ANALYSIS OF THE GROWTH-INHIBITORY EFFECTS OF HYDROCORTISONE ON A MOUSE LYMPHOMA CELL GROWING *IN VITRO*

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WE HAVE previously described a growth-inhibitory effect of certain steroids when assayed upon a mouse lymphoma cell line, ML-388, cultured *in vitro*[1]. The assay system consists of inoculating about 2×10^5 cells in 20 ml, of culture medium into 8-oz prescription bottles, and adding the drug one day later to give the final desired concentration. The steroid is added in 0·2 ml, of a dilute ethanol solution, such that the final concentration of ethanol in the culture medium is never more than 0·05%. Appropriate vehicles added to control flasks had no effect on cellular growth at ethanol concentrations of 0·05% or less. The culture medium consists of Eagle's mixture of salts, vitamins, amino acids, glucose and calf serum[2]. In addition, pyruvate and serine are added for optimal growth. After five days, the supernatant medium is poured off and the cells, which adhere to the floor of the bottle, are suspended by scraping into a known volume of salt solution. They are then counted in an electronic cell counter. Control cultures uniformly multiply 20- to 30-fold under these conditions.

The effects of three active steroids of widely different potency are presented in Fig. 1. It may be seen that the IC_{50} (the concentration of steroid necessary for 50% inhibition of growth) for hydrocortisone is slightly less than 1×10^{-7} M. Many other steroids also possess growth-inhibitory effects. The relative ranking of active steroids is very similar to the relative activity observed in a variety of other bioassay systems such as an anti-inflammatory assay, a glycogen deposition test, or a thymic involution bioassay[3].

Many other steroids are inactive in our system (i.e. they have an $IC_{50} > 10^{-5}$ M). Among the inactive steroids are the sex steroids, 11-keto-steroids, and 11-epihydrocortisone, which differs from hydrocortisone only in the orientation of the hydroxyl group at the 11 position.

It has been possible to develop sub-lines of this cell strain that are resistant to the steroid effect. After incubating cells in a sub-lethal concentration of hydrocortisone for a period of four weeks, a cell line

emerges which grows at a rapid rate in the presence of hydrocortisone concentrations as high as 10^{-5} M. These cells maintain their resistance even after growing for two months in the absence of any added drug, indicating that the emergence of a drug-resistant cell line is probably a mutation-selection phenomenon. These resistant cells were found to be crossresistant to all other active steroids tested.

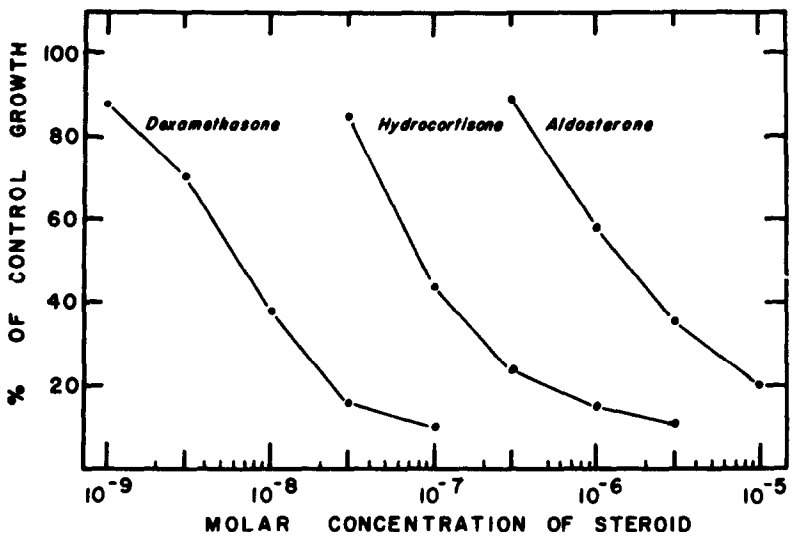

FIG. 1. Effects of steroids on *in vitro* growth of ML-388 cells. The steroids were added the day after inoculation of replicate cultures in 8 oz prescription bottles as described in the text. Each point represents the mean of a group of three bottles.

