Effect of Steroids on Human Cell Cultures; Sustaining Effect of Hydrocortisone.* (28842)

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While studying the effects of various steroid hormones on human cancer cells in tissue culture, it was found that hydrocortisone improved the growth of some cell lines.

Materials and methods. All tissue cultures were established cell lines growing as monolayers on glass, in ordinary culture tubes or Leighton tubes. Incubation was stationary at 35° to 36°C. Nutrient media were those found most suitable for each cell line in our previous experience, since no single medium gave satisfactory growth with all cell lines (Table I). Tubes were planted 24 or 48 hours before steroids were added, using an inoculum of approximately 100,000 cells per tube and sufficient tubes to provide at least 2 for every experimental and control variable. Stock cultures were maintained as monolayers in milk-dilution bottles on the same media as used for the experiment and were passed by standard trypsinization (0.75% trypsin) methods.

Twelve human cell lines were studied. Amnion B(1) was the only cell of non-neoplastic origin. HEp 1 and HEp 2(2) and presumably Detroit 6(3) and RPMI-191(4), are epidermoid carcinoma cells. Ovary 2 and Ovary 3, AdenoCx 1(5), and MAC 21(6) were cultivated from adenocarcinomas of ovary, endocervix, and lung respectively. J-111(7) was grown from peripheral blood of a patient with acute monocytic leukemia. RPMI-212 and RPMI-41(4) are from mesothelioma and osteogenic sarcoma respectively.

The following steroids were studied:

Androsterone (3αhydroxy-androstane-17-one),

Delalutin® (17αhydroxyprogesterone hexanoate),

Droxone® (16α, 17α, dihydroxyprogesterone acetophenide),

Estradiol ($\Delta^{1,3,5(10)}$-estratriene-3, 17β-diol),

Etiocholanolone (3αhydroxyetiocholane-17-one),

Hydrocortisone,

Provera® (6αmethyl-17α-acetoxyprogesterone),

Testosterone.

All steroids were supplied as crystalline preparations without additives. A weighed sample of each steroid, sufficiently large for all contemplated studies, was dissolved in absolute ethanol to give a concentration of 1.0 mg/ml. These solutions were then put into glass ampules in 0.5 ml aliquots and allowed to dry in a covered box at room temperature. Ampules were then flame-sealed and stored in the dark at room temperature. Immediately before use in each experiment an ampule of each of the desired steroids was opened and 0.5 ml of absolute ethanol was added. When completely dissolved a 1:10 dilution was made in complete tissue culture media of the type appropriate to the cell lines to be studied, and from this two further 10-fold dilutions were made in the same nutrient solution. To cultures which had just been refed with 0.9 ml of medium was then added 0.1 ml of one of these steroid solutions in culture media, thus giving final concentrations of 10, 1, or 0.1 γ/ml of culture media. The steroids remained in solution under these conditions as judged by gross and microscopic appearance of the cultures. Ten γ/ml was the highest concentration tested because above this the steroids precipitate out of the aqueous tissue culture medium. However, this is probably well above the concentration expected in body fluids *in vivo* even with administration of pharmacologic doses.

A parallel series of dilutions of pure ethanol in culture media was prepared and added to cultures as controls on the effect of these concentrations of alcohol alone.

Many studies were repeated using concentrations of 1.25, 2.5, 5, and 10 γ/ml. Appropriate alcohol controls were included.

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After the experiment was set up the medium was never replaced or supplemented. The tubes were observed daily for 7 days and every other day thereafter until destruction of both control and experimental cultures was far advanced. The condition of each culture was rated 0 for no cytopathology; ± for minor changes of doubtful significance, + for degenerative changes in scattered cells, ++ for definite cytopathology involving about half of the cells, +++ for advanced degeneration of the cultures but with a few cells remaining attached to the glass, and ++++ for complete destruction. After grading the individual tubes an evaluation of cell damage attributable to the steroid was made by comparing cultures containing steroid against the appropriate alcohol controls. The minimal cytopathogenic concentration was recorded as the lowest test concentration that gave greater cell damage than the appropriate alcohol controls after 7 days of incubation. Other readings were useful for substantiation of the day 7 evaluation.

