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EFFECTS OF STEROID HORMONES IN FETAL BOVINE SERUM ON PLATING AND CLONING OF HUMAN CELLS IN VITRO

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SUMMARY

Fetal bovine sera from each of three different commercial sources were tested for their ability to support cloning of human fibroblastoid cells in vitro. Cloning efficiencies varied according to serum source. Serum (10 samples) from company A did not support growth, while sera (10 samples) from companies B and C provided adequate to excellent conditions for cloning and growth. Cells from neonatal foreskin or embryonic lung responded to each serum similarly. Bovine serum albumin type H7 from company C supported cell growth in media without serum.

Sera containing 1.0 ng per ml or more of progesterone inhibited growth, whereas sera containing less than 1.0 ng per ml supported cloning and growth. In the low progesterone sera, the concentration of 17-β-estradiol exceeded 100 pg per ml. Growth supporting sera could be made non-supportive by adding 0.1 μg per ml of progesterone. The addition to non-supportive sera of 0.1 μg per ml of 17-β-estradiol or hydrocortisone made these sera supportive of cell growth.

Addition of estrogen or hydrocortisone to a culture medium that inhibits growth, with subsequent reversal of the inhibitory effect, implies that these hormones competitively regulate growth of responsive cells in vitro.

Key words: serum; cell cultures; hormones; cloning efficiency; plating efficiency.

INTRODUCTION

In vitro studies in tumor virology (1), chemical carcinogenesis (2), and cellular aging (3, 4), that involve evaluation of growth conditions of cells in vitro, frequently have not taken into account the effects on growth of naturally occurring steroid hormones in the culture media. These effects have not been adequately considered in some studies involving hormone action, such as estrogen effects on cornification of the mouse vagina in vitro (5), or the induction of avidin synthesis by progesterone in chick oviduct cultures (6).

Whereas some other factors in serum affecting cell growth have been investigated (7), the influences of the hormones in fetal bovine sera on cell plating and cloning efficiencies, in the presence of supplemental exogenous hormones, have not been examined. Serial subcultivation in vitro of hormone sensitive cells may be dramatically affected by endogenous hormones present in sera. Hormone added to cell cultures also affects the properties of cellular membranes (8). Some responses attributable to oncogenic virus could be significantly affected by the addition of steroid hormones, especially in the presence of endogenous concentrations of the same hormones.

This report presents a study of the effects of exogenous and endogenous hormones on human cells cultivated in vitro, as measured by plating and cloning efficiencies.

MATERIALS AND METHODS

Cell cultures. The cell lines used in these experiments were: (*1*) human male embryonic lung (HEL-M) (Flow 2000, Flow Labs Inc., Rockville, Md.); (*2*) Detroit 550 (D-550), normal foreskin (American Type Culture Collection, Rockville, Md.); and (*3*) WI-38 human embryonic

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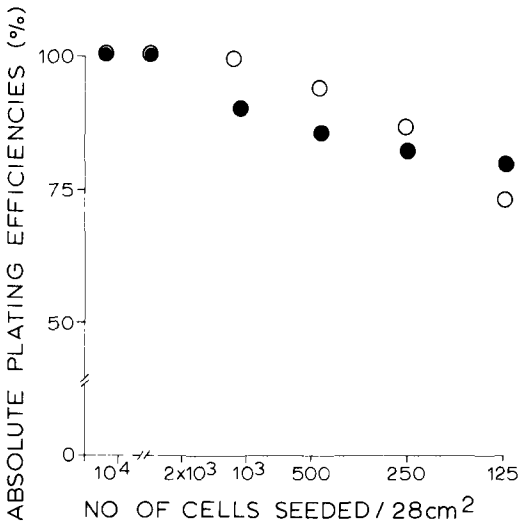

FIG. 1. The open cirles (O O) represent the plating efficiencies for the D-550 cell line between serial subpassages 17 and 24. The filled circles (● ●) represent the plating efficiencies for the HEL-M cell line between serial subpassages 2 and 24. Each point represents the mean values of the P.E.'s for eight wells. Serum from company C was used in these experiments.

lung cells, a gift from L. Hayflick (Medical College, Stanford University, Palo Alto, California).

