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# Growth of embryonal carcinoma cells in serum-free medium

(teratocarcinomas/mammalian development/Pedersen fetuin/PCC.4 aza-1/$F_9$)

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ABSTRACT **Two mouse embryonal carcinoma cell lines, PCC.4 aza-1 and $F_9$, have been grown in serum-free F-12 medium supplemented with Pedersen fetuin, insulin, transferrin, and 2-mercaptoethanol. This medium supports long-term growth of both cell lines. When these cells are transferred from medium containing serum to this serum-free medium, growth continues without any detectable lag. PCC.4 aza-1 grown in this medium for over 20 generations retains the capacity to differentiate *in vivo*. This medium appears to be a general serum-free medium for the growth of embryonal carcinoma cells.**

Little is known about the hormonal requirements of early mammalian development. One approach to this problem has been the cultivation of mouse embryos in a variety of defined media. Unfortunately, this approach has had limited success. Development *in vitro* beyond the blastocyst stage requires serum (1–3), and it has been impractical to test the large number of serum factors directly with embryos. Therefore, a different strategy is necessary. Numerous studies have suggested that mouse embryonal carcinoma cell lines can be used to study early mammalian development (4). These cell lines have been established from malignant tumors known as teratocarcinomas (4). These tumors are composed of pluripotent embryonal carcinoma cells (stem cells) plus a variety of differentiated cell types normally derived from each of the three embryonic germ layers. Although many embryonal carcinoma cell lines exhibit only limited differentiation *in vitro*, some of these cell lines differentiate into a wide range of cell types, both *in vitro* and *in vivo* (4). In addition, a number of clonal embryonal carcinoma cell lines established by Martin and Evans (5, 6) form cellular aggregates, known as embryoid bodies, *in vitro*. These structures morphologically resemble the inner cell mass of the mouse embryo. By day five, the inner cell mass, which gives rise to the embryo proper (plus extraembryonic membranes) is composed of two cell types (7), an outer layer of endoderm and an inner core of undifferentiated cells. Simple embryoid bodies are also composed of two cell types, an outer layer of endoderm that surrounds a core of embryonal carcinoma cells (4). Furthermore, embryonal carcinoma cells are able to participate in normal development when injected into early mouse embryos (8–10). These findings, and others not reviewed here (4), suggest that embryonal carcinoma cells may share other properties with mouse embryos, including hormones required for differentiation. Therefore, characterization of the hormonal requirements of embryonal carcinoma cells should make it feasible to define a serum-free medium for mouse embryos.

Recent work in this laboratory has demonstrated that hormonal requirements of established cell lines can be identified by the gradual replacement of serum with purified hormones, growth factors, and accessory factors such as transferrin. For $GH_3$, HeLa, and B-16 cells, serum was replaced primarily with commercially available hormones such as insulin, progesterone, follicle stimulating hormone, triiodothyronine, and parathyroid hormone (11, 12). These results argue that one of the major roles of serum in cell culture is to provide hormones. Furthermore, these studies suggest that it is feasible to develop serum-free media for other cell lines, including embryonal carcinoma cells. In this article we report that two different embryonal carcinoma cell lines (PCC.4 aza-1 and $F_9$) grow for extended periods of time in a serum-free medium supplemented with insulin, transferrin, 2-mercaptoethanol, and Pedersen fetuin. PCC.4 aza-1 cells maintained in this serum-free medium retain the ability to differentiate *in vivo*.

## METHODS AND MATERIALS

**Cell Culture.** PCC.4 aza-1 and $F_9$ were obtained from M. Sherman at the Roche Institute of Molecular Biology. These cells were maintained on Falcon tissue culture flasks and kept under a mixture of 5% $CO_2$ in air. The medium was composed of 45% Dulbecco's modified Eagle's medium (Gibco HG-21), 45% nutrient mixture F-12 (Gibco H-17), and 5% each horse and fetal calf serum. When cells were switched from serum-containing to serum-free medium, the cells were briefly washed with serum-free F-12 medium. This was done by first removing the serum-containing medium from stock plates and replacing it with serum-free F-12 medium. This medium was then removed, and fresh F-12 medium was added. Cells were removed from the plates by pipetting the medium up and down over the cells with a sterile pasteur pipette. When cells were grown for prolonged periods in EM-1 medium (F-12 medium containing 500 μg of fetuin per ml, 10 μg of insulin per ml, 5 μg of transferrin per ml, and 10 μM 2-mercaptoethanol), the medium was changed nearly every day. Cell growth was determined by counting cells with a Coulter counter.

