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The Induction of Differentiation in Teratocarcinoma Stem Cells by Retinoic Acid

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Summary

Embryonal carcinoma cells, the stem cells of teratocarcinomas, usually undergo extensive differentiation in vivo and in vitro to a wide variety of cell types. There exist, however, several embryonal carcinoma cell lines that have almost completely lost the capacity to differentiate, so that the cells are propagated primarily as the stem cells. Using one such cell line, F9, we have found that retinoic acid at concentrations as low as 10^{-9} M induces multiple phenotypic changes in the cultures in vitro. These changes include morphological alteration at the resolution of the light microscope, elevated levels of plasminogen activator production, sensitivity to cyclic AMP compounds and increased synthesis of collagen-like proteins. The nature of these changes, as well as their independence of the continued presence of retinoic acid, are consistent with the proposition that retinoic acid induces differentiation of embryonal carcinoma cells into endoderm.

Introduction

Analysis of cellular differentiation is often hampered by factors such as insufficient numbers of stem cells, heterogeneity of starting populations and inability to intervene in the developmental processes. Recently, however, the discovery that murine virus-induced leukemia cells behave as erythrocyte precursor cells blocked at the proerythroblast and erythroblast stage (Friend, Patuleia and de Harven, 1966), and the subsequent finding that certain chemicals can induce the blocked cells to develop into mature erythrocytes (Friend et al., 1971), have made available an experimental system which circumvents some of these problems in the study of erythropoiesis.

The investigation into the biochemistry of early mammalian development has suffered from the lack of such a model system. Thus the realization that teratocarcinomas of mice contain stem cells that can differentiate into many diverse tissues (Pierce and Dixon, 1959; Stevens, 1960) and that the stem cells closely resemble primordial germ cells (Pierce, Stevens and Nakane, 1967) and embryonic ectoderm (Damjanov and Solter, 1975) offered promise that the development of these cells could be used as a paradigm for normal embryogenesis. However, although the cells could be obtained in plentiful number, their differentiation was chaotic and unpredictable, and consequently was difficult to study.

Several of the cell lines developed from teratocarcinomas (Bernstine et al., 1973) are analogous in many respects to the Friend virus-infected erythroblasts. In these lines, the ability of the cells to differentiate into diverse cell types has been greatly diminished, and the cells in culture or the tumors in vivo derived from these cultures are composed almost exclusively of embryonal carcinoma cells, the stem cells of teratocarcinomas (Kleinsmith and Pierce, 1964). Whereas the erythroleukemia cell system can rely upon hemoglobin production as a marker for differentiated cells, investigation into factors which may influence embryonal carcinoma cell development has been impeded by the lack of such a suitably defined and convenient marker.

It has recently been demonstrated that endoderm cells derived from the early mouse embryo (Strickland, Reich and Sherman, 1976) or from embryonal carcinoma cells (Sherman, Strickland and Reich, 1976; Topp et al., 1976; Linney and Levinson, 1977) produce the serine protease plasminogen activator. This observation has relevance to studies of teratocarcinoma differentiation, since in many cases endoderm is one of the first cell types to develop from embryonal carcinoma cells (Martin and Evans, 1975). Thus one approach to the study of the differentiation of these cells would be to survey cultures grown under various conditions for plasminogen activator production. This approach has in fact been used successfully to confirm earlier observations (Rosenthal, Wishnow and Sato, 1970; Martin and Evans, 1975) that the aggregation of stem cells can induce limited differentiation (Sherman et al., 1976).

In this study, plasminogen activator has been used as a marker to survey a variety of compounds for their effect on F9 cells, a teratocarcinoma cell line that undergoes very limited differentiation under normal culture conditions. In view of the established influence of vitamin A compounds on differentiation (Wolbach and Howe, 1925) and the induction of plasminogen activator synthesis in chicken embryo fibroblasts by retinoic acid (Wilson and Reich, 1978), we have tested various retinoids, among other compounds. We have found that retinoic acid at low concentrations induces the synthesis and secretion of plasminogen activator in F9 cultures. The morphological and biochemical evidence suggest that this result is a consequence of the differentiation of embryonal carcinoma cells into endoderm.

Results

Morphological Observations

F9 cells grow in culture as tightly packed colonies of embryonal carcinoma cells (Figure 1A). The cell population appears predominantly homogenous,

