Production of Hormones by Human Anterior Pituitary Cells in Serial Culture. (25265)

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The need to develop methods to supply human-type pituitary hormones has been evident since the demonstration(1-5) of effectiveness in humans of growth hormone from humans, and the realization that specific activity of human-derived hormone in man is due to differences in molecular structure of somatotropins(6-9). Cell culture(10-16), has been applied especially in virus and cancer research, but production of specialized secretion products of cells, such as enzymes or hormones, has not heretofore succeeded(17,18). Explants of placental or pituitary tissue, on the other hand, have produced detectable amounts of hormones in tissue, as contrasted with cell culture(19-21).

Methods. The "starter" tissues (stage I*) comprised portions of 9 anterior pituitary glands, 4 from women, 5 from human fetuses varying in age, and fragments of an acidophilic adenoma from a woman with acromegaly. These tissues were obtained incidental to surgical operations for therapeutic purposes. Immediately after excision, the sterile tissue fragments were separated aseptically from surrounding membranes, and minced and prepared as explant cultures in Eagle's basal medium(11) with serum supplements, either human, equine or bovine. *Serial propagation of heterogeneous cell cultures* (stage II) was undertaken after a sufficient outgrowth from tissue fragments had taken place. Then, by serial subculture, a considerable number of stationary cultures were derived from each starter tissue. Whenever practical at time of transfer, permanently stained coverslip preparations of cells were examined. *Propagation of selected epithelial-like cells in stationary culture* (Stage III): Islands of epithelial-like cells eventually appeared among the fibroblast-like cells of early cultures. These were selected for subculture. With several starter cultures, it was possible to pick out epithelial-like cells from the proliferating explant. *Propagation of selected cell lines in a suspension system* (stage IV) was usually successful with cell concentration of 100,000 to 200,000 cells/ml. In a suspension-type of culture the generation time in most cell lines was about 24 hours. However, 2 lines, S-4374-VAM-I and -II have generation times of about 48-72 hours. Cell counts of all these cultures were maintained with a wide range of from 100,000 to nearly a million cells/ml. Clones have been developed from primary cultures as well as from established hormone-producing cultures. To prepare permanent stained sections of cells of suspension cultures, cubes of gelatin sponge† (5x5x5 mm) were introduced into cultures from 48-72 hours, and were subsequently fixed and stained with acid fuchsin aniline-blue or iron-periodic acid-Schiff(22) methods. *Frozen-storage of cells and their recultivation* (stage V) was carried out as follows: Samples of line 4374-M from both stationary- and suspension cultures were frozen and after several months were re-established, employing methods of Stulberg *et al.*(23) with the following modifications: Suspension cultures in the log phase of growth were used, and on day before freezing, were fed with nutrient medium containing 15% serum. Glycerol was added prior to freezing, and they were not centrifuged. *Assay of hormones in cultures* (stage VI) employed standard endocrine bioassays.‡ *Somatotropin* was tested by measuring width of tibial epiphysis in the female rat hypophysectomized at 30 days of age. Subcutaneous injections were started 12 days after hypophysectomy and were made once daily for 4 days, with autopsy on 5th day. Culture medium alone was used as control. A reference somatotropin (bovine) was used as

* Stages I - V were carried out at Microbiological Assoc.; Stages VI, VII at Organon where Stage IV is being continued.

† Gelfoam, Upjohn.