**Preparation of Serum-Free Growth Media.** F-12 medium was prepared from powdered nutrient mixture F-12 (Gibco H-17) at least every 2 weeks. The best results were obtained with fresh powder that was stored at 0–5° and was not older than 6 months. Water quality is also important; the best results were obtained with triple-distilled water. Water distilled only once did not give consistent results. Insulin (Sigma: bovine) was prepared as a 100-fold concentrated stock solution by first dissolving insulin in 0.1 M HCl at 10 mg/ml. After insulin had dissolved, it was diluted with phosphate-buffered saline to 1 mg/ml. Transferrin (Sigma: human) was made up as a 100-fold concentrated solution by adding 500 μg of transferrin per 1 ml of water. Pedersen fetuin (Sigma: type II lot 65C-0068) was made up to 50 mg/ml in water with a pH of 2.5. This pH was maintained by adding 1 M HCl as the fetuin dissolved. This 100-fold concentrated solution was stirred slowly for 10–20 min in order for the fetuin to dissolve. Spiro (Gibco, lot 0459315) and Deutsch (Gibco, lot 0656504) fetuin were made up in the same manner except that Spiro fetuin was treated with Chelex to

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Table 1. Response of PCC.4 aza-1 cells to insulin and transferrin

| Additions* | % of control† |
|---|---|
| 10% FCS | 100 |
| 1% FCS | 16 |
| 0.2% FCS | 6 |
| 0.2% FCS + I | 10 |
| 0.2% FCS + TF | 8 |
| 0.2% FCS + I + TF | 12 |
| I + TF | 1 |
| No additions | 1 |

* Additions to nutrient mixture F-12 (Gibco H-17) were fetal calf serum (FCS), 10 μg of insulin (I) per ml, or 5 μg of transferrin (TF) per ml.

† 100,000 cells were plated per 35-mm culture dish. At the end of 48 hr the medium was removed and 1 ml of phosphate-buffered saline was added. The cells were removed from the culture plate with a pasteur pipette and counted by a Coulter counter. Cells grown in 10% fetal calf serum were chosen as the control; the actual cell number was 651,452.

remove toxic zinc and barium ions. 2-Mercaptoethanol (Sigma: type I) was diluted to the appropriate concentration with distilled water. Stock solutions were filter sterilized with Millex filters (Millipore).

**Production of Tumors.** PCC.4 aza-1 were injected subcutaneously into *nude* mice to determine if they were capable of forming teratocarcinomas. Cells grown in serum or in EM-1 for over 20 generations were used for each injection. After 21 days the tumors were removed and placed in Bouin's solution for 24 hr and then placed in 70% ethanol. After sectioning, the tissue was stained with hematoxylin and eosin.

## RESULTS AND DISCUSSION

### Serum-free growth medium

The cell line PCC.4 aza-1, established by Jakob *et al.* (13), was used to develop a serum-free medium for growth of embryonal carcinoma cells. These cells are normally grown in Dulbecco's modified Eagle's medium containing fetal calf serum. However, rather than using this medium, these studies were initiated with a richer medium, nutrient mixture F-12 (Gibco, H-17). Initially, it was found that approximately 1% fetal calf serum maintained PCC.4 aza-1 cells in culture, but no growth occurred (Table 1). As expected from other studies in this laboratory (11, 12), these cells responded to insulin and transferrin when 0.2% fetal calf serum was present in the medium (Table 1). However, in the absence of serum, insulin and transferrin were unable to maintain these cells. After 48 hr, very few cells had attached to the culture dish, and those cells floating in the culture medium did not exclude trypan blue.