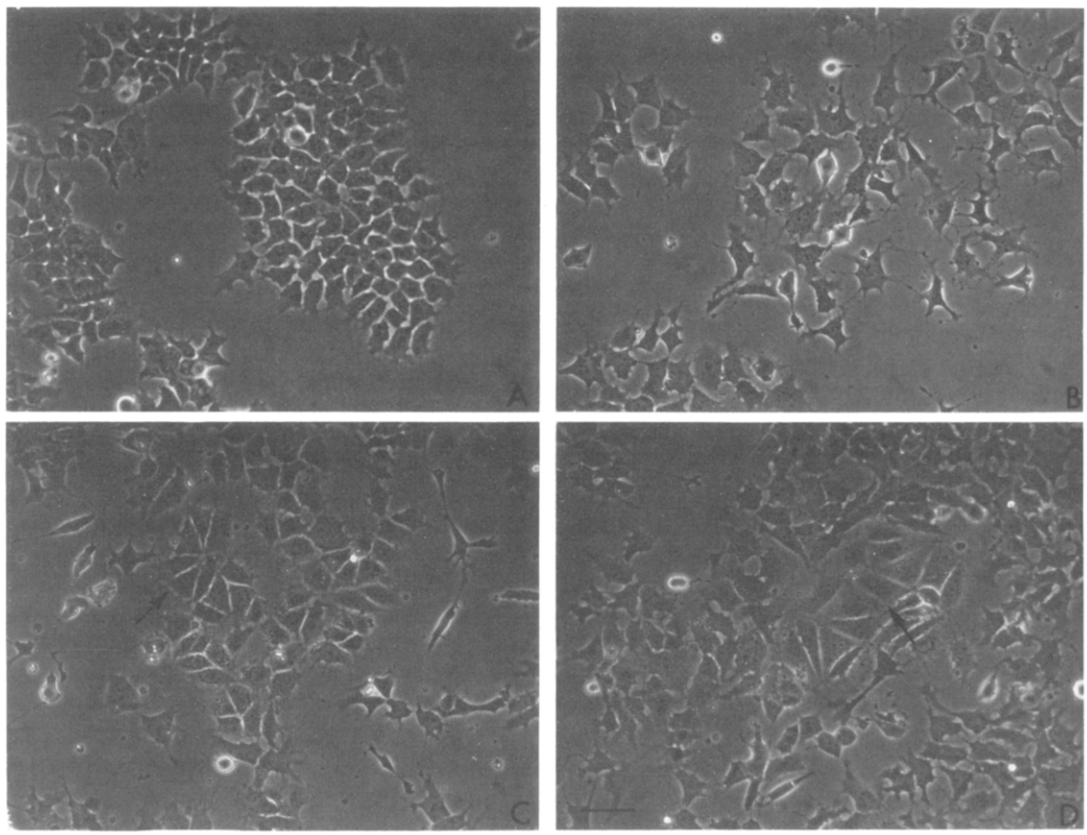

Figure 1. Morphology of F9 Cells Treated With Retinoic Acid

F9 cells were plated at a density of 5×10^5 per 100 mm petri dish in the presence or absence of 10^{-7} M retinoic acid. (A) Control cells after 72 hr; (B) retinoic acid-treated cells after 48 hr; (C) and (D) retinoic acid-treated cells after 72 hr. Arrows indicate regions that have endoderm-like morphology. Marker (in D) = 50 μ.

although patches of cells with different morphology are occasionally observed. Upon addition of 10^{-7} M retinoic acid to the culture, a pronounced morphological change occurs during the next several days. Within 24 hr, the cells move apart from one another and the colonies become much less compact (Figure 1B). Then the cellular morphology alters gradually in many colonies to triangular, flat cells with cytoplasmic granules (Figures 1C and 1D). The appearance of these cells is strikingly similar to that of the differentiated cells derived by allowing F9 cells to form aggregates (Sherman et al., 1976) and closely resembles the presumptive endoderm derived from teratocarcinoma embryoid bodies (Martin and Evans, 1975). Under standard conditions, primary parietal endoderm cells from normal embryos attach to the substrate but do not spread, and have a refractile morphology.

These results suggested that endoderm cells might be accumulating in the cultures upon treatment with retinoic acid. This interpretation is supported by an examination of biochemical markers which have been reported to distinguish endoderm from embryonal carcinoma.

Plasminogen Activator Production

Endoderm cells synthesize and secrete plasminogen activator. [The primitive endoderm which first arises in embryogenesis and the more differentiated parietal endoderm from mid-gestation conceptuses both secrete plasminogen activator, whereas differentiated visceral endoderm does not (Strickland et al., 1976). For this reason, and since any finer distinction is not central to this paper, the term endoderm will be used to designate those cells producing plasminogen activator, implying that they are primitive or parietal but not visceral.] This property was first demonstrated in primary cultures of parietal endoderm and in cultures derived from blastocysts and isolated inner cell masses (Strickland et al., 1976). It has also been shown that embryonal carcinoma cells do not secrete appreciable levels of this enzyme, but that the endoderm cells which differentiate from these cells do (Sherman et al., 1976; Topp et al., 1976; Linney and Levinson, 1977).

In addition, the production of plasminogen activator by teratocarcinoma-derived endoderm has been reported to be increased by treatment of the

cells with reagents that increase the intracellular concentration of cAMP (Linney and Levinson, 1977). We have corroborated this result using primary cultures of parietal endoderm. Table 1 demonstrates that protease production by these cells is enhanced approximately two fold by treatment with dibutyryl cAMP (DBcAMP). This effect is not due to mitogenic activity of the DBcAMP since the cell number in treated and control cultures is equivalent. Retinoic acid alone or in combination with DBcAMP has no effect on protease production (Table 1).

F9 cells grown under standard conditions produce very low amounts of plasminogen activator. The amount of enzyme does increase slightly but reproducibly in these cultures as the cell density increases (Figure 2). Addition of retinoic acid to the cultures, however, increases plasminogen activator secretion more than 10 fold in 3 days of culture (Figure 2). It should be noted that the effect of retinoic acid on protease production is not apparent until approximately 48 hr of treatment; thus the changes in morphology (Figure 1) and enzyme secretion are roughly parallel in time.

In light of the effect of cAMP on endoderm described above, it was of interest to examine the sensitivity of the cultures to cAMP. DBcAMP alone has little effect on F9 cultures; DBcAMP and retinoic acid taken together, however, have an apparent synergistic effect on protease secretion (Figure 2). The action of DBcAMP in cultures treated with retinoic acid is not immediate but requires several days to become manifest.

Thus using morphology, plasminogen activator production and sensitivity to DBcAMP as criteria for identification, the cell type generated upon treatment of F9 cells with retinoic acid is similar to endoderm. The following experimental observations are relevant to the interpretation of these results:

Table 1. Effect of Retinoic Acid and Dibutyryl cAMP on Plasminogen Activator Production by Primary Cultures of Parietal Endoderm

Treatment	Substrate Solubilized (%)	Final Cell Number
None	13.5	4.4×10^4
10^{-3} M Dibutyryl cAMP	30.6	3.5×10^4
10^{-7} M Retinoic Acid	12.7	4.0×10^4
10^{-3} M Dibutyryl cAMP and 10^{-7} M Retinoic Acid	28.2	5.0×10^4

Parietal endoderm cells were plated at a density of 8×10^4 per Microliter II well in the presence of the indicated compounds. After 48 hr, the conditioned media were collected and assayed for plasminogen activator, and the cells were counted. No fibrinolytic activity could be detected in the absence of plasminogen.

—None of the effects described can be explained on the basis of cell proliferation, that is, selective toxicity resulting in enrichment for differentiated cells. Figure 3 demonstrates that the growth rate is essentially identical in control cultures and in those treated with retinoic acid, DBcAMP, or both.

—The increased proteolytic activity is not due to the release of lysosomal proteases. As shown in Table 2, an assay of representative lysosomal enzymes (for example, N-acetyl-β-glucosaminidase) present intracellularly or released into the medium reveals no difference between control and retinoic acid-treated cultures.

—The enzyme produced in retinoic acid-treated cultures is similar in general properties to other plasminogen activators. The fibrinolytic activity of the conditioned medium is completely dependent upon the presence of plasminogen in the assay and the conditioned medium is quantitatively inhibited by diisopropyl-fluorophosphate (data not shown).