In most individual experiments either a single cell line was tested against several steroids, or a single steroid was used in several cell lines. However, a few studies were performed with 2 or 3 steroids, each in several cell lines, and in almost all experiments there was repetition of at least one previously tested combination.

Results. All of the cell lines propagated well in culture for at least one week after transplantation, without refeeding. Thereafter early signs of impaired cell nutrition appeared and steadily progressed. There was a considerable variation in the duration of vigorous growth in control cultures in the many experiments, depending both on cell line and culture medium, but in general control cultures showed definite deterioration during the second week, and degeneration was usually complete by 3 or 4 weeks.

The alcohol toxicity controls usually grew just as well as the complete controls, even at the highest level tested (1% initial concentration). The exceptions were Ovary 2 and Ovary 3 which showed up to ++ damage, and RPMI-212 and RPMI-191 which showed + damage, in some experiments after 5 to 7 days in media with 1% alcohol. The only cell line showing damage in media containing 0.5% alcohol was Ovary 2, and none showed evidence of toxicity from 0.25% alcohol. Since toxicity from these concentrations of alcohol appeared late, if at all, it was probably due to effects on cell nutrition rather than direct cytotoxicity.

None of the tested steroids caused immediate cell damage at the maximum concentration studied (10 γ/ml). When toxicity did occur it was first evident after 3 to 7 days of incubation. Such cultures showed an earlier and more rapid deterioration, but the type of damage was indistinguishable from that eventually seen in control cultures, as judged simply by microscopic observation of the living cultures.

Minimal cytopathogenic concentrations of the 8 steroids on the 12 cell lines are summarized in Table I, based on the 10-fold series of concentrations. Results at intermediate doses are omitted here (see below) because such data were not obtained routinely.

Reproducibility in repeated tests was generally good, but there were inexplicable discrepancies with AdenoCx 1 in Delalutin, Ovary 3 in testosterone, and Ovary 3 in hydrocortisone.

Among the 30 tests in which 2-fold dilution steps were used for the steroid concentrations, only 7 showed a different end point than was found with the 10-fold dilution steps. Six of these were only 2-fold differences (toxicity at 5 γ/ml), but Droxone was toxic for Ovary 3 at 2.5 γ/ml.

Of the 80 combinations of steroids and cells tested, 31 showed no cytotoxicity at 10 γ/ml, the highest steroid concentration tested, and 43 showed cytotoxicity at 1 γ/ml. None showed toxicity at 0.1 γ/ml. No consistent pattern of cytological, endocrinological, or chemical characteristics was discernible (Table I) that could be related to relative cytotoxicity of these steroids for these cell lines.

Comparison of the various steroids showed that testosterone and androsterone were toxic for the greatest number of cell lines at the 10 γ/ml level. Hydrocortisone was the

least frequently toxic—only 3 lines (the adenocarcinomas of ovary and endocervix) showing cytotoxicity at the 10 γ/ml level.

Comparison of the several cell lines revealed that the 3 gynecological adenocarcinomas and the mesothelioma were the only cell lines consistently damaged by all of these steroids at the 10 γ/ml level. The mesothelioma (RPMI-212) was most sensitive, but this observation is based on only one study. Detroit 6 showed no evidence of toxicity from any of the steroids at the 10 γ/ml concentration.

The most striking observation was the sus-

TABLE I. Minimal Cytopathogenic Doses (γ/ml) of Steroids in Tissue Cultures. (See text for criteria and technics.) Each entry indicates result of one experiment.