Serum. Ten samples of fetal bovine serum (FBS) per supplier were obtained in 500 ml quantities from different pooled 80 liter lots from three commercial suppliers, A, B and C. These sera were procured by the suppliers by aseptic heart puncture from the fetuses immediately after parturition. Even though these sera were collected aseptically, a sample from each lot was routinely tested for the presence of mycoplasmas (13). The samples tested were negative.

Each serum sample collected from the three individual fetuses separated on the basis of sex was treated and processed in the same manner described for pooled lots.

Bovine serum albumin. Bovine serum albumin designated as fraction H-7 (BSA) was dissolved in 1X minimum essential medium with Earle's salts at pH 7.0. A 10% stock solution was stored at 12°C until used in the following experiments.

Calcium determinations. Calcium in the sera was measured by atomic adsorption spectrophotometry (9), (Perkin Elmer 303, Atomic Adsorption Spectrophotometer). The values were reported as pg per ml. The lower limits of sensitivity were 1 to 5 ng per ml of calcium.

Cortisol determination. Cortisol in serum was determined by an established fluorometric procedure for the estimation of free 11-hydroxycorticoids in plasma (10). The cortisol was extracted from the serum with methylene chloride. The organic phase was separated and transmission read at its excited wavelength with a mercury lamp, using a primary filter (Chance 0B10 blue filter) and secondary filters (Chance 0Gi and 043) with a final peak transmission at 540 nm. Sensitivity is 10 to 50 pg per ml.

Progesterone, estrogen (estrone E1; estradiol E2; estradiol E3) determinations. The assay procedure according to Powell and Stevens (11) employs a simplified radioimmunoassay for determination of free forms of the steroids. Samples were prepared by extraction of the serum with ether, followed by column chromatography and resolution of the steroids by radioimmunoassay. The sensitivities of the assays were: E1, 20 pg per ml; E2, 10 pg per ml; E3, 30 pg per ml; progesterone, 12 pg per ml.

Plating efficiency. Cultures were trypsinized as described below to achieve high plating and cloning efficiencies. Plating efficiency (P.E.) was defined as the percentage of cells seeded that attached to the bottom of a culture dish within 24 hr and assumed a typical fibroblastic morphology. Cloning efficiency (C.E.) was defined as the percentage of seeded individual cells which gave rise to colonies of 100 cells or more in 8 to 11 days when seeded at a low cell density (i.e. less than 10,000 cells per cm^2).

Media. Growth medium for D-550 cells contained 1X minimum essential medium with Earle's salts (Flow Labs., Rockville, Md.) (1X MEM-E), with 50 μg per ml of gentamicin sulfate (Gentocin) (50 mg per ml, Schering Corp., Bloomfield, N.J.), 2 mM of glutamine (100 mM glutamine, GIBCo., Grand Island, N.Y.), 1X non-essential amino acids (100X, Microbiological Associates Inc., Bethesda, Md.), 1 mM of sodium pyruvate (100 mM, Microbiological Associates Inc., Bethesda, Md.), 2.2 mg per ml of sodium bicarbonate, and supplemented with 10% of FBS.

The growth medium for serial subcultures of HEL-M or WI-38 cultures was 1X Eagle's basal medium, with Hanks' or Earle's salts (12).

Attachment and cloning medium for all cells was 1X-MEM-E, with 50 μg per ml of Gentocin, 2 mM of glutamine, 4X non-essential amino acids, 2X vitamins (100X MEM-Eagle, Flow Labs.

