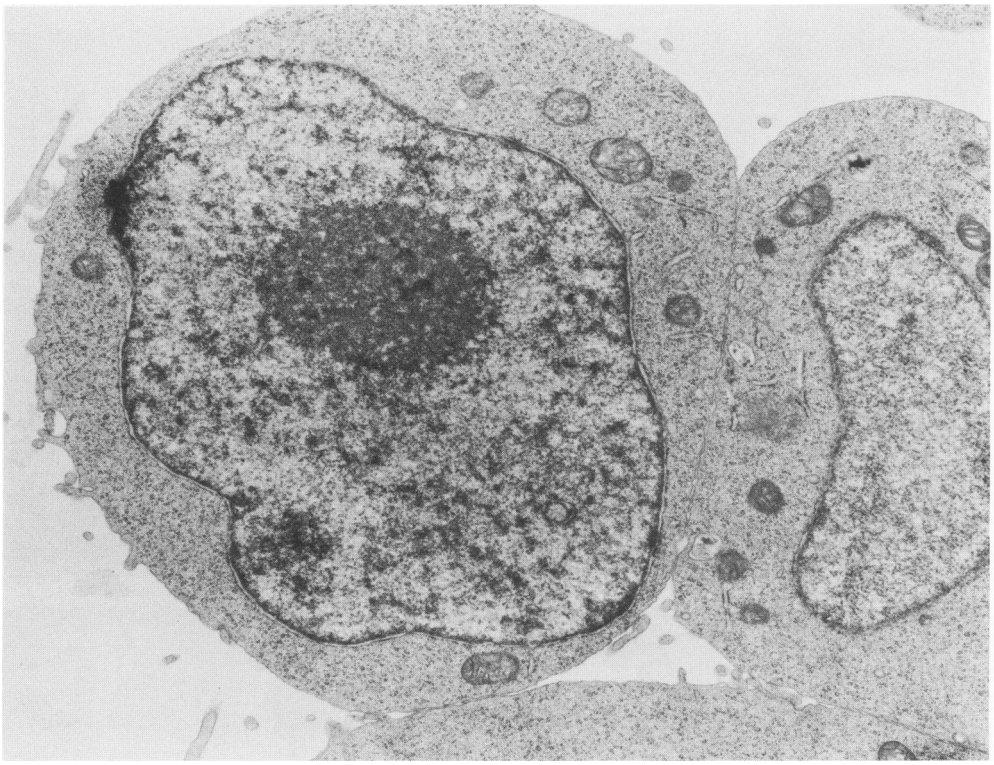
Figure 3—EC colony with minimal differentiation produced by growing untreated EC cells in suspension for 4 days and plating the resulting aggregates as a monolayer for 4 days. (MGG, ×80) **Inset**—EC cells. (May–Greenwald Giemsa, ×500) **Figure 4**—Portion of colony with nearly complete differentiation into F-L cells. Note the small central cells, spindle cells, and flattened peripheral cells. EC cells were treated in suspension with 10 mM DMA for 4 days and the resulting aggregates plated in untreated medium for 4 days as a monolayer. (May–Greenwald Giemsa, ×50) **Inset**—Spindle form of F-L cells. (May–Greenwald Giemsa, ×500)



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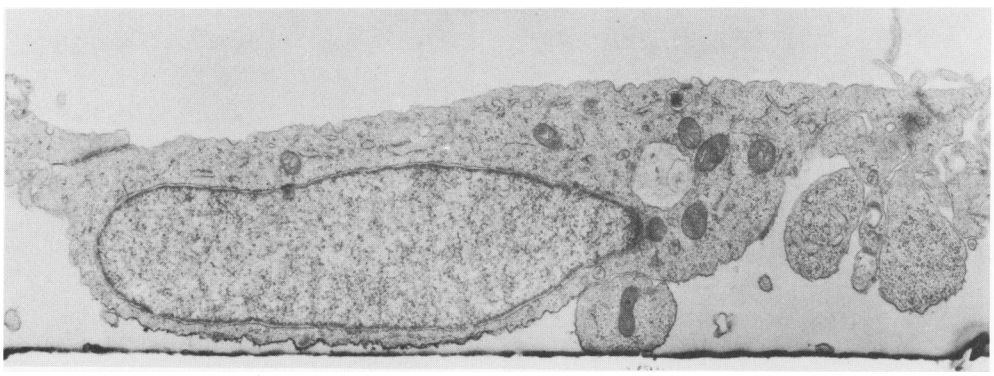

