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Cell Biology

Growth and maintenance of HeLa cells in serum-free medium supplemented with hormones

(growth factor/hormone stimulation)

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ABSTRACT **HeLa cells grow in a nutritionally complete synthetic medium (Ham's F12) supplemented with insulin, transferrin, hydrocortisone (aldosterone), fibroblast growth factor, and epidermal growth factor. This hormone-supplemented medium supports clonal growth, long-term cultivation, and a growth rate equal to that of serum-supplemented medium. The omission of any one of the five components results in less than maximal cell growth.**

The definition of the constituents of media necessary for the survival and proliferation of cultured animal cells has been an active field of investigation since the tissue explants of Harrison in 1906–1907 (1). Development of synthetic media began with simple salt solutions to which various biological fluids were added. With the definition of the amino acid and vitamin requirements of mammalian cells in culture by Eagle and his coworkers (2, 3), a relatively complete, synthetic medium for the growth of cells came into existence. Several cell lines have been serially propagated in synthetic, serum-free media. But these cells required extensive periods of adaptation and may also have a decreased growth rate (4–7).

Synthetic media are usually supplemented with serum. Our laboratory is currently investigating this requirement, with the hypothesis that a main function of serum is to provide hormones (8). We have recently reported the growth of a rat pituitary cell line, GH_3, and a mouse melanoma cell line, M2R, in serum-free synthetic medium, supplemented with hormones and transferrin (9, 10). This paper presents further evidence in support of our hypothesis: the serum component in the culture medium of HeLa cells can be replaced by insulin, transferrin, hydrocortisone (aldosterone), epidermal growth factor (EGF), and fibroblast growth factor (FGF).

MATERIALS AND METHODS

Cells and Culture. The HeLa cell line designated HeLa-S was obtained from John Holland of the University of California, San Diego. Stock cultures were kept in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium supplemented with sodium bicarbonate at 1.2 g/liter, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), penicillin at 192 units/ml, streptomycin at 200 μg/ml, ampicillin at 25 μg/ml, 5% (vol/vol) horse serum, and 2.5% (vol/vol) fetal calf serum. The cells were grown on plastic tissue culture dishes in a humidified atmosphere of 95% air/5% CO_2 and subcultured every 3 or 4 days.

Experimental Media. Serum-free Ham's F12 medium (SFF12) containing sodium bicarbonate at 1.2 g/liter, antibiotics (see *Cells and Culture*), 15 mM Hepes, and trace elements (Table 1) was used in all experiments (11, 12).

Table 1. Trace elements

Compound	Concentration, nM
$MnCl_2 \cdot 4H_2O$	0.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.5
$NiSO_4 \cdot 6H_2O$	0.25
H_2SeO_3	15
$Na_2SiO_3 \cdot 9H_2O$	250
$SnCl_2$	0.25
$Na_3VO_4 \cdot 4H_2O$	2.5
$CdSO_4$	50

Experimental Procedure. For each experiment, exponentially growing cells were trypsinized (0.1% trypsin solution with 0.03% EDTA), suspended in SFF12 containing soybean trypsin inhibitor, and centrifuged. The supernatant was removed and the cell pellet was resuspended in SFF12 and plated into the various experimental conditions at the indicated inoculum.

Experiments of greater than 5 days duration required a medium change on the 5th day. This entailed carefully removing the medium from each plate and replacing it with fresh medium that had been fully equilibrated in the cell culture incubator.

Cell number was determined by counting with a hemacytometer. Experimental cell counts were obtained in duplicate with a Coulter counter after trypsinization.

Single-cell plating experiments, originally developed by Puck and Marcus (13), were performed by plating the cells at the specified density in the manner indicated above; the medium was changed on the 5th day; on the 9th day, the colonies were fixed in methanol and stained with crystal violet (0.1%).

Hormone Preparations. Bovine crystalline insulin, human transferrin (99% iron free), aldosterone, and hydrocortisone were obtained either from the Sigma Co., St. Louis, MO or Calbiochem, La Jolla, CA. The following hormones were generous gifts: FGF, Denis Gospodarowicz of the Salk Institute, La Jolla, CA, and Tom Maciag, Collaborative Research, Waltham, MA; luteinizing hormone preparation NIH.LH.B9, the National Institute of Health Hormone Distribution Program, Bethesda, MD; EGF, Stanley Cohen, Vanderbilt University, Nashville, TN, and Collaborative Research; purified luteinizing hormone, Harold Papkoff, University of California School of Medicine, San Francisco, CA; purified human transferrin

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Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SFF12, serum-free Ham's F12 medium.