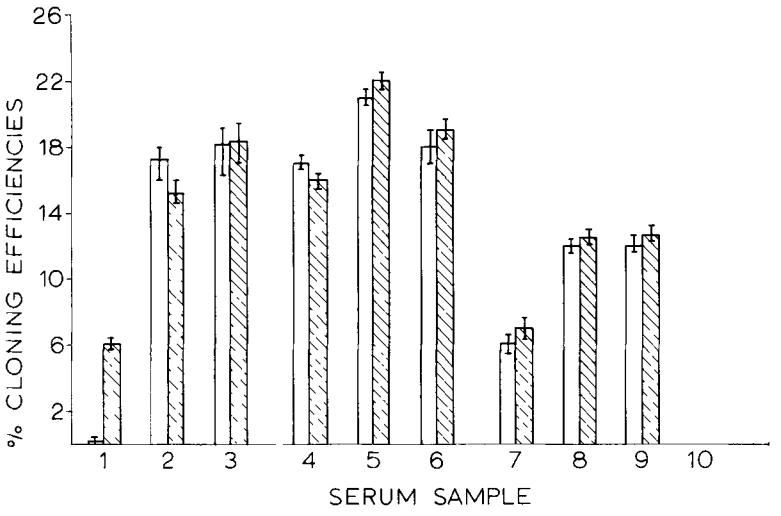

FIG. 2. Each serum sample from 1–10 represents either fetal bovine serum or bovine serum albumin.

Sample 1 is FBS from company A at a 20% concentration in the cloning medium (see Materials and Methods). Sample 2 is 20% FBS of mixed source from company B. Sample 3 is 20% FBS of mixed source from company C. Sample 4 is 20% C FBS from a male fetus. Sample 5 is 20% C FBS from a female fetus. Sample 6 is 10% C from a mixed source supplemented with 1% C Bovine Serum Albumin fraction type H-7. Sample 7 is 3% C Bovine Serum Albumin fraction type H-7. Sample 8 is 10% C FBS. Sample 9 is a 10% B FBS. Sample 10 is 10% A FBS. The open bars represent cloning efficiencies values for Detroit 550 cells between passages 17 and 25 and the slash bars represent C.E. values for HEL-M cells between passages 2 and 24. The vertical bar represents one standard deviation value for the cloning efficiency in 16 wells.

The clones were counted under a binocular microscope at 7×.

Inc., Rockville, Md.), 1 mM of sodium pyruvate, 2.2 mg per ml of sodium bicarbonate, and supplemented with the appropriate concentration of FBS and/or BSA.

Cell suspensions. Cell suspensions were prepared in the following manner. The growth medium from a 120 cm^2 bottle was removed and the cell sheet washed three times with 10 ml of 1X Hanks' balanced salt solution (HBSS). One ml of 0.1% trypsin (1.0% sterile trypsin (lyophilized trypsin 3680 BAEE U per mg, Worthington Biochemical Corporation, Freehold, N.J.)) in 1X MEM-E, pH 6.8, was added to the cell sheet. The cells were usually detached in 45 to 90 sec. The cell suspension was diluted to 10 ml with 1X MEM and the suspension pipetted vigorously against the bottom of the vessel. At this time, 3 to 8 ml of complete MEM-E or Eagle's basal medium with Hanks' salts (BME-H), was added and the cells pipetted vigorously three or six times. Cell suspensions were then transferred to sterile 35 ml plastic centrifuge tubes at cell densities of 100,000 cells per ml. Ten plates were seeded at cell densities of 1,000 cells per 28 cm^2 well. The cells were cloned in a 5 to 7% CO_2 in air environment at 37°C and 95% relative humidity for 8 days at cell densities of more than 5000 cells per well, or 11 days at cell densities of less than 5000 cells per well.

Fixation, staining and enumeration of clones. The medium was decanted and the cultures were washed once with 1X MEM-H. Cells were fixed with 2% formalin in phosphate buffered saline at pH 7.33 (Dulbecco). The fixed colonies were stained with Ehrlich's glycerin alum hematoxylin and eosin Y and examined and counted under a dissecting microscope (25 X).