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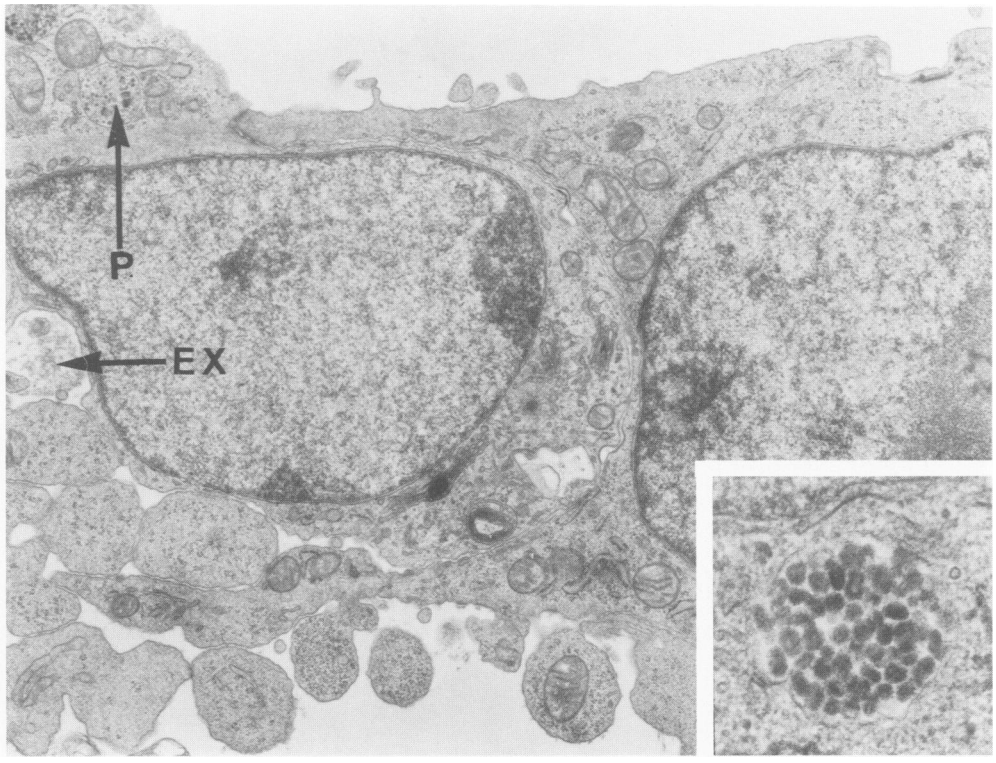
Figure 5—Transmission electron micrograph of untreated EC cells after 4 days in monolayer culture. Note the large nucleus in proportion to the cytoplasm, the prominent nucleolus, and the simple cytoplasm. (×8400) **Figure 6**—Transmission electron micrograph of EPI induced by 10 mM DMA for 4 days in monolayer culture. Note the flattened profile, junctional structures, and microvilli. The dark line represents the interface with plastic substratum. (×9900)



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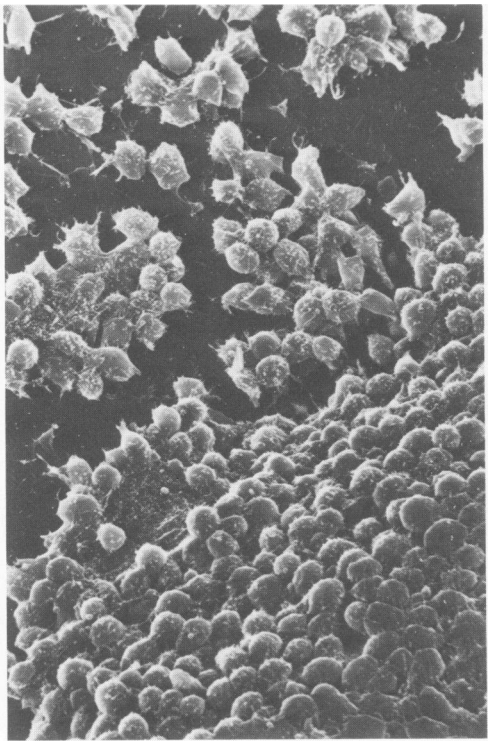