—Treatment of the cells with retinoic acid alone or in combination with DBcAMP results in an increase in intracellular plasminogen activator which parallels the enhanced secretion of this enzyme (Table 3). This result indicates that these compounds do

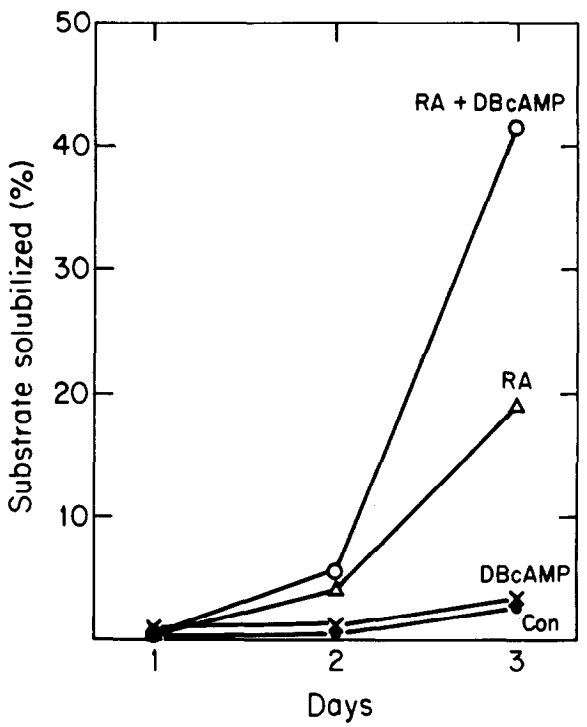

Figure 2. Effects of Retinoic Acid and Dibutyryl cAMP on the Production of Plasminogen Activator by F9 Cultures

F9 cells were plated at a density of 5×10^4 per Linbro well in the presence of the indicated compounds. Six wells were prepared for each treatment. Each day the conditioned media were removed from duplicate wells and assayed for plasminogen activator. (Con) Control cells, no additions; (RA) 10^{-7} M retinoic acid; (DBcAMP) 10^{-3} M dibutyryl cAMP.

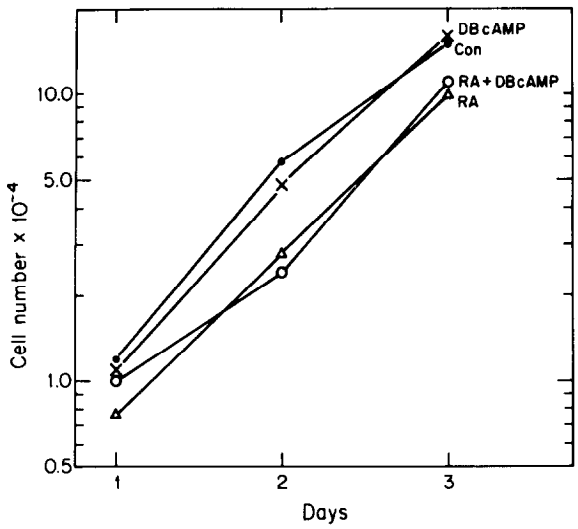

Figure 3. Growth Curve of F9 Cells in the Presence of Retinoic Acid and Dibutyryl cAMP

F9 cells were plated at a density of 10^4 cells per Linbro well in the presence of the indicated compounds. Each day cells were removed from duplicate wells for each condition and counted. Abbreviations and concentrations as in Figure 2.

Table 2. Effect of Retinoic Acid and Dibutyryl cAMP on N-Acetyl-β-Glucosaminidase in F9 Cell Extracts and Conditioned Media

Treatment	Conditioned Medium (U/g of Protein)	Cell Extract (U/g of Protein)
None	1.90	2.35
10^{-3} M Dibutyryl cAMP	1.87	2.36
10^{-7} M Retinoic Acid	2.46	2.37
10^{-3} M Dibutyryl cAMP and 10^{-7} M Retinoic Acid	1.27	2.37

Cells were prepared as described in Figure 2. The assay was performed as described in Experimental Procedures. One unit of activity is defined as the amount of enzyme required to hydrolyze 1 μm of substrate per min at 37°C.

not simply increase secretion of preformed plasminogen activator but induce and stimulate the production of active enzyme.

—Both the effect of retinoic acid on F9 cells and the effect of DBcAMP on retinoic acid-treated F9 cells are dose-dependent. Retinoic acid in concentrations as low as 10^{-9} M results in differences in plasminogen activator levels from control cultures. The molecule is increasingly effective as the concentration is raised to 10^{-6} M; above this amount it is toxic (Figure 4). At all concentrations of retinoic acid, the cultures can be stimulated by DBcAMP to increase secretion of plasminogen activator, which suggests that the two molecules are not acting via the same pathway to stimulate enzyme production. The effect of DBcAMP on retinoic acid-treated cultures is detectable at 10^{-4} M.

—The structural requirements for the effect of retinoic acid are stringent. Figure 5 illustrates that modification of the acid function at carbon-15 by reduction to the aldehyde or alcohol decreases the effectiveness of the molecule approximately 1000 fold. In contrast, the trimethylmethoxyphenyl analog of retinoic acid (TMMP), in which the 5,6-cyclohexenyl ring system has been replaced with a substituted aromatic ring, retains substantial activity. In keeping with the importance of the carboxylic acid function suggested above, conversion of the TMMP-acid to the ethyl ester or substituted amide completely abolishes its activity.

—The effect of DBcAMP on the retinoic acid-treated cultures is due to its cAMP activity. Figure 6 shows that other means of increasing the intracellular level of cAMP, for example, addition of cholera toxin or the cyclic phosphodiesterase inhibitor methyl-isobutylxanthine, have similar effects to DBcAMP. Cholera toxin has previously been shown to increase plasminogen activator synthesis by teratocarcinoma-derived endoderm cells (Linney and Levinson, 1977), and it affects primary cultures of parietal endoderm in a similar manner (data not shown). Since butyrate is a common contaminant of DBcAMP preparations and also a potent inducer of erythroleukemia cell differentiation (Leder and Leder, 1975) it is of interest that this compound has no effect (see below).

—Retinoic acid increases the percentage of colonies secreting plasminogen activator from < 10% to almost 100% (Table 4). Thus this compound is not simply inducing a small number of cells in the population to produce large amounts of enzyme. We estimate, based on the data in Table 4 and the average colony size at time of assay (about 5 cells), that a minimum of about 20% of the cells are differentiating (this estimate assumes only one active cell per colony). A more direct measure of the percentage of differentiated cells can be obtained by replating control or treated cells at low density and determining the number of plasminogen activator-producing cells by the fibrin-agar overlay. Data obtained in this manner suggest that the percentage in control cells is very low (<1%) and that a 24 hr treatment with retinoic acid increases this number to approximately 2.5%, and a 48 hr treatment to approximately 15%. We do not yet know the maximum differentiation that can be obtained with prolonged treatment.