RESULTS

The effects of different fetal bovine sera on P.E.'s were determined for the cell lines. Consistent P.E.'s were obtained with 250 to 10^4 cells per 28 cm^2. Each serum lot examined affected the growth characteristics of the cells, but did not influence cell P.E. when as few as 125 cells per 28 cm^2 for each cell line were used (Fig. 1). The P.E. values for WI-38 cells at low passage

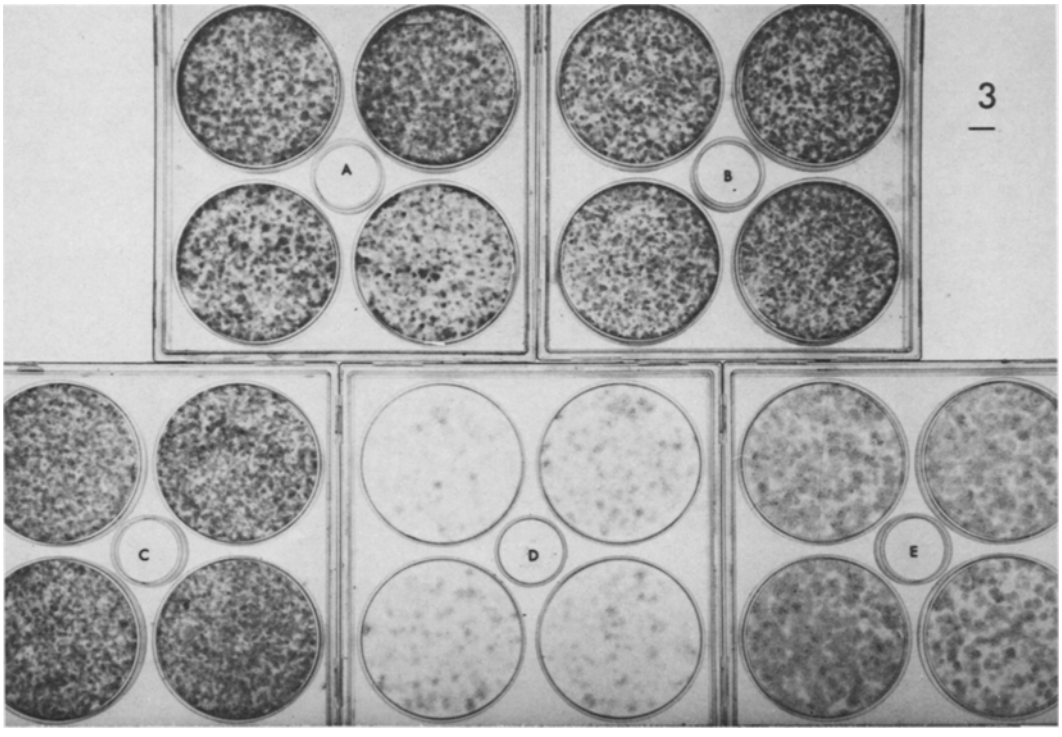

FIG. 3. The clones in Fig. 3 were enumerated after seeding the wells with D-550 cells at passage 12, cloning, fixing and staining the cultures.

Dish A represents the growth of the cells in the clone under: 10% C, FBS, Dish B 10% C, supplemented with 1% Bovine Serum Fraction H-7; Dish C 20% C, FBS; Dish D 20% A, FBS; Dish E 20% B, FBS. (Surface area of a well is 28 cm^2) Magnification, ×1.

levels (12 to 17), were similar to the values obtained for HEL-M cultures.

The ability of each serum supplement to support cloning varied between the suppliers of the serum (Fig. 2). Cloning efficiencies also varied with the serum source (Figs. 2–4). Serum from company A provided inadequate support for growth at the cell densities between 10^3 and 10^4 cells per 28 cm^2 (Figs. 3 and 4).