‡ With assistance of Endocrine Labs., Madison, Wis.

standard in doses of 25 and 40 μg, 25 μg being smallest dose that caused a clearly positive effect. Each hypophysectomized rat usually tolerated doses of about 200 mg of the acetone-dried preparations, and only about 100 mg of freeze-dried preparations. *Gonadotropin* screening tests were made in 21-day-old female mice, each injected subcutaneously twice daily for 4 days with total dose of 5 or 10 mg of crude preparations, sacrificed at 100 hours then ovaries and uteri were weighed. Additional tests were made in 21-day-old female rats and in 21- and 26-day-old§ hypophysectomized male rats, in which weights of ventral prostate and testis differentiate effects of ICSH and FSH(24). To determine whether observed effects were caused by estrogenic type of substance present in serum of the media, sera and media were tested in castrated immature female rats or in castrated immature female mice, injecting doses larger than or equal to those used in screening tests. *Thyrotropin* was tested in 1-day-old chicks‖ which tolerated doses of 15 mg of acetone-dried harvests. *Corticotropin* was tested by ascorbic acid depletion method in hypophysectomized rats.‖ In harvesting, to prevent enzymatic destruction of hormones, cultures were treated immediately as follows: Some were frozen and aliquots were freshly thawed for each injection; others were frozen, lyophilized and further dried over P_2O_5; and others were precipitated with acetone and precipitates were rewashed with acetone, then ether, dried *in vacuo* and over P_2O_5. The method of choice appeared to be an adjustment of pH to 5 with HCl, followed by acetone precipitation at 92%. Large doses of crude materials had the disadvantage of causing toxic effects and local edema in animals with possibly faulty absorption. To reduce toxicity some samples were extracted(25-27) but the possibility of losses and the cumbersomeness of processing numerous samples led us to rely on tests of crude material in moderate doses.

Results. Hormonal assays of heterogeneous cell cultures. Assays indicated gonadotropin, corticotropin and somatotropin activity was present in some cultures in amounts greater than found in sera contained in the media (Table I). *In selected epithelial cell cultures,*

TABLE I. Hormones Detected in Heterogeneous Cultures.

Culture No.	ACTH	TSH	Gon.	STH
4371-A-23 + A-17	0	0	0	+
4373X-P9	+	0	+	±
4373X-2A	+	0	0	+

gonadotropin and trace amounts of somatotropin were detected (Table II). *In suspension systems,* reached thus far with 15 cell lines, 2 (S4374-M; S4374 VAM-I) showed production of gonadotropin. The gonadotropic action represented combined effects of FSH and ICSH (Tables III to IX). Sera employed in media contained small amounts of uterotropic (estrogen-like) substance (Tables V, VI). Washing of crude powders with acetone and ether did not always eliminate this estrogen-like contaminant. The amount of gonadotropin in harvests such as #051801 was considerably greater than would have been contributed by serum of the media. Gonadotropin was concentrated by ammonium sulfate precipitation followed by ethanol precipitation (Table IX). The amount of FSH present/ml of culture harvest #051801 was approximately that contained in 1 mg of a reference sample of gonadotropin from human menopausal urine (HMG20A). The amount of FSH caused an increase of about 50% in weight of H-rat testes, and this increase we have adopted as our criterion of 1 U of FSH. The amount of ICSH in proportion to FSH in harvest #051801 was roughly the same as as in the menopausal urine preparation, HMG20A, but #051801 had considerably

TABLE II. Hormones Detected in Selected Cell Cultures.

		No. of positive tests/No. of samples tested	
Cell line	Serum	Gonadotropin	Somatotropin
4373-X	Human	+ 1/1	
4374-M	"	+ 3/4	
" -V	"	+ 1/3	
4375	"	+ 1/4	+ 2/5
"	Calf	+ 2/3	

Tests for thyrotropin and corticotropin were negative in all samples tested.

§ Rats 26 days old are preferred to 21-day-old rats because more survive hypophysectomy, and yet are sensitive to gonadotropin.

‖ USP XV.

more FSH than did *Gonadophysin*, a sheep-pituitary preparation. Our criterion of a unit of ICSH is an increase of 100% in weight of the ventral prostate of hypophysectomized 26-day-old rats. The apparent lack of ovarian stimulation with the immature female test animal may be explained by quantities of ICSH found, which may act before the ovarian follicles have had an opportunity to become stimulated by FSH.