—The effect of DBcAMP does not require the continued presence of retinoic acid. Table 5 shows that when cultures are treated first with retinoic acid, washed well, and then treated with DBcAMP, cells secrete much more plasminogen activator than if they are incubated with retinoic acid followed by control medium. This result would be

Table 3. Effect of Retinoic Acid and Dibutyryl cAMP on Intracellular Plasminogen Activator Levels in F9 Cultures

	Substrate Solubilized (%)		
Treatment	Day 1	Day 2	Day 3
None	0.7	0.6	2.4
10^{-3} M Dibutyryl cAMP	0.8	0.8	3.6
10^{-7} M Retinoic Acid	0.3	8.0	8.8
10^{-3} M Dibutyryl cAMP and 10^{-7} M Retinoic Acid	1.4	11.3	26.3

F9 cells were plated as in Figure 2 in the presence of the indicated compounds. At the designated times, the conditioned media were collected, the cells were washed twice with PBS and extracted with 0.1% Triton X-100 in H_2O. The conditioned media and extracts (10 μg of cellular protein) were assayed for plasminogen activator as described in Experimental Procedures. The results are the average of triplicate cultures.

predicted from the proposition that retinoic acid effects a transition of embryonal carcinoma cells into a cell type with a stable phenotype, that is, response to dibutyryl cAMP. This finding is also consistent with the temporal pattern of stimulation (Figure 2); the action of dibutyryl cAMP is not significant until retinoic acid has induced sufficient differentiated cells to accumulate.

—The migration in a sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) of the plasminogen activator from retinoic acid-treated F9 cultures is very similar to that from parietal endoderm cultures (Figure 7). Estimates of apparent molecular weight from Figure 7 yield a value of 79,000 daltons for the parietal endoderm enzyme and 78,000 daltons for the retinoic acid-induced enzyme. Trophoblast, another cell type of the early mouse embryo that secretes plasminogen activator, produces an enzyme with an apparent molecular weight of 42,000 daltons (S. Strickland, unpublished observation).

Synthesis of Collagen-Like Protein

Parietal endoderm cells synthesize, in vivo (Pierce et al., 1962) and in vitro (Clark et al., 1975b), a thick basement membrane, Reichert's membrane, which is known to contain between 20 and 50% collagen in the rat (Clark et al., 1975a). For this reason, we have examined F9 cultures in the presence and absence of retinoic acid for the synthesis of collagen-like proteins. Figure 8 shows SDS-PAGE autoradiograms derived from cultures which had been metabolically labeled with ^{14}C-proline and from incubation fluids from these cultures. It is clear that treatment with retinoic acid results in autoradiograms in which two bands are substantially increased (arrow; compare lane B with lane A); these two bands are proximate on the gel at an apparent molecular weight of 165,000 daltons. When such radiolabeled cultures are incubated in

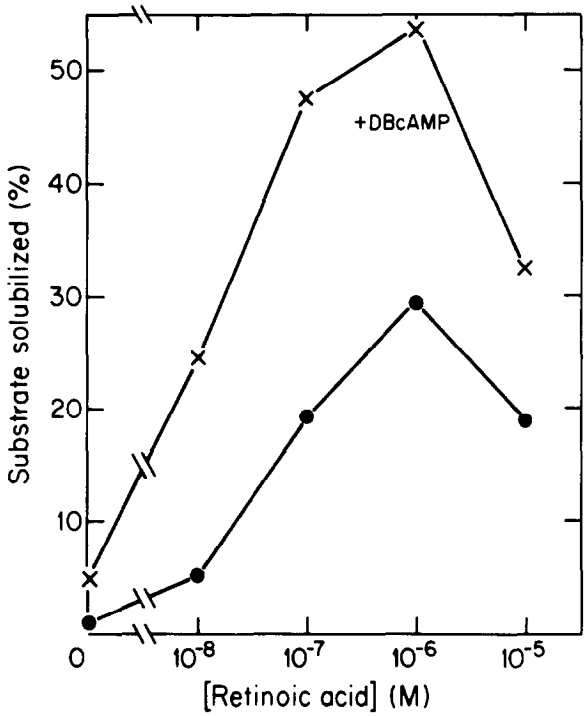

Figure 4. Dose Dependence of the Effect of Retinoic Acid on F9 Cultures

F9 cells were plated as in Figure 2 in the presence of the indicated concentration of retinoic acid, with or without 10^{-3} M dibutyryl cAMP. After 72 hr, the conditioned media were collected and assayed for plasminogen activator.

a small volume of PBS, many of the proteins can be detected in the incubation fluid (lane a), including the two bands induced by retinoic acid (lane b). This suggests that the proteins responsible for the doublet are secreted. Furthermore, these proteins are digested by a protease-free collagenase under conditions in which the rest of the proteins remain essentially unchanged (compare lane d, with collagenase, to lane b, without). In separate experiments (not shown), parietal endoderm cells were also shown to synthesize and secrete proteins which migrate as a doublet at 165,000 daltons; these proteins were also sensitive to collagenase digestion.

Alkaline Phosphatase

It has been suggested that decreases in the specific activity of alkaline phosphatase can serve as an indicator of differentiation of embryonal carcinoma cells. Although solid experimental justification for this concept is lacking (see Discussion), we have examined the alkaline phosphatase content of F9 cultures under varying conditions. Table 6 shows that there is a 20 fold increase in the specific activity of alkaline phosphatase in control cultures over three days of growth. Using the other criteria for differentiation mentioned above, there was extremely limited differentiation occurring in these