HEL-M cells serially subpassaged from passage levels 6 through 12 which were seeded at a cell density of 10^3 cells per 28 cm^2 in the presence of 20% FBS from company A, cloned at 6%; from company B at 16%, and from company C at 19% (Fig. 2). D-550 cells, treated in the same manner in 1X MEM supplemented with 20% FBS from company A, cloned at 1.0%; from company B at 18.0%, and from company C at 19.0% (Fig. 2). WI-38 cells between passage levels 19 and 31 seeded at the same cell densities as the HEL-M cells gave similar cloning values as the HEL-M cells in each medium supplemented with 20% FBS from companies A, B, or C. Lowering of the FBS in the cloning media from 20% to 10% gave a corresponding decrease in cloning efficiency. Supplementation of the cloning medium, containing 10% serum from company B or C, with 1% BSA increased the cloning efficiency to the same level as that obtained with 20% FBS supplement. Cloning media that contained only 3% BSA supported limited growth of the D-550 and HEL-M cells; however, the cloning efficiency was only 7%.

Fetal bovine sera from a male and a female fetus yielded cloning efficiencies similar to sera from a mixed source for both cell lines.

Hormonal analysis (Tables 1 and 2) revealed that sera that would support cloning of D-550

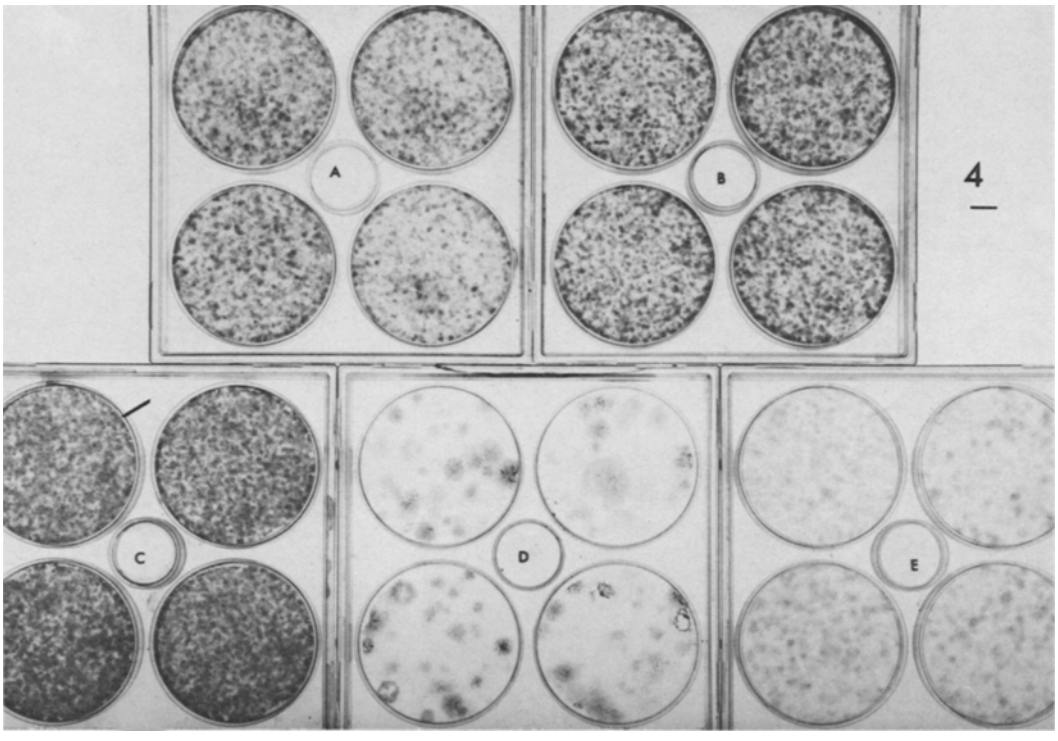

FIG. 4. The clones in Fig. 4 were enumerated after seeding the cells with HEL-M at passage 7, cloning, fixing and staining the cultures.

The cells in dish A, B, C, D, and E were cloned as described in the test with supplements described in the legend of Fig. 3.