Cell line	Nutrient medium*	Estradiol	Provera	Delalutin	Droxone	Testosterone	Androsterone	Etiocholanolone	Hydrocortisone
Amnion B	20% calf in E	>10	10,10	>10	10	10,10	10,10	>10	>10
HEp 1	"	>10	10	>10	10	10	>10	>10	>10,>10
HEp 2	"	10	>10			>10,10	10		>10
Detroit 6	"	>10	>10	>10	>10	>10	>10	>10	>10
RPMI-191	20% calf in P						10		>10
AdenoCx 1	10% human in E	10	10,10	>10,10, 1	10	10,10	10,10	10,10, 10	10,10, 10
Ovary 2	"	10		10		10	10	10	10,10
Ovary 3	"	1	10	>10,10, 10		10,1	10	10	10, 1
MAC 27	"		>10	>10,10		10	10	>10	>10
J-111	20% calf in E	10,10	>10,>10, 10	>10,>10	10,10	>10,>10, >10	>10,10, 10	>10,>10	>10,>10
RPMI-212	20% calf in P	1	10	1		1		1	>10,10
RPMI- 41	"		10	1	10	10	10	10	>10,>10, >10,>10, >10

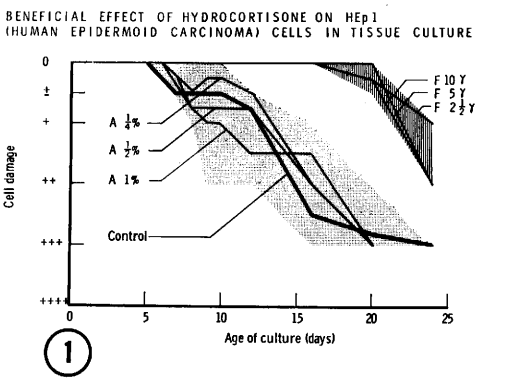
* Calf serum was either fetal calf serum or a-gamma calf serum from Hyland Laboratories, Los Angeles, Calif. Human serum was a commercial pool supplied by Hyland Laboratories. E indicates Eagle's basal medium with glutamine and vitamins (Baltimore Biological Laboratory, West Chester, Pa.). P indicates Puck's N-16 medium (Cappel Laboratories, West Chester, Pa.).

taining effect of hydrocortisone on 2 of the cell lines, RPMI-41 and HEp 1. In these initial survey tests these cultures remained in excellent condition without refeeding for more than 21 days whereas all controls, with or without alcohol, were deteriorating by day 14. A repeat test with HEp 1 using 2-fold dilution steps and 8 tubes for each variable confirmed the observation (Fig. 1). Eight separate studies were made with RPMI-41 and hydrocortisone. The steroid never caused toxicity, and the "sustaining" effect at 5 or 10 γ/ml was observed in 6 of the 8 studies. The duration of cultures in excellent condition, or to the development of ++ cytopathology was extended from 25 to 100% as compared with controls. One "blind" study was carried out with 6 replicate tubes for each variable. The experiment was set up in the usual manner and appropriately labeled by one person. Code numbers were then selected from random number tables by a second person and substituted for the original labels. The tubes were then rearranged into numerical order. Daily observations were made independently by the original investigator and a third person. The code was not revealed until the 30th day of observation and the results presented in Fig. 2 were obtained.

Discussion. Action of steroid hormones on cells in tissue culture has been studied previously in a variety of experimental conditions, but no general pattern of effects has emerged.

Gillette and Buchsbaum(8) found marked cytological changes in chick and mouse fibroblasts exposed to 35 to 70 γ/ml of 11-desoxycorticosterone or 17-hydroxy 11-desoxycorticosterone ("compound S"). Grossfeld and Ragan(9) reported reversible inhibition of chick heart fibroblasts by 200 to 500 γ/ml of hydrocortisone in the tissue culture medium, while growth of intestinal epithelial cells was stimulated. Geiger *et al*(10) reported a reversible inhibition of dog fibroblasts by cortisone at 10 to 100 γ/ml.