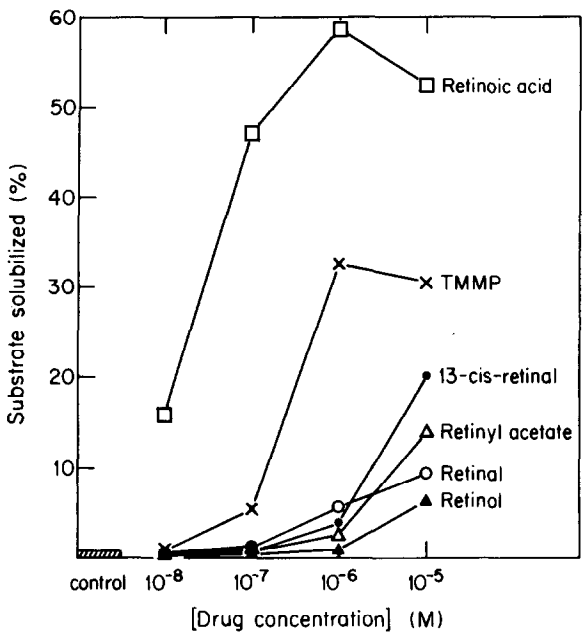

Figure 5. Effect of Derivatives of Vitamin A on F9 Cultures

F9 cells were plated as in Figure 2 in the presence of the indicated compounds. After 72 hr, the conditioned media were removed and assayed for plasminogen activator. The final cell number in each of the wells was not significantly altered by any of the treatments. (TMMP) Trimethylmethoxyphenyl-3,7-dimethyl-2,4,6,8,-nonatetraenoic acid. Derivatives which had no effect were retinyl palmitate, the ethyl ester of TMMP and the N-ethyl amide of TMMP.

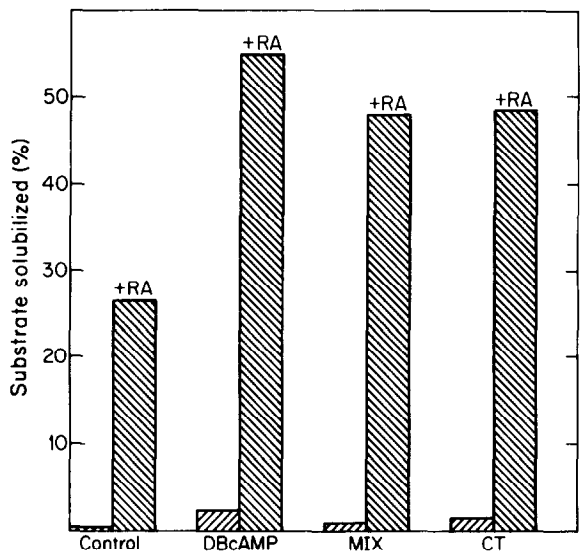

Figure 6. Effect of Compounds That Elevate Intracellular cAMP Concentration on the Retinoic Acid-Treated F9 Cultures

F9 cells were plated as in Figure 2 in the presence of the indicated compound alone (left box) or in combination with 10^{-7} M retinoic acid (right box, +RA). The conditioned media were collected and assayed for plasminogen activator as in Figure 4. (Control) No additions; (DBcAMP) 10^{-3} M dibutyryl cAMP; (MIX) 10^{-4} M methylisobutylxanthine; (CT) 10^{-9} M cholera toxin.

cultures. The presence of retinoic acid results in an approximately 2 fold increase in the treated cells when measured at 1, 2, or 3 days after plating. It should be noted that in contrast to the effect on plasminogen activator levels, a combination of retinoic acid and DBcAMP does not have an apparent synergistic effect on alkaline phosphatase levels.

Other Observations

Cell lines maintained in vitro often undergo phenotypic and genotypic changes with the consequence that eventually the cells bear little resemblance to the starting population. For this reason, the F9 cells used in these experiments were injected subcutaneously into mice, the resultant tumor was excised two weeks later, and a culture was established from this tumor. All the results presented above could be quantitatively reproduced using this fresh isolate of F9 cells.

To test the generality of the response of embryonal carcinoma cells to retinoic acid, the cell line PCC4.aza 1 was tested in a manner identical to that described for F9 cells in Figure 2. Retinoic acid was toxic to the cells at concentrations $\geq 10^{-8}$ M. At 3×10^{-9} M and below, no increase in plasminogen activator was observed during three days of culture. However, cells with an altered morphology were observed. Since the first cell type to differentiate from PCC4.aza 1 embryonal carcinoma cells has a fibroblast morphology (Sherman, 1975) rather than the epithelial appearance of endoderm, it is possible that differentiation is occuring, but the cells produced do not secrete protease.

We have also tested other compounds for their effect on F9 cells. The most notable of these are the following. Other fat-soluble vitamins such as vitamin E, vitamin K_1 and vitamin D_2 had no effect. Steroids such as progesterone, estradiol and dihydrotestosterone were also inactive. Compounds known to increase plasminogen activator in other cell types, such as phorbol myristate acetate and prostaglandins, had no effect. Molecules which induce differentiation in the Friend-virus infected cells, such as butyrate, dimethylsulfoxide and hexamethylene bis-acetamide, were toxic to F9 cells at the concentration recommended for the erythroleukemic cells and were not effective at lower concentrations.

Finally, although the molecular weight of the plasminogen activator produced (Figure 7) and the cellular morphology (Figure 1) made it improbable that retinoic acid was inducing the formation of trophoblast, we have also analyzed the cultures for the presence of $\Delta^5,3\beta$-hydroxysteroid dehydrogenase. This enzyme, which is involved in progester-

Table 4. Effect of Retinoic Acid and Dibutyryl cAMP on the Number of F9-Derived Colonies Producing Plasminogen Activator

Treatment	Number of Colonies	Number of Lytic Zones
None	168	8
	152	18
10^{-7} M Retinoic Acid	163	159
	129	124
10^{-7} M Retinoic Acid and 10^{-3} M Dibutyryl cAMP	156	156
	160	160

F9 cells were plated at a density of 200 cells per 60 mm petri dish in the presence of the indicated compounds. After 48 hr, the cells were overlaid with 1.5 ml of fibrin-agar mixture as described in Experimental Procedures. The number of colonies and lytic cell zones were scored by visual inspection and phase microscopy.

Table 5. Plasminogen Activator Production of F9 Cultures Treated Sequentially with Retinoic Acid and Dibutyryl cAMP

First Treatment	Second Treatment	Substrate Solubilized (%)
Control	Control	0.6
Control	DBcAMP	1.1
Control	Retinoic acid	12.9
Control	DBcAMP and retinoic acid	27.1
Retinoic acid	Control	2.6
Retinoic acid	DBcAMP	32.9
Retinoic acid	Retinoic acid	20.2
Retinoic acid	DBcAMP and retinoic acid	81.5

F9 cells were plated at a density of 10^4 per Linbro well in the presence of the compounds indicated under First Treatment. After 48 hr, the cultures were washed twice with PBS and incubated for 4 hr in growth medium with no additions; this procedure facilitates removal of excess retinoic acid. The cultures were again washed twice with PBS, and growth medium containing the compounds listed under Second Treatment was added. After a further 72 hr, the conditioned media were collected and assayed as described in Experimental Procedures. Dibutyryl cAMP and retinoic acid were used at 10^{-3} M and 10^{-7} M, respectively. The results are the average of duplicate determinations. It should be noted that the cell density in this experiment is five fold lower than in most other experiments and accounts for the small response to retinoic acid after 48 hr (retinoic acid-control).

one biosynthesis and is characteristic of trophoblast cells (Marcal et al., 1975), was not detected in F9 cultures subjected to any of the treatments described in this report.