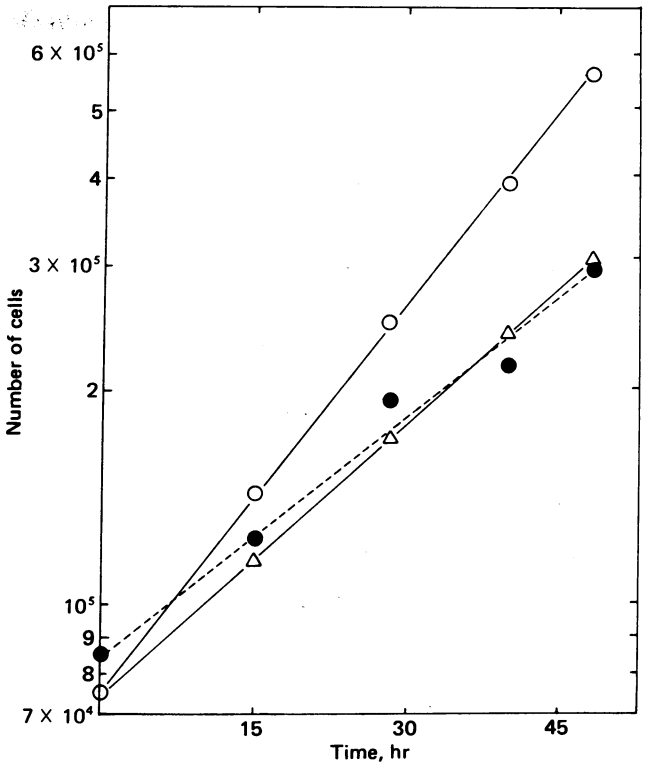


FIG. 1. Growth rates of PCC.4 aza-1 cells in serum-containing and serum-free media. The growth rates of PCC.4 aza-1 cells in medium containing 10% fetal calf serum or a serum-free medium (insulin, transferrin, Pedersen fetuin, and 2-mercaptoethanol) were compared. Cells grown in medium containing 10% fetal calf serum were washed with F-12 medium (lacking serum) and transferred to plates with F-12 medium plus serum (○) or to the serum-free F-12 medium described above (△). The growth rate of PCC.4 aza-1 cells that had been grown in this serum-free medium for 11 weeks has also been included. These cells were transferred to plates containing the above-described serum-free medium (●).

Table 2. Response of PCC.4 aza-1 cells to serum factors and 2-mercaptoethanol

| Additions* | % of control† |
|---|---|
| 10% FCS | 100 |
| 5% FCS | 76 |
| P. fetuin | 4 |
| P. fetuin + I | 6 |
| P. fetuin + I + TF | 13 |
| P. fetuin + I + TF + ME | 72 |
| P. fetuin + I + TF + DTT | 53 |
| P. fetuin + I + TF + Cys | 65 |
| D. fetuin + I + TF | 1 |
| S. fetuin + I + TF | 1 |
| No additions | 1 |

* Additions to nutrient mixture F-12 (Gibco H-17) were 500 μg of Pedersen fetuin (P. fetuin) per ml, 10 μg of insulin (I) per ml, 5 μg of transferrin (TF) per ml, 10 μM 2-mercaptoethanol (ME), 10 μM dithiothreitol (DTT), 1 mM cysteine (Cys), and 5% or 10% fetal calf serum (FCS). Spiro fetuin (S. fetuin) and Deutsch fetuin (D. fetuin) were also added at 500 μg/ml.

† 85,000 PCC.4 aza-1 cells were plated in 35-mm culture dishes. After 48 hr, the experiment was terminated, medium was removed, and 1 ml of phosphate-buffered saline was added to remove the cells. Cells were counted by a Coulter counter. These numbers are an average of three separate plates. Cells grown in 10% fetal calf serum were chosen as the control; the actual cell number was 526,836.

These results suggested that serum factor(s) are required for PCC.4 aza-1 to attach to the culture dish. Therefore, we tested the effectiveness of the serum glycoprotein, fetuin, which has been reported to enhance the attachment of HeLa cells (14). When fetuin (Pedersen fetuin: Sigma type II) was added to medium lacking serum, a significant fraction of the cells plated attached to the culture dish, and the combination of insulin, transferrin, and Pedersen fetuin resulted in the attachment of most cells (Table 2). However, little or no growth occurred. These results suggest that Pedersen fetuin contains one or more factors necessary for the attachment of these cells. However, the actual function of Pedersen fetuin remains a mystery. Several studies (15) have suggested that the attachment activity of Pedersen fetuin may be a serum factor copurifying with fetuin. Unfortunately, this has been proven to be a difficult