Identification of cells (stage VII): Cells of early cultures resembled fibroblasts, but in later cultures epithelial-like types also extended in some areas from the edge of explants. In the heterogeneous cell population stage, the fibroblast type which appeared preponderant showed during early transfers remarkable nuclear changes with increased chromatic material, and there were numerous giant cells and imperfect daughter cells. After further transfers, there was less nuclear activity; the nucleoli became more regular in size

TABLE III. Gonadotropin Tests in #051801, #708901, and #709901. Test animals: 21-day-old female mice, 5/group.

Preparation	Dose total/mouse, mg	Body wt, g	Ovarian wt avg, mg	Uterine wt avg, mg	Vagina
#051801*	4.8	13.4	4.4	36.0	5 open
(S-4374-V4-6)	2.4	12.4	4.8	21.0	3 "
Controls	0	"	4.9	11.3	All closed
#708901† (S-4374-M-47-53)	10	13.2	4.2	35.6	3 open
#709901‡ (S-4374-M-45-51)	10	15.1	4.6	52.1	5 "
Controls	0 (saline)	12.6	4.3	12.5	1 "

* 1 ml of suspension culture of S-4374-V4-6 at the time of this harvest contained 300,000 cells and, when acetone-dried, weighed 10.5 mg. It contained 10% human serum.

† 1 ml of suspension culture at time of this harvest contained 350,000 cells and, when acetone-dried, weighed 9.2 mg. It contained 10% of horse serum (lot #101).

‡ 1 ml of suspension culture at time of this harvest contained 285,000 cells and, when acetone-dried, weighed 9.4 mg. It contained 10% of horse serum (lot #101).

TABLE IV. Gonadotropin Test in #051801. Test animals: 21-day-old female rats, 5/group.

Preparation	Dose total/rat, mg	Body wt avg, g	Ovarian wt avg, mg	Ovarian response	Uterine wt avg, mg	Vagina
#051801	20	64	23.7	A few c.l.* in all	104.5	All open
(S4374-V-4-6)	10	63	17.4	A few c.l. in one	83.8	" "
Controls	0	68	19.9	Follicles in all	31.0	All closed

* Corpora lutea.

TABLE V. Estrogen (Uterotropic) Test. Test animals: castrated, 21-day-old female mice, 3/group.

Preparation	Dose total/mouse, mg	Body wt, g	Uterine wt avg, mg	Vagina	Interpretation
#051801 (gonadotropin	4.8	13.8	29.7	All open	Estrogen +
positive)	1.2	14.3	10.9	None "	—
#081801* (gonadotropin	4.8	14.3	31.2	All "	+
negative)	1.2	13.9	14.1	2 "	+
Controls (saline only)	0	14.3	10.5	3 closed 1 open	—

* This acetone-dried harvest of a culture of a cell line which did not produce gonadotropin, as shown by tests in the intact immature rats and mice, is representative of many which contained small amounts of estrogen-like substance. However, there were many such harvests which did not show positive results in these tests.

TABLE VI. Estrogen (Uterotropic) Test. Test animals: castrated, 21-day-old female rats, 4/group.

Preparation	Dose	Uterine wt avg, mg	Vagina	Interpretation
#051801	50 mg equiv. to 4 ml (about) culture	41.1	All closed	Estrogen ±
"	25 mg equiv. to 2.5 ml culture	27.6	*Idem*	0
Medium* (#050801)	30 mg equiv. to 3 ml culture	25.6	"	0
Human serum†	.25 ml‡	30	"	0
" "	.125 ml	35	"	0
No treatment		33	"	0

* Sterile medium containing 10% of human serum was employed for the culture of #051801, and was dried in the same way.

† This serum was employed in the medium #050801 and the culture prior to time of its harvest as #051801.