We have found only two previous reports of steroid hormones in human cell cultures. Kline *et al*(11) studied the response of 4 human cell lines to hydrocortisone at concentrations of 10, 100, and 1000 γ/ml. They



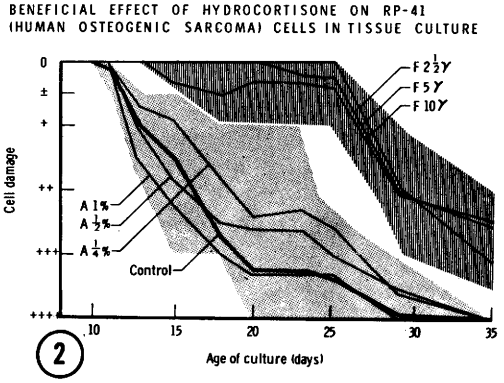


FIG. 1. Sustaining effect of hydrocortisone on growth of HEp 1 cells (human epidermoid carcinoma of Cervix) in tissue culture. Experimental media were added on day 0 and were never removed nor replenished. Curves indicate average rate of development of cellular degeneration. (Eight cultures for each experimental variable.) F and A refer to hydrocortisone and ethanol concentrations, respectively. Shading indicates extreme range of all cytotoxicity readings in control and hydrocortisone-treated groups.

FIG. 2. Sustaining effect of hydrocortisone on growth of RPMI-41 cells (human osteogenic sarcoma) in tissue culture. Each curve is the average of 6 cultures. See legend of Fig. 1 for other details.

observed no acute cytotoxicity, but after 3 or more days some degree of cell damage occurred in all cell lines at all dose levels. A fibroblastic line (D-189) showed earlier, more severe, and more persistent damage than the 3 epithelial lines. Wheeler *et al*(12), studying the effect of hydrocortisone on production of herpes simplex virus in HeLa cell cultures, noticed that hydrocortisone delayed and decreased non-specific degenerative changes in the uninfected cultures. This is probably the same phenomenon which we have observed with HEp 1 and RPMI-41.

We have not investigated the mechanism of this "sustaining effect." Casual examination of the living cultures did not reveal any slowing of growth due to hydrocortisone, but this possibility deserves investigation since conditions which retard cell metabolism may improve longevity of cultured cells when the medium is not renewed(13), and inhibition of mitotic rate by about 50% has been reported with prednisolone at 1 to 10 γ/ml in short term human leukocyte cultures(14).

The initial objective of these studies was to see if the synthetic progestins would cause a selective inhibition of the AdenoCx 1 cell line, since these drugs have clinical value in treatment of endometrial cancer(15) and this cell line is presumably a neoplastic endometrial (endocervical) cell. No such selective effect was observed, but generalization must be avoided since the exact nature of this cell line is not certain and the clinical responsiveness of the patient from whom it came is unknown.

Summary. The effect of 8 steroid hormones on 12 lines of human cells was studied in tissue culture. The steroids included estrogens, androgens, progestins, and hydrocortisone. The cell lines included amnion, epidermoid carcinomas, adenocarcinomas, and sarcomas. Cytotoxic effects were not acute, but developed after several days if at all. No consistent relationship was apparent between minimum cytotoxic dose and endocrinologic characteristics of the steroids or type of cell. Testosterone and androsterone were toxic to most cell lines at 10 γ/ml. The ovarian and cervix adenocarcinoma cells were sensitive to most of the steroids at 10 γ/ml. Toxicity at 1.0 γ/ml was rare. Hydrocortisone at 2.5 to 10 γ/ml greatly extended the duration of good growth in cell lines HEp 1 and RPMI-41.

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Influence of Repeated Subtotal Hepatic Resections on *in vitro* Survival of Rat Liver Cells. (28843)

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Subtotal hepatic resection in rats is followed by rapid regrowth of liver tissue, apparently due to an increased rate of cellular division in the hepatic remnant(1). Such liver regeneration has been studied by changes in gross liver size(2), liver weight (3) and liver DNA and RNA content(4,5, 6). Rate of liver regrowth varies inversely