TABLE 1

STEROID HORMONE AND CALCIUM CONTENTS OF SELECTED FETAL BOVINE SERA AND OF BOVINE SERUM ALBUMIN

Factor Assayed		Fetal Bovine Sera				Bovine Serum Albumin
		A	B	C	C	
					(dialyzed)	*(Fraction Type H-7)*
Estrone (pg/ml)	Range	18–32	41–54	48–92	76–80	110–124
	$\bar{X}$	26.4	51.2	75.3	78.0	116
17β Estradiol (pg/ml)	Range	58–174	112–134	121–232	190–200	162–210
	$\bar{X}$	66.3	128.3	188.5	195.0	180
Estriol (pg/ml)	Range	9–16	20–28	24–48	36–42	35–52
	$\bar{X}$	13.3	25.6	37.6	39.0	42
Progesterone (pg/ml)	Range	1000–1500	50–100	40–70	50–90	40–80
	$\bar{X}$	1250	80	50	70	60
Cortisol (ng/ml)	Range	12–31	21–54	19–45	41–49	16–33
	$\bar{X}$	16	39	28	45.3	19
Calcium (mg/100 ml)	Range	9–14	10–14	10–16	11–17	10–14
	$\bar{X}$	11.07	12.19	12.40	14.92	11.8

The hormone values for Estrone E_1, Estradiol E_2, and Estriol E_3, progesterone, cortisol and the calcium concentrations for mixed serum samples were determined by radioimmunoassay as described in the text. Each letter at the heading of the column A, B and C represents the sources of the serum from different commercial suppliers. The number of replicates of samples was 10.

The apparent increase in values following dialysis was a reflection of the samples dialyzed and not an actual increase.

TABLE 2

STEROID HORMONE CONTENTS OF FETAL BOVINE SERA FROM MALE AND FEMALE FETUSES

Factor Assayed		Male C	Female C
Estrone, E1 (pg/ml)	Range	42–115	49–59
	$\bar{X}$	92.4	54.2
17β Estradiol, E2 (pg/ml)	Range	106–378	49–59
	$\bar{X}$	242.2	51.0
Estriol, E3 (pg/ml)	Range	20–75	25–31
	$\bar{X}$	47.4	27.2
Progesterone (pg/ml)	Range	400–500	400–500
	$\bar{X}$	400	400
Cortisol (ng/ml)	Range	2.0–3.1	4.4–2.7
	$\bar{X}$	2.8	3.6

The hormone values in this table are from individual lots for different sexes. The number of replicates for the samples was three. See the legend for Table 1 for additional detail.

and HEL-M cells were consistently low in progesterone (less than 1.0 ng per ml) and high in estradiol (E_2), (100 pg per ml). The mean progesterone content in these samples was 0.07 ng per ml. The concentration of E_2 was 12.0 pg per ml and of cortisol 35 ng per ml.

DISCUSSION

Progesterone and E_2 could act in a regulatory manner exerting constraints, or releasing constraints, over growth of human cells in vitro. Dialyzing (14) FBS did not reduce the hormone content significantly. The levels of hormones in the fetal bovine sera varied from lot to lot and between sexes (Tables 1 and 2). The extreme variation did not appear to affect the cloning efficiencies, except when the level of progesterone in the FBS exceeded 1.0 ng per ml, and when the E_2 fraction was decreased below 60 pg per ml. As long as the progesterone in the FBS