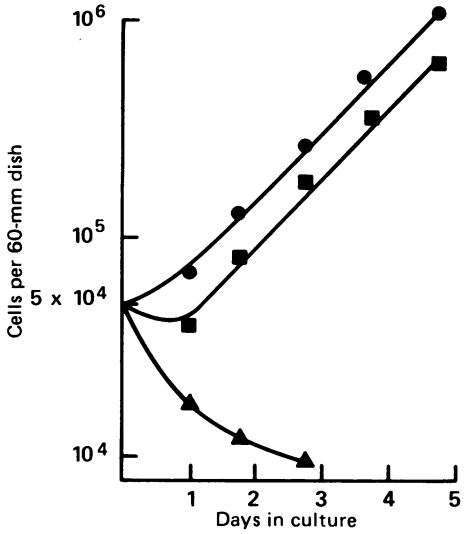

FIG. 1. Growth curve of HeLa cells. Cells were inoculated at 5 × 10^4 cells per 60-mm tissue culture dish containing F12 medium supplemented with: ●, 10% fetal calf serum; ■, insulin (5 μg/ml), transferrin (5 μg/ml), EGF (5 ng/ml), hydrocortisone (100 nM), NIH.LH.B9 (1 μg/ml); ▲, no addition.

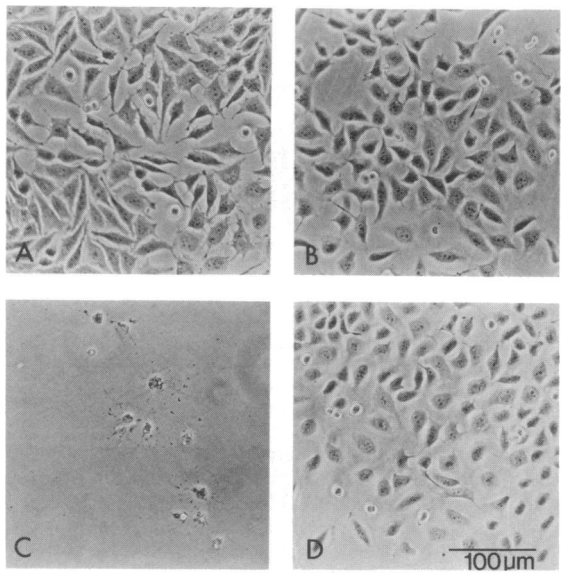

FIG. 2. Photomicrographs of HeLa cells. Cells were plated at 5 × 10^4 per 60-mm tissue culture dish containing F12 medium supplemented with: (*A*) 10% fetal calf serum; (*B*) insulin (5 μg/ml), purified transferrin (0.5 μg/ml), EGF (50 ng/ml), FGF (100 ng/ml), hydrocortisone (50 nM); (*C*) no addition; (*D*) as in *B*, but the cells have been kept in this medium for 6 months, having been subcultured every 4–6 days.

(original source: Behring Diagnostics), R. W. Holley, Salk Institute, San Diego, CA. The trace elements were a gift from R. G. Ham, University of Colorado at Boulder, CO.

RESULTS

In SFF12, HeLa cells degenerate and die. If SFF12 is supplemented with insulin (5 μg/ml), hydrocortisone (30–100 mM), NIH.LH.B9 (1 μg/ml), EGF (5–30 ng/ml), and transferrin (0.5–5 μg/ml), growth is comparable to that in SFF12 supplemented with 10% fetal calf serum. Fig. 1 presents a typical growth curve. The single difference (observed in all growth curves in the absence of serum) is a 24-hr time lag between inoculation of the cells and the onset of growth. Morphologically, the cells in the hormone-supplemented medium appear quite similar to those in serum-supplemented medium. Some cytoplasmic retraction does occur in the absence of serum. Only cell debris remains in SFF12 alone (see Fig. 2).