‡ This dose is approximately the amount contained in 2.5 ml of culture harvest (10% serum).

and were usually 4 in number. When these cultures were incubated for from 6 to 10 days we usually observed in intercellular spaces filaments of pale blue staining material which resembled collagen with acid fuchsin aniline-blue stain. In most cell lines, at intervals in the heterogeneous stage, we noted clusters or whorls of fibroblast-like cells which tightly enclosed a clump of nuclear chromatic material, the details of which were obscure. We determined that these represented nests of epithelial-like cells, for when these clusters were picked out and subcultured, epithelial-like cells proliferated. Islands of epithelial-like cells which appeared could be easily differentiated microscopically from the fibroblast type.

TABLE VII. Differentiation of Type of Gonadotropin in Harvest #051801. Test animals: hypophysectomized, 26-day-old male rats, 4/group.

Preparation	Dose total/rat	Ventral prostate avg, mg	Testes avg, mg
#051801*	30 mg	48.8	611
	15	31.2	511
	7.5	18.8	439
Human menopausal gonadotropin† HMG20A	2 IU (2 mg)	33.4	567
	1 IU (1 mg)	32.5	390
Gonadophysin (Searle)	2 U	42.2	311‡
	1 U	32.5	390§
Controls (untreated)	0	13.1	358

* 1 ml of harvest #051801 yielded 10.5 mg acetone-dry powder, and contained 300,000 cells.

† Kindly supplied by Dr. J. B. Dekanski of Organon Laboratories Ltd., Newhouse by Motherwell, Lanarkshire, Scotland.

‡ Only 2 animals survived, and 1 of these had unduly small testes.

§ 1 rat had unduly large testes (504 mg) although no remnant of the pituitary could be found. Only 3 rats survived.

TABLE VIII. Differentiation of Type of Gonadotropin in Harvests #708901 and #709901. Test animals: hypophysectomized, 26-day-old male rats, 3/group.

Preparation	Dose total/rat	Ventral prostate avg, mg	Testes avg, mg
#708901	50 mg	19.4	411
	37.5	36.7	324
#709901	50	26.5	390
	37.5	34.4	574
Medium*	5 ml (equiv. to about 50 mg #708901)	14.7	317
"	3 ml	9	265
Serum #101 (horse)	.5 (equiv. to 5 ml medium)	12.1	301
Idem	.3	15.3	266
Controls (saline)		11.7	270

* This medium is a sample of that employed for the cultures that yielded harvests #708901 and #709901 and they all contained serum #101 (equine) in the amount of 10%.

1 ml of #708901 = 9.4 mg.
1 " " #709901 = 9.2 ".

The cell which appears to be typical of the gonadotropin-secreting line (S4374-M), when stained by the iron-PAS method, contains in the cytoplasm oval or round masses which appear pink in bright field illumination and peacock blue in phase. These cells growing on glass frequently extend pseudopods, and they do not remain as firmly attached as do many other types of cells. Under phase microscopy, the unstained living cells of the gonadotropin-producing line of suspension cultures have numerous oval or round highly translucent masses irregularly placed in their cytoplasm which also contains clusters of fine dark granules best visualized by a green or blue filter.

TABLE IX. Partial Purification of Gonadotropin. Test animals: hypophysectomized, 26-day-old male rats, 3/group.

Preparation	Dose total/rat, μg	Ventral prostate avg, mg	Testes avg, mg
#731B901*	400	31.7	473
	120	17.7	383
	75	20.5	348
	0	11.6	280

* #731B901 was prepared as follows: Harvest #051801 (Table VIII) was subjected to ammonium sulfate precipitation, and portion which precipitated between 33% and 75% saturation was freed of ammonium sulfate by dialysis. This solution, clarified, was precipitated with ethanol at 80% concentration. Precipitate was taken up in 0.5 M ammonium acetate solution at pH 5.0 and ethanol was added to a concentration of 40%. Precipitate was removed and ethanol added to 80% concentration. This precipitate was freed of ammonium acetate by dialysis, precipitated with ethanol at 80% concentration, washed with ethanol and ether and dried over P_2O_5. This was sample #731B901.