We next turned our attention to experiments attempting to explore the mechanism of action of steroids inhibiting the growth of these cells. Carbohydrate, lipid, protein and nucleic acid metabolism were examined by measuring these parameters along with cell counts, over a growth period of several days, in the presence and absence of added steroid.

The procedure used was to inoculate as many as 200 replicate 8-oz prescription bottles with cells. At the desired times, a group of bottles was removed from the incubator. Cell counts were obtained individually on 4 bottles as already described. The remaining bottles, after pouring off the supernatant medium, were rinsed with a balanced solution. Four of these washed bottles were utilized for another cell count determination, since the washing procedure resulted in the loss of some cells from the wall of the bottle. The cellular material in the remaining bottles was chilled and treated according to the flow diagram in Fig. 2. The actual number of bottles used depended on the number of cells present. Thus, the day after inoculation, when the cell count is about 2×10^5 cells per bottle, 20–30 bottles were required to provide enough

material for the chemical analyses. After 5 or 6 days of growth, however, 6 bottles provided ample material.

Chemical assays were performed in duplicate on the following final extracts: the *medium,* which was assayed for lactate[4] or glucose by the glucostat procedure[5]; the *cold-acid soluble fraction,* which was assayed for total ninhydrin reacting material[6] as a measure of the free amino acid

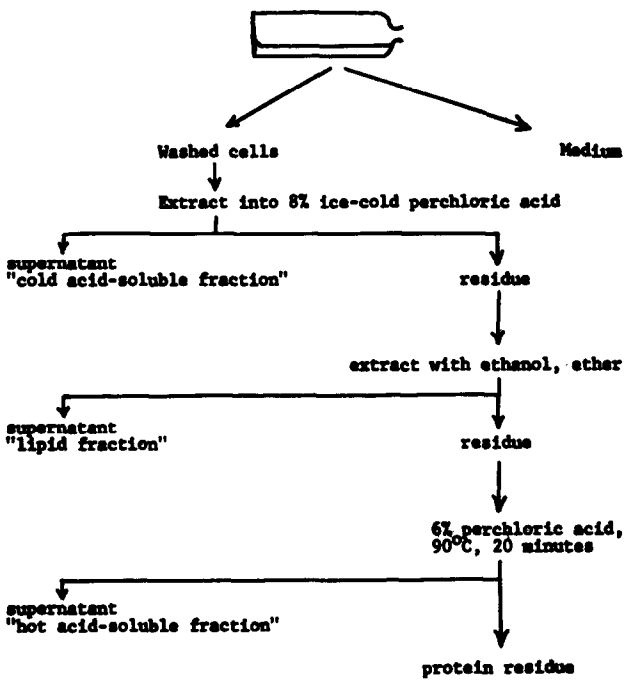

FIG. 2. Fractionation procedure for cells grown *in vitro.* The lipid extraction is performed with succesive portions of 5 ml of ethanol-water (4 : 1 by volume); ethanol-ether (3 : 1); and ether.

pool in the cell; the *lipid fraction,* which was analyzed for cholesterol[7] employing the ferric chloride-sulfuric acid color reaction; the *hot-acid soluble fraction,* which was analyzed for ribose[8] and deoxyribose[9] as measures of RNA and DNA; and a final *protein residue,* which was analyzed for cellular protein[10]. Results can be expressed either as accumulation of each of these materials with time per bottle or they can be related to the amounts of each of these materials per cell as growth proceeds.

Fig. 3 illutrates the results of such an experiment in which we examined the effects of 5×10^{-6} M hydrocortisone. Control bottles and hydrocortisone-treated bottles were done simultaneously. It may be seen that within 12 hr cell count was depressed slightly in the hydrocortisone-treated group and they subsequently failed to grow as rapidly

as the controls. The amount of DNA per cell stayed relatively constant. Ribonucleic acid, protein, and the amino acids appearing as total ninhydrin-reacting material in the cold acid-soluble fraction, were depressed as early as 12 hr. This depression was both in total amount per bottle, and also when calculated on a per cell basis. Results of the glucose,