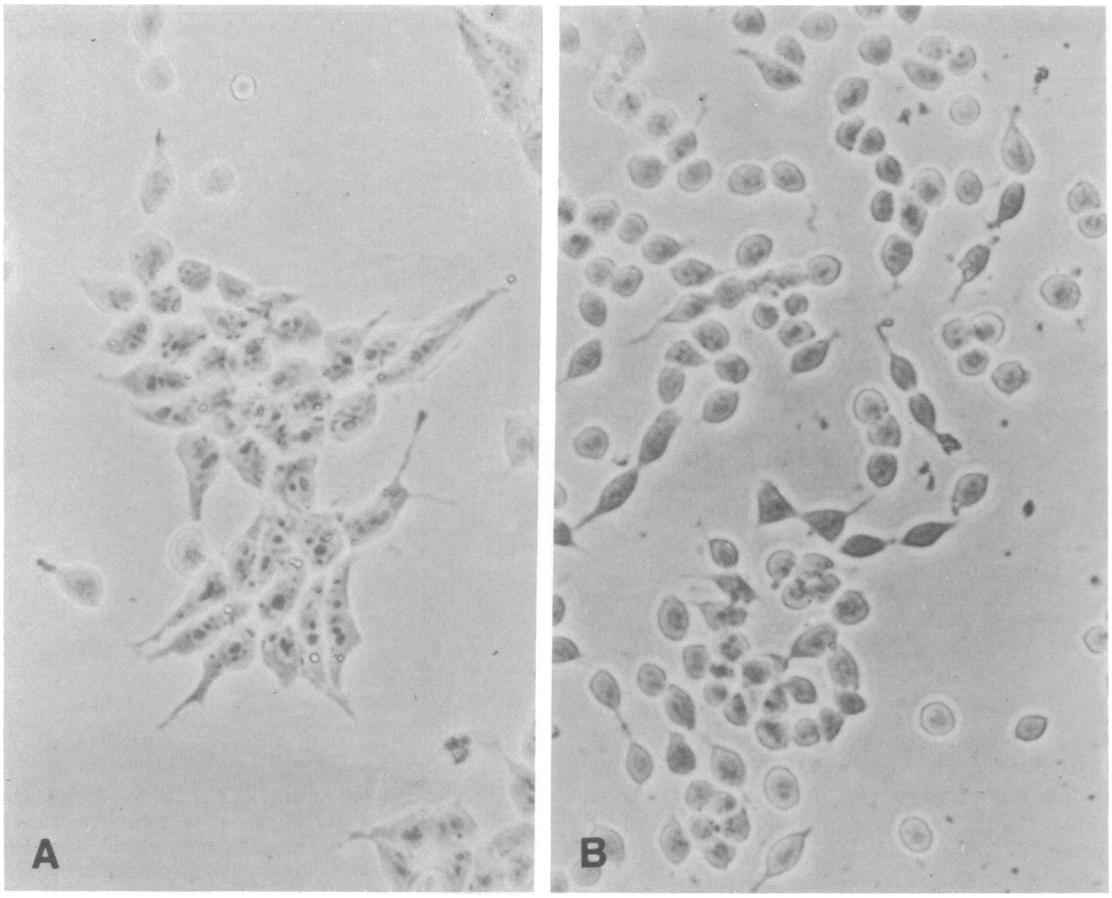


FIG. 2. Morphology of PCC.4 aza-1 cells in serum-containing and serum-free media. (*A*) PCC.4 aza-1 cells in F-12 medium containing 10% fetal calf serum. (×70.) (*B*) PCC.4 aza-1 cells in serum-free F-12 medium containing Pedersen fetuin (500 μg/ml), insulin (10 μg/ml), transferrin (5 μg/ml), and 2-mercaptoethanol (10 μM). (×70.)

problem and has yet to be resolved. Furthermore, Spiro (16) and Deutsch (17) have prepared fetuin by two other methods, and these preparations obtained from Gibco are without effect. Since these preparations are relatively pure (Spiro fetuin is reported by Gibco to be 99% pure), we believe that PCC.4 aza-1 cells are responding to a serum factor that copurifies with Pedersen fetuin. However, the possibility does exist that fetuin is responsible for attachment and that its activity is destroyed during its isolation by the Spiro and Deutsch procedures. Experiments are now in progress to resolve this matter. Thus far we have found that the activity of Pedersen fetuin is destroyed by heating at 100°, is stable at pH 2.5 for over 24 hr, and is nondialyzable.