Omission of any single hormone results in less than maximum cell growth; optimal concentrations were determined by measuring cell growth as a function of concentration of each hormone (Figs. 3 and 4). It has been reported that the growth-promoting effect of NIH.LH.B9 may be attributed to FGF present in the preparation, rather than to the luteinizing hormone itself (14–16). We examined the growth activities of these two components of the NIH.LH.B9. Fig. 4 shows that the activity found in NIH.LH.B9 can be replaced by purified EGF. Purified luteinizing hormone has no activity. Highly purified transferrin gave an identical dose response curve and the same ability to form colonies as the partially purified transferrin preparation (Sigma) (data not shown). Other steroids (aldosterone, progesterone, estrogen, testosterone) have been tested in lieu of hydrocortisone. Only aldosterone (50–100 mM) has equivalent activity (data not shown).

The substitution of serum by the five components was tested further by examining colony formation at low density. Colonies form readily in SFF12 supplemented with, but not in the absence of EGF, FGF, insulin, purified transferrin, and hydrocortisone (Fig. 5).

A final measure of the extent to which serum could be replaced by the hormone-supplemented SFF12 is the ability to support long-term growth. HeLa cells have been grown in the defined medium (SFF12 + insulin, transferrin, EGF, hydrocortisone, NIH.LH.B9) for 6 months with subculturing every 4–6 days (depending on inoculum). Representative of such long term growth is the curve in Fig. 6, showing growth of HeLa cells through 11 doublings and subcultures. 2

DISCUSSION

We have found that a set of five components—EGF, FGF, transferrin, insulin, hydrocortisone (aldosterone)—can replace the serum in the culture medium of HeLa cells. This set is characteristic of these HeLa cells; requirements of other cell lines, for instance GH_3 and M2R, differ significantly, although some components, such as insulin and transferrin, appear to be common requirements. Other hormones have been tested for growth-promoting activity, including: hypothalamic releasing factors, pituitary hormones, calcitonin, parathyroid hormone, progesterone, estrogen, testosterone, relaxin, ceruloplasmin, prostaglandins, and thyroxin; none have shown activity.

These components can be categorized as classical hormones, growth factors, or accessory factors. Their specific roles in an *in vitro* system such as this one are unknown. However, the possibility cannot be overlooked that these components serve functions analgous to their ones *in vivo*. In this regard the effective concentrations *in vitro* are within the physiological range. (Insulin is an exception, but this may be due to its rapid breakdown in cysteine-containing medium.)

In a previous report from this laboratory, we reported the growth of HeLa cells in serum-free medium supplemented with a large number of hormones, growth factors, and accessory factors such as transferrin and ceruloplasmin (9). In the present study we have made the following specific advances: (*i*) the components have been reduced to those that are essential; (*ii*) the serum-free hormone-supplemented medium has been shown to support continuous long-term growth and colonial growth from single cells; and (*iii*) purified components were