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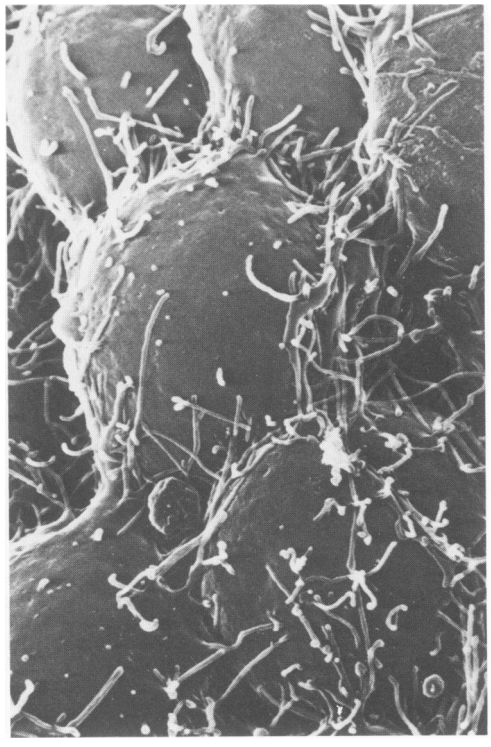

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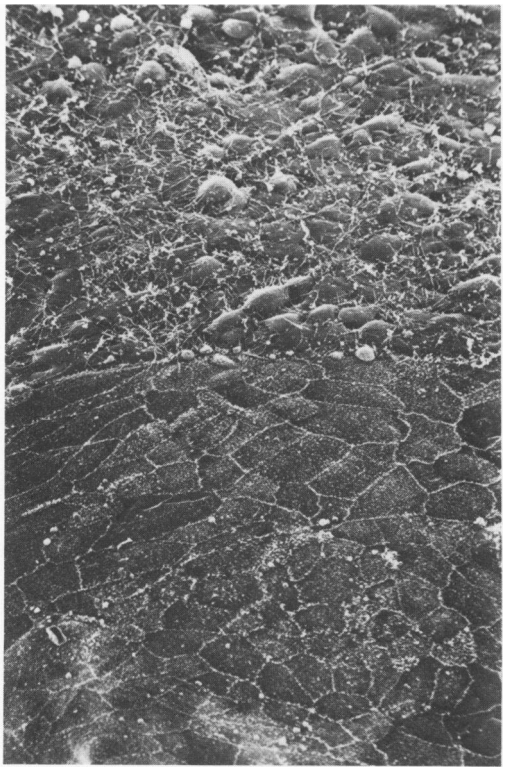
Figure 7—Transmission electron micrograph of F-L cells induced with 10 mM DMA for 4 days in suspension, followed by 4 days in monolayer in untreated medium. Note extracellular material (*EX*) and intracisternal particles (*P*). (×10,500) **Inset**—Intracisternal particles interpreted as "A-type" viral particles. (×52,000) **Figure 8**—Scanning electron micrograph of untreated EC cells grown in suspension for 4 days and in monolayer for 4 days. (×300) **Figure 9**—Same cells as Figure 8. Scanning electron micrograph. (×3200)



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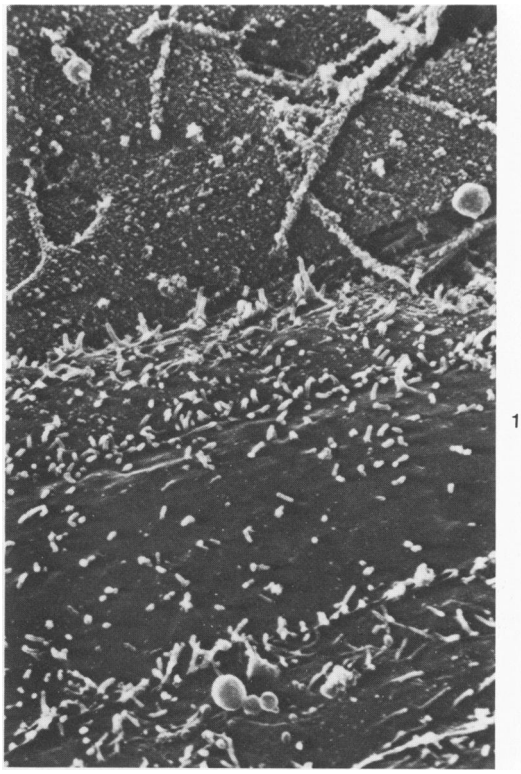