The last addition to the culture medium necessary for growth of PCC.4 aza-1 is 2-mercaptoethanol. In a period of 48 hr, PCC.4 aza-1 cells increase 3-fold in F-12 medium supplemented with Pedersen fetuin, insulin, transferrin, and 2-mercaptoethanol (Table 2). 2-Mercaptoethanol can be replaced with dithiothreitol and cysteine (Table 2). The optimal concentration of 2-mercaptoethanol was 10 μM. At higher concentrations, 2-mercaptoethanol is inhibitory, and at 1 mM all cells are killed. Although the addition of 2-mercaptoethanol may seem unusual, other cells (18), including B-16 cells grown in serum-free medium, also respond to 2-mercaptoethanol (J. Mather, personal communication); R. Oshima (personal communication) has found that the embryonal carcinoma cell lines established by Martin and Evans (5, 6) normally carried on feeder layers can be grown at low density in the absence of feeder layers provided that 2-mercaptoethanol is added to the medium. Furthermore, the response of embryonal carcinoma cells to 2-mercaptoethanol is interesting in light of the observation that high concentrations of cysteine enhance the *in vitro* cultivation of mouse embryos (M. Sherman, personal communication).

## Long-term growth in serum-free medium

F-12 medium plus Pedersen fetuin, insulin, transferrin, and 2-mercaptoethanol (EM-1 medium) was used to culture PCC.4 aza-1 cells for 17 weeks (over 60 generations). However, the growth rate in this medium is slower than in medium containing 10% fetal calf serum (Fig. 1) and about equal to the growth rate of cells cultured in medium containing 5% fetal calf serum (Table 2; unpublished results). The growth rate of PCC.4 aza-1 cells changed very little during growth in EM-1 medium for 11 weeks (Fig. 1). More importantly, no lag of growth was observed when there was a change from medium containing serum to EM-1 medium (Fig. 1).

Besides the slower growth rate, the morphology of PCC.4 aza-1 cells in EM-1 medium differs from that of cells growing in medium containing serum (Fig. 2). In EM-1, the cells are somewhat smaller and do not grow in close association with one another. However, these cells have not lost their capacity to differentiate *in vivo*. Cells grown in this medium for 32 days (over 20 generations) formed tumors when injected into *nude* mice. Four mice were each injected with $10^6$ PCC.4 aza-1 cells that had been grown in EM-1 medium, and two other mice were injected with $10^6$ PCC.4 aza-1 cells grown in medium containing serum. In each case a large tumor formed within 3

Table 3. Response of $F_9$ cells to serum factors and 2-mercaptoethanol

| Additions* | % of control† |
|---|---|
| 10% FCS | 100 |
| P. fetuin | 5 |
| P. fetuin + I | 8 |
| P. fetuin + TF | 10 |
| P. fetuin + I + TF | 15 |
| P. fetuin + I + TF + ME | 89 |
| I + TF | 1 |
| No additions | 1 |

* Additions of 500 μg of Pedersen fetuin (P. fetuin) per ml, 10 μg of insulin (I) per ml, 5 μg of transferrin (TF) per ml, and 10 μM 2-mercaptoethanol (ME) were made to nutrient mixture F-12 (Gibco, H-17).

† 15,000 $F_9$ cells were plated onto a 35-mm culture dish. After 48 hr, the experiment was terminated, medium was removed, and 1 ml of phosphate-buffered saline was added to remove the cells. Cells were counted by a Coulter counter. These numbers are an average of two separate plates. Cells grown in 10% fetal calf serum were chosen as the control; the actual cell number was 68,642.

weeks. The size and appearance of all six tumors were very similar. No differences were noted in the rate of growth of these tumors and, most importantly, the histology of all tumors was essentially the same. In each case the tumor was composed of roughly equal amounts of embryonal carcinoma and primitive glandular tissue (Fig. 3) plus a small amount of other cell types. Thus, EM-1 medium supports the long-term growth of cells, retaining their ability to differentiate *in vivo*.