The nucleus is a clear area in which the nucleoli are seen as dark masses, best visualized with a red filter.

A chromosome count was made with cells of line S4374-M-32B when serially cultured for nearly 8 months. This particular line was started on 7/3/58 with a fragment of human fetal pituitary gland, and was cultivated for 6 weeks in the heterogeneous cell stage, and for 20 weeks in the selected cell stage; thereafter it was in suspension culture for 5 weeks, at which time a division of the culture was submitted for a chromosome count. Bio-assay at this time showed that gonadotropin production was continuing. The count on the fixed, squashed preparation after colchicine treatment(28) was:

Chromosome No.	No. cells	Chromosome No.	No. cells
76	2	79	11
77	1	80	5
78	3	81	3
		Total	25

This was interpreted as unmistakably primate. The cell strain is heteroploid and the presence of a few telocentric or acrocentric chromosomes indicates that chromosome rearrangement has occurred in the strain's history.¶ This chromosome count was repeated 3 months later (after frozen storage and recultivation), with the finding of a high peak at 79-80 chromosomes per cell.

¶ We are indebted to Dr. T. C. Hsu, Univ. of Texas, Houston, Texas, for this interpretation of his chromosome counts.

Summary. Serial cultivation of cells obtained from the human pituitary is described. Somatotropin, corticotropin, and gonadotropin have been detected in these cultures. Two of the cell lines have produced gonadotropin, both FSH and ICSH, when in suspension culture, and one, S4374-M, has been frozen and recultivated in both stationary- and suspension types of culture with media containing 10% of human, equine or bovine serum, with resumption of production of hormone. The gonadotropin-producing cells have the microscopical appearance of a chromophobe type of pituitary cell and have a characteristic appearance when stained by the iron-PAS method. These cells are heteroploid with a chromosome count in narrow range with modality at 79.

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Mechanism of Tumor Inhibition by Estrogen.*† (25266)

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It has been shown that estrogen enhances the development of immunity against transplantable tumors in mice(1) and the postulate has been advanced that inhibition of growth of transplantable tumors during pregnancy might be due to hyperestrogenemia(2). The administration of estrogen also increases production of endometrial secretions (uterone)(3,4) and enhances biosynthesis in the endometrium(5). Since endometrial secretions were shown to inhibit growth of transplanted tumors in mice under certain conditions(6), it was logical to hypothesize that some of the tumor-inhibiting effects observed during induced or physiological hyperestrogenemia might be mediated through the uterus (6). The present paper deals with a study to determine the possible role of the uterus in tumor inhibition by estrogens.

Materials and methods. Forty immature AKD2F_1 (AKS x DBA/2) female mice, obtained from the Jackson Memorial Laboratory, housed in air-conditioned animal rooms throughout the experiment, were fed Purina Laboratory Chow Checkers and tap water *ad libitum*. The animals were divided into 4 groups of 10 individuals, and surgery was performed on all 4 groups (under nembutal anesthesia) according to the protocol shown in Table I. At operation, estradiol benzoate pellets of known weight were implanted subcutaneously in Groups II and IV. All animals were given achromycin in their drinking water for 5 days following surgery, and on the eleventh postoperative day tumor transplants were made. Sarcoma 1 cells from an AKS female host (obtained from Jackson Memorial Lab.) were suspended in chilled Ringer's solution according to the cytosieve method of Snell(7) and adjusted to a high cell concentration (17%) as measured by means of a Wintrobe hematocrit tube. All animals were injected subcutaneously with 0.2 cc of the same tumor suspension. The order of the injections was arranged so that an identical num-

TABLE I.

Groups of 10 mice	Surgical condition	Treatment
I	Intact (sham operation)	
II	*Idem*	Subcut. estradiol benzoate pellet
III	Hysterectomy & castration	
IV	*Idem*	*Idem*

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