Discussion

The data presented here are consistent with the interpretation that F9 embryonal carcinoma cells can be induced by retinoic acid to differentiate into endoderm. Clearly, further examinations (for example, ultrastructural analysis) should be performed to test this tentative conclusion. With this reservation in mind, the results should be considered from several points of view: first, an analysis of the biochemical evidence that differentiation into endoderm is occurring; second, the implications for the control of development; and third, the relationship of previous knowledge concerning retinoic acid.

Several lines of evidence imply that the cell type which accumulates in retinoic acid-treated F9 cultures is endoderm. The similarities between this cell type and "native" endoderm are: the production of plasminogen activator, the apparent molecular weight of the protease, the sensitivity of enzyme production to elevated levels of cAMP, and the synthesis and secretion of a collagen-like protein. The observation that treatment of F9 cells with retinoic acid increases the specific activity of alkaline phosphatase runs counter to the concept that differentiation of embryonal carcinoma cells invariably results in a decrease in alkaline phosphatase. However, the experimental foundation of this concept bears close examination.

Embryonal carcinoma cells do have high levels of alkaline phosphatase (Damjanov, Solter and Skreb, 1971; Bernstine et al., 1973). The endoderm cells that surround the embryonal carcinoma cells in embryoid bodies were initially reported to be devoid of histochemically detectable alkaline phosphatase (Bernstine et al., 1973; Martin and Evans, 1975); recent work, however, has detected this enzyme in the endoderm cells using unfixed preparations and has suggested that the lack of enzyme activity previously noted may have been due to the fixation procedure (Wada et al., 1976). Furthermore, analysis of the data of Bernstine et al. (1973) on several teratocarcinoma cell lines indicates no correlation between the capacity to differentiate and the alkaline phosphatase level.

Biochemical assay of parietal endoderm cell extracts has shown these cells to have levels of alkaline phosphatase lower than embryonal carcinoma cells (data not shown). These cells, however, are derived from mid-gestation tissue and therefore represent differentiated endoderm; there could be significant differences between parietal endoderm and the primitive endoderm as it first differentiates from precursor cells.

Thus the concept that an immediate decrease in alkaline phosphatase content must accompany differentiation of embryonal carcinoma into endoderm appears open to question, and thus the effect of retinoic acid on the levels of this enzyme in F9 cultures does not necessarily contradict the conclusions reached using other criteria. It should be mentioned that the results presented here support earlier conclusions (Sherman et al., 1976) that a low level of differentiation occurs spontaneously

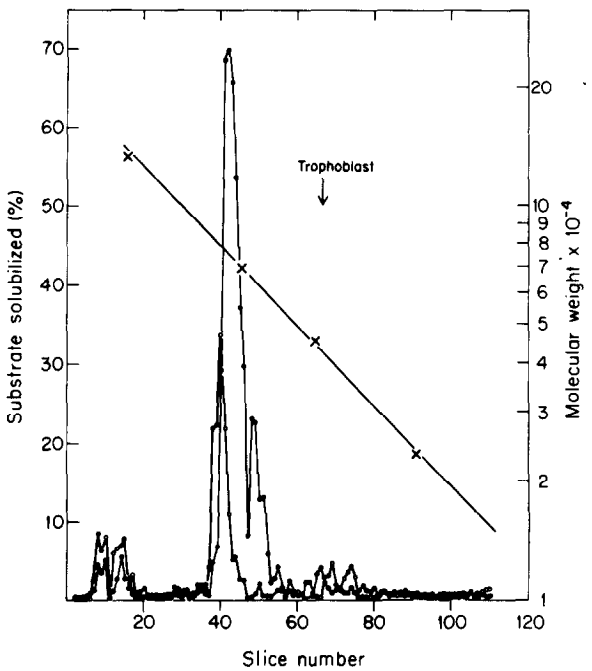

Figure 7. Gel Electrophoresis Analysis of Plasminogen Activator from Retinoic Acid-Treated F9 Cultures and Parietal Endoderm Cells

F9 cells were plated at a density of 2 × 10^5 per 60 mm petri dish and treated for 72 hr with 10^{-7} M retinoic acid. Parietal endoderm cells obtained from 10 conceptuses were plated at a density of 5 × 10^5 per 35 mm petri dish and allowed to attach for 16 hr. Both cultures were then incubated in 1 ml of Dulbecco's modified Eagle's medium containing 0.5% plasminogen-depleted fetal bovine serum. The conditioned media were analyzed as described in Experimental Procedures on a 9% SDS-polyacrylamide slab gel. Molecular weight markers: β-galactosidase, 132,000; human serum albumin, 68,000; ovalbumin, 45,000; chymotrypsinogen, 23,600 daltons. (○) Parietal endoderm; (●) treated F9 cells.

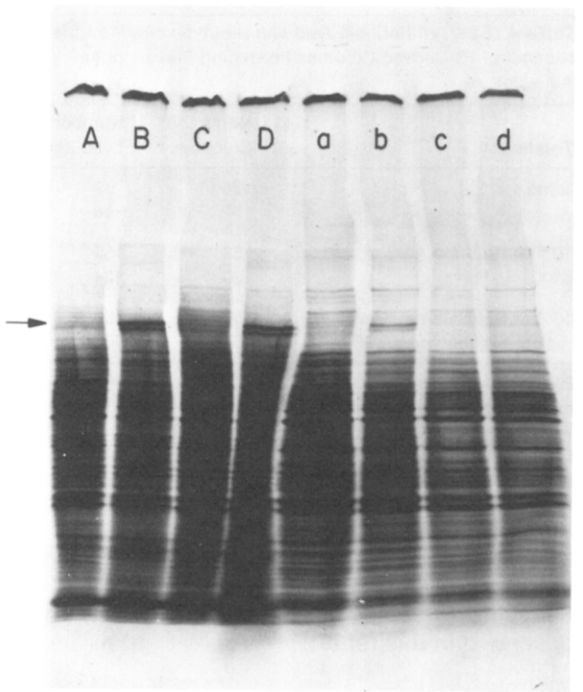

Figure 8. Autoradiogram of ^{14}C-Proline-Labeled F9 Cell Extracts and Incubation Media Analyzed by SDS-Polyacrylamide Gel Electrophoresis