## Growth of $F_9$ cells in EM-1 medium

EM-1 medium also supports the growth of the embryonal carcinoma cell line $F_9$ (19). The response of $F_9$ to Pedersen fetuin, insulin, transferrin, and 2-mercaptoethanol is very similar to that of PCC.4 aza-1 (Table 3). In particular, the absence of fetuin resulted in the death of all $F_9$ cells even when insulin, transferrin, and 2-mercaptoethanol were present. As with PCC.4 aza-1, $F_9$ can be grown in EM-1 medium for prolonged periods of time. Growth was continuous for 5 weeks (over 20 generations) before these cultures were discontinued. We have also noted that the morphology of $F_9$ cells in EM-1 medium differs somewhat from cells grown in medium containing serum (Fig. 4).

## Concluding remarks

Although Pedersen fetuin is impure, its use has enabled us to monitor the growth response of embryonal carcinoma cells to insulin, transferrin, and 2-mercaptoethanol. Furthermore, the response of these cells to Pedersen fetuin is quite significant since mouse embryos are also affected by it. Gwatkin (20) has reported that mouse embryos cultured in the absence of serum develop somewhat further in the presence of fetuin. Preliminary results (A. Rizzino and M. Sherman) indicate that, in the presence of serum albumin, Pedersen fetuin, insulin, and transferrin, mouse embryos attach to the culture dish and exhibit outgrowth of trophoblast and differentiation of the inner cell mass (cells with the morphology of endoderm are found). In contrast, embryos cultured in serum-free media containing serum albumin may attach to the culture dish but fail to develop further. Therefore, it appears that both embryonal carcinoma cells and mouse embryos respond to fetuin or to a serum factor that copurifies with it.

In each of the experiments reported here, the concentration of fetuin was 500 μg/ml. The concentration was nearly optimal; however, at 2 mg/ml, a slight increase (5%) in cell attachment was observed (unpublished results). These concentrations are within the concentration range of fetuin in medium containing 10% fetal calf serum (1–2 mg/ml) (21). The optimal concentrations of insulin and transferrin were 10 μg/ml and 5 μg/ml, respectively (unpublished results). These results were expected since other cells cultured in serum-free media respond opti-

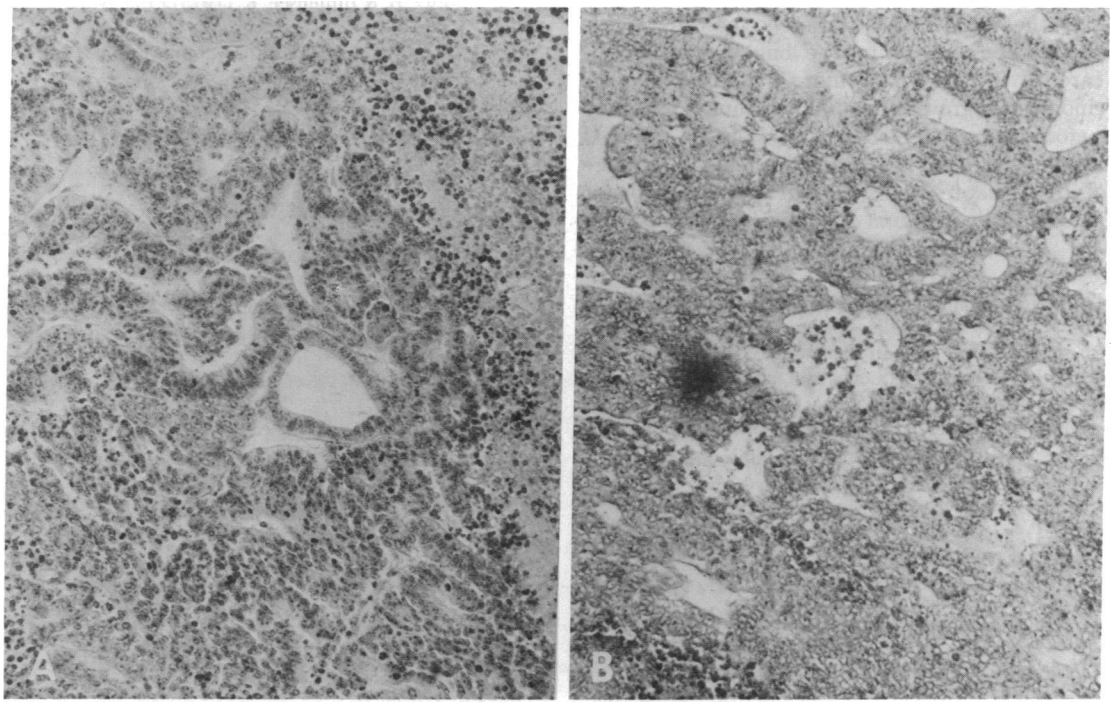


FIG. 3. Hematoxylin- and eosin-stained sections of tumors from PCC.4 aza-1 cells. (*A*) Histology of tumors derived from PCC.4 aza-1 cells grown in serum-free medium (described in the legend to Fig. 2) for over 20 generations. (×58.) (*B*) Histology of tumors derived from PCC.4 aza-1 cells that were grown in medium containing serum. (×58.)