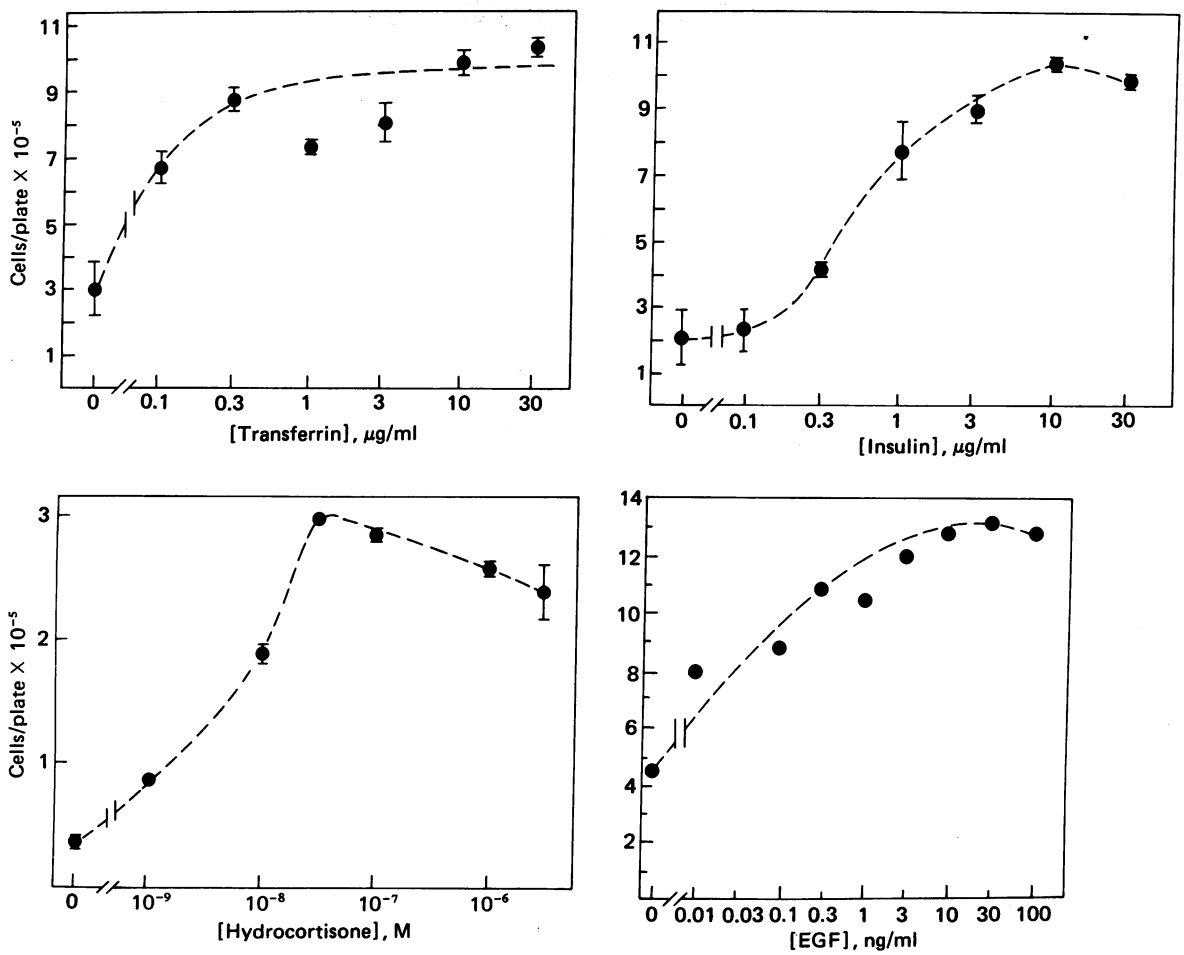

FIG. 3. Growth response as a function of hormone concentration. In each experiment the concentration of four of the five components was constant while the concentration of the fifth was varied over the indicated range. The concentrations of each of the components when in the basal group were 5 μg/ml for insulin, 100 nM for hydrocortisone, 1 μg/ml for NIH.LH.B9, 5 μg/ml for transferrin, 5 ng/ml for EGF. The cells were inoculated at 5×10^3 cells per 100-mm tissue culture dish; the medium was changed on the 5th day; on the 8th (hydrocortisone) or 9th (transferrin, insulin, EGF) day, the cells were trypsinized and counted.

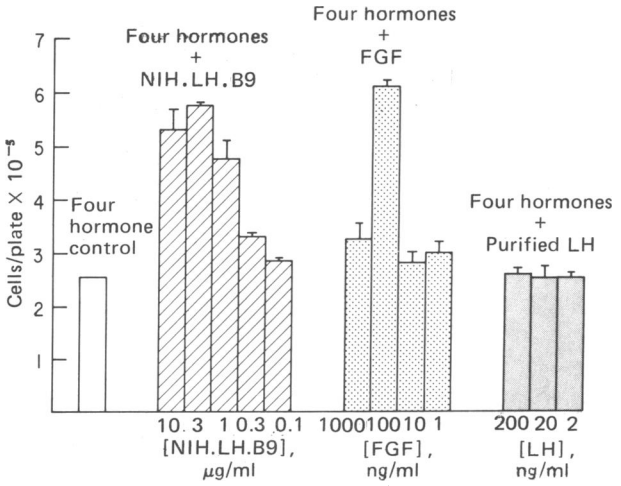

FIG. 4. Comparison of the growth of HeLa with NIH.LH.B9, purified FGF, and purified luteinizing hormone (LH). SFF12 was supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), EGF (50 ng/ml), and hydrocortisone (100 nM); to this were added various concentrations of either NIH.LH.B9, purified FGF, or purified luteinizing hormone as indicated. Experimental procedures were the same as those given in Fig. 3.

used in this study, while in the previous study the somatomedin and transferrin were only partially purified. Furthermore, in our hands, no adaptation of selection was necessary; all assays were carried out with HeLa cells harvested from serum-supplemented cultures.