F9 cells were plated, exposed to retinoic acid and dibutyryl cAMP, labeled with ^{14}C-proline and digested with collagenase as described in Experimental Procedures. (A) F9 cell extract after incubation in control digestion medium (no enzyme) for 3 hr at 37°C; (a) control digestion medium. (B) F9 cell extract after treatment for 72 hr with 10^{-7} M retinoic acid and 10^{-3} M dibutyryl cAMP and incubation in control digestion medium for 3 hr at 37°C; (b) control digestion medium. (C) As in (A) except treated with collagenase for 3 hr; (c) collagenase digestion medium. (D) As in (B) except treated with collagenase for 3 hr; (d) collagenase digestion medium.

in the F9 cultures. For example, the levels of plasminogen activator increase slightly as the cell density increases in untreated cultures (Figure 4); there are cells with endoderm morphology; and the untreated cultures exhibit a low but variable response to DBcAMP (Figure 4).

A prevailing concept in mammalian embryogenesis is that the position of stem cells determines, to a large extent, the ultimate differentiative pathway into which the cells will proceed (Tarkowski and Wroblewska, 1967). This idea is supported by studies on teratocarcinoma cell differentiation since the formation of three-dimensional aggregates appears to be one way to induce development of embryonal carcinoma cells in vitro (Martin and Evans, 1975). The effect of retinoic acid on F9 cells, while in no way diminishing the positional hypothesis, suggests that given the correct chemical stimuli, topography is not a necessary prerequisite. With this in mind, it should be of interest to determine whether retinoic acid also influences blastocyst or inner cell mass development in vitro, and whether other physiologically relevant compounds can exert an effect upon embryonal carcinoma cell differentiation.

Several in vitro systems have been developed in which a biological activity of retinoid compounds can be detected. These are the prevention of squamous metaplasia in organ cultures of mouse prostate (Lasnitzki, 1962) and hamster trachea (Clamon et al., 1974), and an increased content of cellular RNA in mouse epidermal cell culture (Sporn, Dunlop and Yuspa, 1973). The response of chicken embryo fibroblasts (Wilson and Reich, 1978) and F9 cells to retinoic acid provides two additional cell types in which vitamin A derivatives can be tested. It should be noted that although the end result of retinoic acid stimulation of F9 cells and chicken embryo fibroblasts is the same (that is, increased plasminogen activator production), the nature of the response is very different. In the fibroblasts the cells respond in a few hours by synthesizing elevated levels of protease, and the presence of reti-

Table 6. Effect of Retinoic Acid and Dibutyryl cAMP on Alkaline Phosphatase in F9 Cultures

Treatment	Activity (U/mg × 10^2)		
	Day 1	Day 2	Day 3
None	1.0	7.1	21.9
10^{-3} M Dibutyryl cAMP	1.1	7.6	22.1
10^{-7} M Retinoic Acid	1.8	11.9	35.4
10^{-3} M Dibutyryl cAMP and 10^{-7} M Retinoic Acid	2.1	8.1	18.6

F9 cells were plated as in Figure 2 in the presence of the indicated compounds. At the designated times, the cultures were washed twice with PBS and extracted with H_2O. The extracts (10 μg of cellular protein) were assayed for alkaline phosphatase by the method of Lowry et al. (1954). One unit of enzyme activity is defined as that required to hydrolyze 1 μm of p-nitrophenyl phosphate (molar extinction coefficient at 410 nm, 17,500 $M^{-1}cm^{-1}$) per ml per min at 37°C.

noic acid is required for increased protease production, whereas the F9 cells respond over days to yield progeny cells which produce this enzyme constitutively.

The influence of retinoic acid on the differentiation of embryonal carcinoma cells is noteworthy due to the fact that vitamin A has been implicated in differentiation for over fifty years (Wolbach and Howe, 1925; Yuspa and Harris, 1974). Interest in retinoids has also been heightened by the suggestion that these compounds might prove useful in preventing certain epithelial cancers (Sporn et al., 1976). Previously, investigations into the regulation of plasminogen activator secretion by other cell types have shed light on the molecular basis and hormonal control of ovulation (Beers, Strickland and Reich, 1975; Strickland and Beers, 1976), implantation (Strickland et al., 1976) and inflammation (Vassalli, Hamilton and Reich, 1976). Thus it seems reasonable to suggest that studies of the chemically induced transition of embryonal carcinoma into other cell types might provide insights into the mechanisms involved in differentiation, and into the possible role of vitamin A derivatives in these mechanisms.

Experimental Procedures

Materials

Materials were purchased from the following sources: NCS strain mice from the Laboratory Animal Research Center (The Rockefeller University); fetal bovine serum from Reheis; Dulbecco's modified Eagle's medium from Grand Island Biological; trypsin from Nutritional Biochemists; gelatin, lab grade, from Fisher; plastic petri dishes from Falcon; multiwell culture plates (24 wells per plate, 16 mm diameter) from Linbro; collagenase (CLS III) from Worthington; collagenase, Type III (chromatographically purified) from Sigma; L-proline ^{14}C, 250 mCi/mM, from Schwartz/Mann, retinoic acid from Eastman; all other compounds from Sigma. Derivatives of retinoic acid not commercially available were supplied by B. A. Pawson (Hoffman-La Roche, Inc.). Plasminogen-depleted fetal bovine serum (Ossowski et al., 1973), purified human plasminogen (Deutsch and Mertz, 1970) and protease-free collagenase (Peterkofsky and Diegelman, 1971) were prepared as previously described. The purified collagenase had no proteolytic activity against casein or ^{125}I fibrin; the specific activity using bovine tendon collagen as a substrate was 25.5 μm of amino acid produced per mg of enzyme per hr at 37°C.