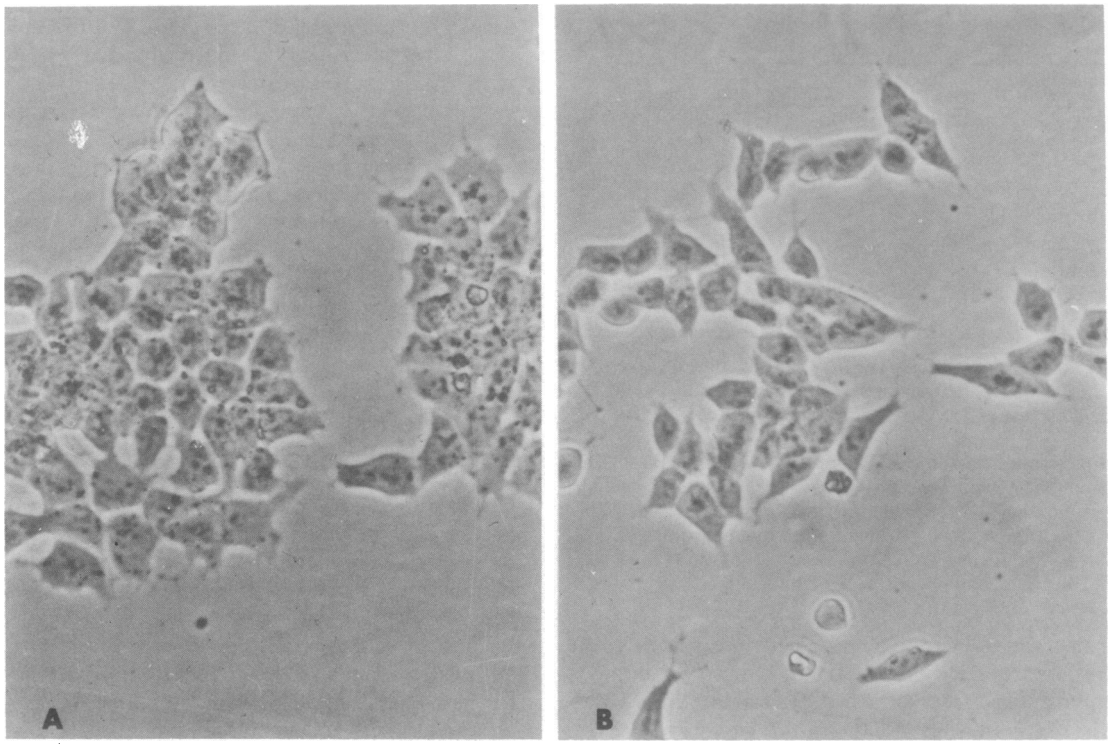


FIG. 4. Morphology of $F_9$ in serum-containing and serum-free media. (*A*) $F_9$ cells in F-12 medium containing 10% fetal calf serum. (×56.) (*B*) $F_9$ cells in the serum-free medium described in the legend to Fig. 2. (×56.)

mally to insulin and transferrin at similar concentrations (11, 12). Medium containing 10% serum contains approximately 100–150 μg of transferrin and somewhat less than 1 ng of insulin per ml (22). At the present time, it is a mystery why insulin is required at such high concentrations by cells in serum-free media. However, part of the reason may be the inactivation of insulin by cysteine present in F-12 medium (I. Hayashi and J. Larner, unpublished results).

In conclusion, the results presented here suggest that EM-1 is a general serum-free medium for the long-term growth of embryonal carcinoma cells. Furthermore, it appears that embryonal carcinoma cells grow in medium containing relatively few serum factors, as do other cell lines (11, 12). Last, it should be emphasized that EM-1 is not considered defined, since there exists the possibility that fetuin itself is not responsible for the response of embryonal carcinoma cells reported here.

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