TABLE 3

CLONING EFFICIENCIES OF HEL-M AND D-550 CELLS IN MEDIA SUPPLEMENTED WITH DIFFERENT HORMONES

Hormone	Concentration	Detroit 550		HEL-M	
		A	C	A	C
	μg/ml	*% Cloning Efficiency ± S.D.*		*% Cloning Efficiency ± S.D.*	
Progesterone	0	6 ± 1.0	18 ± 2.0	1.0 ± 0.5	17 ± 2.0
	0.0001	6 ± 0.8	17 ± 3.0	0	13 ± 1.3
	0.001	6 ± 0.7	17 ± 1.8	0	10 ± 1.2
	0.01	4 ± 0.3	12 ± 2.4	0	0.1
	0.1	1	9 ± 0.8	0	0
	1.0	0	0	0	0
	10.0	0	0	0	0
Estradiol (E_2)	0	6 ± 1.3	18 ± 3.1	0.5	16 ± 1.7
	0.0001	5 ± 1.2	18 ± 2.6	1.0	17 ± 1.3
	0.001	8 ± 0.8	20 ± 3.1	3.0 ± 0.61	18 ± 1.2
	0.01	7 ± 0.2	14 ± 1.8	5.0 ± 0.21	9 ± 3.1
	0.1	11 ± 2.1	16 ± 3.2	12.0 ± 2.1	23 ± 3.8
	1.0	18 ± 1.7	18 ± 2.4	18.0 ± 3.4	TNTC
	10.0	10 ± 4.1	12 ± 3.6	TNTC	TNTC
Cortisol	0	5 ± 0.8	17 ± 1.6	0.3	15 ± 0.8
	0.0001	5 ± 0.6	17 ± 0.8	0.2	16 ± 1.3
	0.001	6 ± 1.2	16 ± 0.7	0.4	17 ± 2.6
	0.01	7 ± 1.1	14 ± 1.2	1.3 ± 0.6	15 ± 3.1
	0.1	11 ± 1.2	18 ± 2.1	6.0 ± 0.6	18 ± 1.7
	1.0	17 ± 3.1	19 ± 1.7	12.0 ± 1.1	21 ± 2.8
	10.0	12 ± 2.8	10 ± 3.1	18.0 ± 2.6	TNTC

The cultures were seeded at 1000 cells per 28 cm^2 well, cloned and grown for 11 days as described in the Materials and Methods. ± S.D. represents one standard deviation and TNTC means that the clones, at the end of 11 days, were too numerous to count in these wells. The values here were determined on six separate wells for each concentration of hormone. The designations A and C represent the sera obtained from two commercial suppliers.

was less than 1.0 ng per ml and the E_2 fraction was 60 pg per ml or more, cloning efficiencies were not adversely affected (Table 2 and Fig. 2).

The wide range of concentrations of each hormone in the fetal bovine sera up to the critical values did not appear to affect the growth properties of the three cell lines, although with other cells such as endocrine responsive tissue, it could be important to have a high progesterone content in the FBS. Cell culture growth might then be inhibited by a low progesterone and high E_2 content. Apparently the cortisol content, while varying significantly from lot to lot, does not affect the growth characteristics of the fibroblast cultures.

The presence of cortisol as an additive to human cultures at pharmacological concentrations has been reported to extend their life span (8) in vitro. This extension of life span could be altered by wide variations in concentration of hormones in the media or FBS (15).

The addition of three different hormones (Table 3) to the cloning media at concentrations from physiological to pharmacological ranges in sera from companies A and C indicated that these hormones could enhance or inhibit the cloning of the cells in vitro. Progesterone completely inhibited cloning of HEL-M cells at 0.1 μg per ml or greater and inhibited cloning of D-550 cells at 1.0 μg per ml.

Estradiol did not inhibit the cloning efficiencies of D-550 cells until a concentration of 10 μg per ml was reached, whereas HEL-M cells did not exhibit a toxic reaction to the presence of added E_2 until 25 μg per ml had been reached. Moreover, 17-β-estradiol could overcome the inhibitory effect of serum from company A at concentrations of 0.1 to 1.0 μg per ml. In addition, cortisol was effective in overcoming the inhibitory effect of the sera from company A at 1.0 to 10.0 μg per ml on D-550 or HEL-M cells, respectively (Table 3). This compound was toxic to the cells at 25 μg per ml or greater. Since both 17-β-estradiol and cortisol would overcome the inhibitory effect of serum A at the lower concentrations, as stated above, it would appear that progesterone and the other steroids act competitively in the cell systems. None of the added hormones affected the attachment of the cells in the wells until concentrations of 25 μg per ml were reached.

Morphological differentiation of cells in monolayer culture does not appear to be affected by the hormone content. However, physiological differentiation and function could be affected. The loss of primary function of cells producing hormones in vitro, such as pituitary, adrenals, etc. could be altered by endogenous estrogen hormone ratios in the feeding medium.