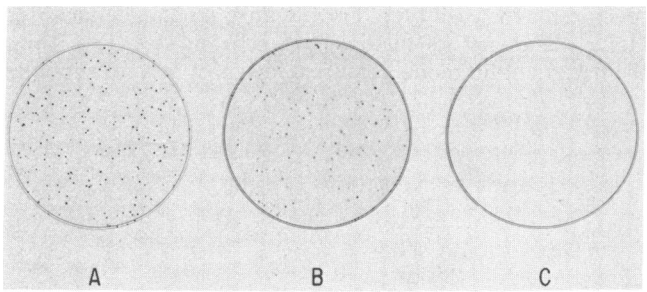

FIG. 5. Colony formation of HeLa cells. The cells were inoculated at 500 cells per 100-mm tissue culture dish in F12 medium containing: (*A*) 10% fetal calf serum; (*B*) EGF (50 ng/ml), FGF (100 ng/ml), transferrin (5 μg/ml), hydrocortisone (100 nM), insulin (5 μg/ml); (*C*) no addition. The medium was changed on the 5th day; on the 9th day the cells were fixed in methanol and stained with crystal violet (0.1%).

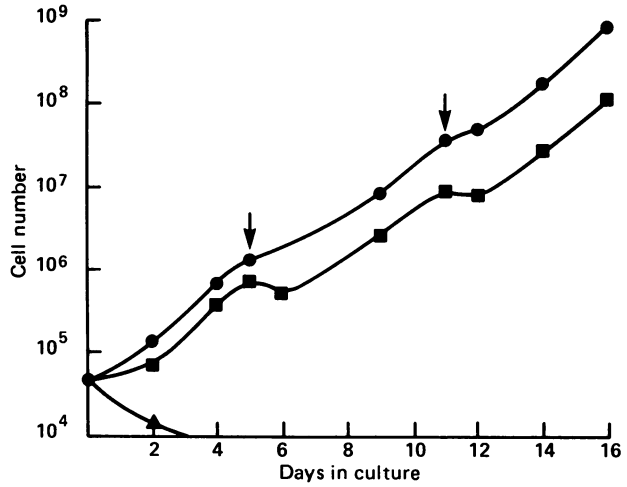

FIG. 6. Long-term growth of HeLa cells. ●, 10% fetal calf serum; ■, insulin (5 μg/ml), purified transferrin (0.5 μg/ml), EGF (50 ng/ml), FGF (100 ng/ml), hydrocortisone (50 nM); ▲, no addition. Cells were subcultured on the days indicated by the arrows.

The feasibility of defining the growth media of cells was dependent upon the development of nutritionally complete, synthetic medium such as Ham's F12. Because the nutritional requirements have been met, removal of the serum from the medium reveals hormone requirements. It is possible that some hormone requirements may be a function of the nutritional composition of the medium.

Early attempts to develop a satisfactory low serum or serum-free medium including the supplementation of that medium with hormones (18–21) were probably unsuccessful because of deficiencies in the basal medium and unavailability of essential growth factors which have only recently been discovered (FGF, EGF, somatomedin, etc.) (16, 22–24).

Because serum-free systems have been developed for three cell lines, we are optimistic that a serum-free system can be developed for any cell line. Such simplified systems will aid in our understanding of how cellular growth is controlled and may lead to the ability to control that growth.

It should be emphasized that the single-cell plating method is the most stringent test for the adequacy of the defined media. The fact that the serum-free, hormone-supplemented media support colony formation with efficiencies comparable to that of serum-supplemented media reinforces our contention that functions previously attributed to serum macromolecules are served mainly by hormones or hormone-like substances.

The constellation of hormones adequate for a given cell is specific for that cell type. For instance, media adequate for HeLa cells are not adequate for GH_3 cells and vice versa. Thus, the pattern of hormonal requirements must be a reflection of the differentiated state of the cell and raises the difficult problem of how such a complex and elaborate pattern of hormonal control functions at the level of integrated physiology.

In addition to the theoretical possibilities, the substitution of hormones for serum in cell culture medium will permit more controlled and definitive experiments in many areas of cell biology.

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