Methods

Cell Culture

The F9 teratocarcinoma cell line, obtained from K. Artz (Sloan-Kettering Institute), was cultured on surfaces that had been coated with gelatin (0.1% gelatin in H_2O for 2 hr at 4°C, then washed with H_2O and stored at room temperature). The cells were plated for maintenance at a density of 2 × 10^5 per 100 mm petri dish, and subcultured twice a week using phosphate buffered saline (PBS) containing 0.25% trypsin and 5 × 10^{-4} M EDTA. Dulbecco's modified Eagle's medium (4.5 g glucose per l) containing 15% fetal bovine serum was used for all maintenance cultures, whereas the serum supplement was 15% plasminogen-depleted fetal bovine serum for all experimental cultures. Retinoid solutions at 10^{-3} M were made fresh in absolute ethanol and diluted further in medium with serum.

Parietal endoderm primary cultures were prepared by dissecting Reichert's membrane with its associated cells from 11 day conceptuses (the morning on which the sperm plug was found was considered the first day of pregnancy), and digesting this tissue for 20 min at 37°C in PBS containing 0.5 mg/ml collagenase and 0.25% trypsin. The cells were cultured as described for F9 cells.

Assays for Plasminogen Activator

—Assays for plasminogen activator in conditioned medium were performed by measuring the plasminogen-dependent fibrinolysis in multiwell dishes coated with ^{125}I-fibrin (Unkeless et al., 1973; Strickland and Beers, 1976). The conditioned medium was acid-treated (Strickland et al., 1976) to reduce protease inhibitor activity, and 25 μl was added to 0.25 ml of 0.1 M Tris-HCl (pH 8.1) containing 8 μg/ml of purified human plasminogen. Assays were conducted for 2 hr at 37°C. No fibrinolysis was observed in the absence of plasminogen. The results are expressed as percentage solubilized of the total radioactive substrate.

—The fibrin-agar overlay assay was performed by a modification of the method previously described (Jones et al., 1975). Cells grown in 60 mm dishes were washed twice with PBS and overlaid with 1.5 ml of a fibrin-agar mixture (0.5 ml Dulbecco's modified Eagle's medium with 4.5 μg/ml human plasminogen and 2.5 μg/ml thrombin; 0.5 ml 2.5% purified agar in H_2O; and 0.5 ml of 10 mg/ml purified fibrinogen in 0.3 M NaCl). The assay was allowed to develop for 18 hr at 37°C and terminated by the addition of 7% acetic acid in 50% methanol containing 2.5% Coomassie brilliant blue.

—Intracellular plasminogen activator was determined by washing the cultures twice with PBS, extracting the cells in a minimum volume of 0.1% Triton X-100 in H_2O with scraping, and then homogenizing. The mixture was centrifuged at 1000 × g for 10 min and the supernatant was assayed as described for conditioned media.

—For estimation of the molecular weight of plasminogen activator, cells were incubated for 16 hr in medium containing 0.5% plasminogen-depleted fetal bovine serum. The conditioned medium was adjusted to 0.1 M Tris-HCl (pH 6.8) 15% glycerol, 2% SDS, and electrophoresed on 9% SDS-polyacrylamide slab gels (Laemmli, 1970). The lanes were cut transversely into 1.1 mm slices and each slice was assayed for plasminogen activator. Proteins of known molecular weight were run in parallel, stained and used to calculate molecular weights.

Analysis of Collagen-like Protein

F9 cells were plated at a density of 5 × 10^4 per 35 mm petri dish and incubated with or without 10^{-7} M retinoic acid and 10^{-3} M DBcAMP. 24 hr after plating, 10 μCi/ml of ^{14}C-L-proline were added and the cells were cultured for a further 48 hr. The cells

were then washed three times with PBS and treated with a protease-free collagenase for 3 hr at 37°C. The digestion medium contained: 200 μl PBS; 30 μl of 20.8 mM N-ethyl maleimide (as a protease inhibitor) and 4.2 mM $CaCl_2$; 20 μl of 0.4 mg/ml purified collagenase, 1.5 M HEPES buffer (pH 7.2) and 0.5 M Tris-HCl. Control incubations were performed which contained all these components except collagenase.

The digestion media and cells were adjusted to 0.1 M Tris-HCl (pH 6.8), 15% glycerol, 2% SDS and 0.1 M dithiothreitol and electrophoresed on an SDS or slab gel containing a linear gradient from 5-13% polyacrylamide. After fixing, staining with Coomassie brilliant blue and destaining, the gel was dried and exposed to Kodak X-Omat R film.

Other Methods

Diisopropyl-fluorophosphate (DFP) inhibition of plasminogen activator was performed as follows. 1 ml of conditioned medium from F9 cells treated with 10^{-7} M retinoic acid for 72 hr was made 30 mM in DFP by three consecutive additions at 1 hr intervals. The sample was extensively dialyzed against 0.1 M Tris-HCl (pH 8.1) and assayed for fibrinolytic activity. The treated sample was shown to be free of unreacted DFP by its failure to inhibit fibrinolysis by untreated samples.

Lysosomal enzyme activity was measured on cells extracted with 0.1% Triton X-100-PBS and on conditioned medium by the colorimetric assay described by Bowers, Finkenstaedt and De Duve (1967). The reaction, in a final volume of 0.5 ml, was performed in 0.05 M acetate buffer (pH 5.0), 0.1% Triton X-100 with 20 μg of cell protein for 16 hr at 37°C and stopped by the addition of 3 ml of 0.13 M glycine buffer (pH 10.7).

Alkaline phosphatase activity was measured on cell extracts (Lowry et al., 1954) prepared as described for intracellular plasminogen activator assays, except that H_2O was used for the extraction. The reaction contained, in a final volume of 0.5 ml, 10 mM p-nitrophenyl phosphate, 2 mM $MgCl_2$, 0.5 M 2-amino-2-methylpropanol-HCl buffer (pH 10), and 10 μg of cell protein. The reaction was incubated for 30 min at 37°C and terminated by the addition of 0.5 ml of 0.75 N NaOH.

Assays for Δ^5, 3β-hydroxysteroid dehydrogenase were performed as descirbed by Marcal et al. (1975) using radioimmunoassay to detect progesterone production. Ovaries from pregnant rats were used as a source of enzyme for positive controls.

For in vivo passage of F9 cells, 3×10^6 cells were resuspended in 0.3 ml of PBS and injected subcutaneously into a male 129 SV/SL mouse (Jackson Laboratory). Two weeks later the mouse was sacrificed and the tumor was removed, cut into 1 mm^3 pieces, and digested in Dulbecco's modified Eagle's medium containing 1 mg/ml collagenase for 20 min at 37°C.

Protein determinations were performed as described by Udenfriend et al. (1972) using bovine serum albumin as a standard.

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