Use of hormones as additives to growth media, without concern for the endogenous hormone concentration in the medium, could introduce uncontrolled variables into a system. Recent reports of the effect of 17-β-estradiol in prevention or inhibiting toxicity of carcinogens (16) suggest that hormones in these systems may alter attachment sites on cytoplasmic membranes. This competitive selection for attachment sites (15), and the interaction between estrogens and progesterone, have demonstrated that the concentrations of progesterone and estradiol in the medium can affect such biological expressions as cell multiplication, biological function and morphology. The growth of normal human cells in vitro before exposure to external etiological agents, chemical or biological, and biochemical studies with these cells, can be affected by their past exposure to growth media containing different amounts of progesterone and estrogen in the FBS. Furthermore, if membrane studies on cells with selective attachment sites cultured in vitro are to be meaningful, rigid control of the quantities of hormones in the medium must be exercised. Standardization of the hormones in FBS for growth of specific cells in vitro would be an important consideration.

REFERENCES

1. Fong, C. K., and N. Ledinko. 1970. Enhancement by estrogens of adenovirus or spontaneous transformation of hamster cell cultures. Cancer Res. 30: 889–892.
2. DiPaolo, J. A., P. J. Donovan, and R. L. Nelson. 1971. Transformation of hamster cells in vitro by polycyclic hydrocarbons without cytotoxicity. Proc. Natl. Acad. Sci. U.S.A. 58: 2958–2961.
3. Turk, B., and G. Milo. 1974. An in vitro study of senescent events of human embryonic lung (WI-38) cells. I. Changes in enzyme activities of cellular and membrane associated enzymes of untreated and cortisone acetate-treated cultures during senescence. Arch. Biochem. Biophys. 161: 46–53.
4. Milo, G., and K. Lewis. 1971. Stimulation of

human cell multiplication in vitro with cortisone. In Vitro 5: 397.
5. Hardy, M. H., J. P. Biggers, and P. J. Claringbold. 1953. Vaginal cornification of the mouse produced by oestrogens *in vitro*. Nature 172: 1196–1197.
6. O'Malley, B. W., and P. O. Kohler. 1967. Studies on steroid regulation of synthesis of a specific oviduct protein in a new monolayer culture system. Proc. Natl. Acad. Sci. U.S.A. 58: 2359–2366.
7. Boone, C. W., N. Mantel, T. D. Caruso, E. Karzan, and R. E. Stevenson. 1972. Quality control studies on fetal bovine serum used in tissue culture. In Vitro 1: 174–189.
8. Cristofalo, V. J. 1970. In: E. Holečková and V. J. Cristofalo (Eds.), *Aging in Cell and Tissue Culture*. p. 83, Plenum, N.Y.
9. Willis, J. B. 1960. The determination of metals in blood serum by atomic absorption spectroscopy. I. Calcium. Spectrochim. Acta 16: 259–164.
10. Mattingly, D. 1962. A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma. J. Clin. Pathol. 15: 374–379.
11. Powell, J. E., and V. C. Stevens. 1973. Simple radioimmunoassay of five unconjugated ovarian steroids in a single sample or serum or plasma. Clin. Chem. 19: 210–215.
12. Milo, G. 1973. Enhancement of senescence in low passage human embryonic lung cells by an agent extracted from phase III cells. Exp. Cell Res. 79: 143–151.
13. Barile, M., and J. Kern. 1971. Isolation of *Mycoplasma arginini* from commercial bovine sera and its implication in contaminated cell cultures. Proc. Soc. Exp. Biol. Med. 138: 432–437.
14. Milo, G. E., J. P. Schaller, and D. S. Yohn. 1972. Hormonal modification of adenovirus transformation of hamster cells, in vitro. Cancer Res. 32: 2338–2347.
15. Leung, B. S., and G. H. Sasaki. 1973. Prolactin and progesterone effect on specific estradiol finding in uterine and mammary tissues in vitro. Biochem. Biophys. Res. Commun. 55: 1180–1187.
16. Schwartz, A. G. 1973. The protective effect of estradiol-17β against polycyclic hydrocarbon cytotoxicity. Cancer Res. 33: 2431–2436.

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