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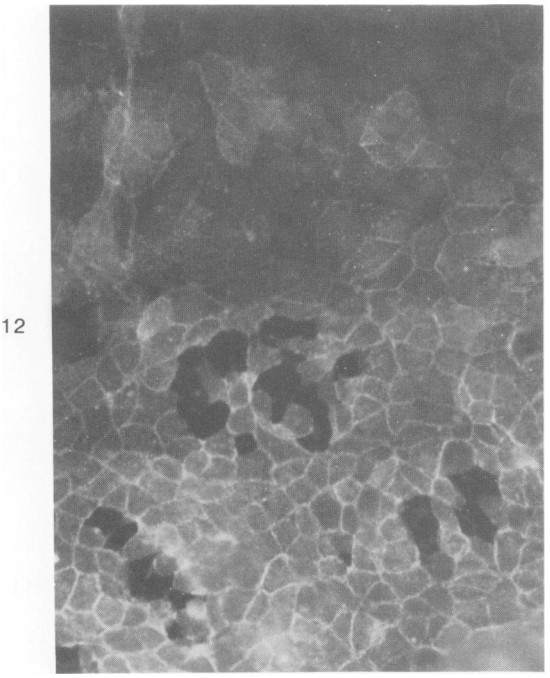
Figure 10—Scanning electron micrograph of F-L (*above*) and EPI cells juxtaposed in monolayer culture subsequent to 10 mM DMA treatment in suspension. EPI cells are characterized by surface microvilli with clear demarcation of cell borders. F-L cells are characterized by longer processes and relatively indistinct cell borders. (×300) **Figure 11**—Same cells as in Figure 10. Scanning electron micrograph showing indirect hemocyanin labeling with absorbed F-L antiserum. F-L cells (*above*) are uniformly coated with hemocyanin granules; EPI cells show trace labeling. (×3800)



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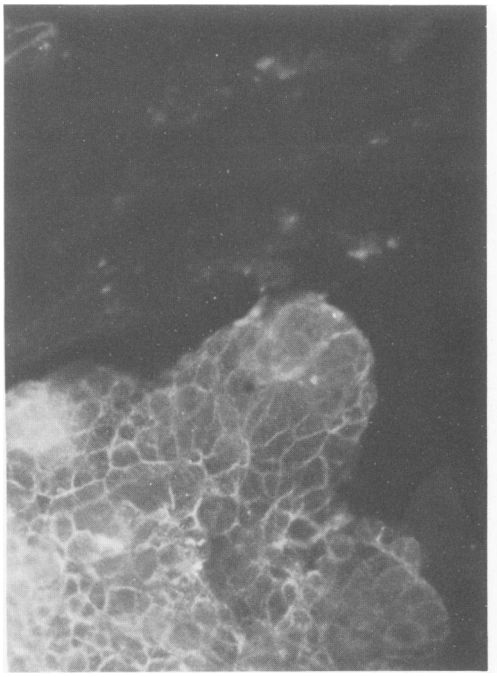

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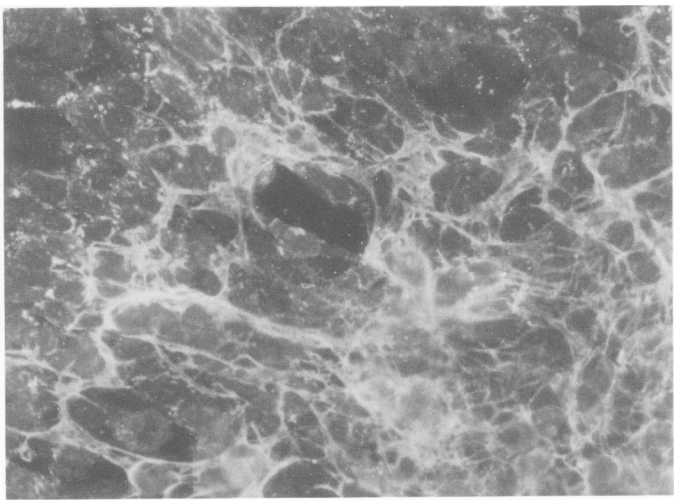

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Figure 12—Indirect immunofluorescence of EC and EPI cells labeled with absorbed anti-EC serum. The EC cells are positive, the well-differentiated EPI cells are negative, and there appear to be morphologic and antigenic intermediates. (×350) **Figure 13**—Indirect immunofluorescence of EC and F-L cells labeled with absorbed EC antiserum. The EC cells are positive, and the F-L cells are negative. The distinct interface between cell types is not a fixation artifact. (×350) **Figure 14**—Indirect immunofluorescence suspension induced F-L cells labeled with fibronectin antiserum. Note the linear external and granular internal stain. (×350)