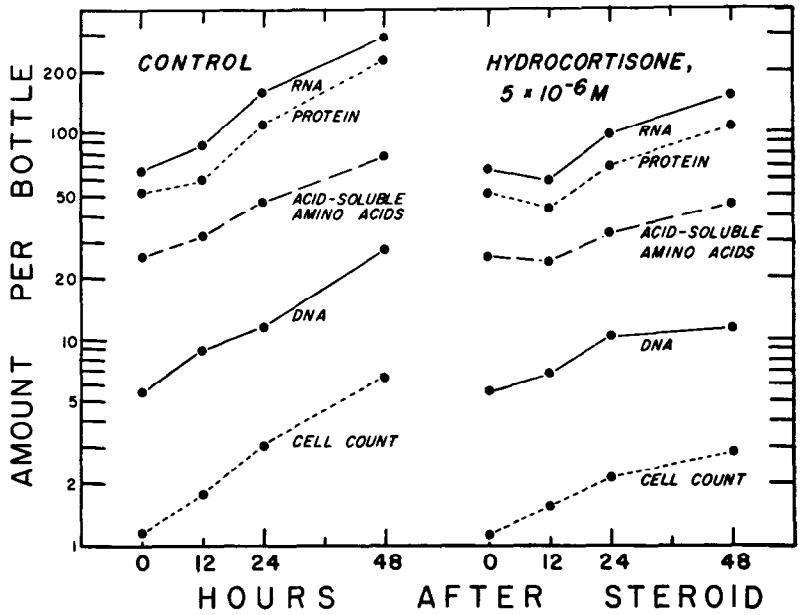

FIG. 3. Effects of hydrocortisone on protein and nucleic acid accumulation in cultures of ML-388 cells growing *in vitro*. Cell counts are the means of groups of 4 bottles at each time. The ribose, deoxyribose, protein, and acid soluble amino acids are values obtained from pooled groups of bottles, as described in the text. The units are as follows: cell counts $\times 10^{-5}$; DNA — mμ moles deoxyribose in hot acid-soluble fraction; RNA — mμ moles ribose in hot-acid soluble fraction; amino acids — mμ moles of ninhydrin-reacting material (as leucine-equivalents) in cold acid-soluble fraction; protein — μg protein nitrogen.

lactate and cholesterol assays are not presented since these values showed no early differences between control and treated groups. We believed then, from these results, that one of the earliest effects of the steroid was to inhibit protein synthesis, perhaps by decreasing the pool of amino acids available.

We attempted to verify this hypothesis by incubating cells in radioactive leucine, so that they incorporated some radioactivity as ^{14}C-leucine into their cellular protein. The cells were then washed in normal medium containing no radioactive amino acid, allowed to continue growing, and subsequently hydrocortisone was added. It could be demonstrated that within 12 hr of steroid addition, the treated cells failed to synthesize

as much new protein as did control flasks, since the specific radioactivity of the cellular protein of the hydrocortisone-treated group did not decline as rapidly as that of the control group. This difference in protein specific activity could not be due to an inhibition of protein turnover, since at the same time the steroid-treated cells released as much (or more) radioactive leucine into the culture medium as the control group.

Since we had already shown that the amino acid pool available to these cells in the cold acid-soluble fraction was depressed upon treatment with hydrocortisone, we decided to analyze this fraction a little more closely. Individual amino acids were analyzed on a Beckman-Spinco automatic amino acid analyzer according to the procedure of Spackman *et al.*[11]. Cells were grown out as previously described, and 12 hr after the addition of hydrocortisone at 5×10^{-6} M, the cells were harvested and fractionated according to the procedure already described.

It was found that the quantity of most of the amino acids per cell, were the same as control values 12 and 24 hr after hydrocortisone treatment, with three exceptions. These were the two dicarboxylic amino acids, aspartate and glutamate, which were depressed, and alanine, which was higher in the steroid-treated group than in the controls. Since these are the amino acids involved in transamination reactions, it is of interest to recall that Blecher and White[12] reported a stimulation of transamination reactions in the lymphoid tissue after steroid treatment, and more recently, Rosen and coworkers[13] have reported increases in transamination reactions of lymphatic tumor tissue in mice after steroid treatment. Our finding of a decrease of the glutamic acid concentration and an increase in the alanine concentration within the cell is consistent with a stimulation of activity of the transaminase system. Such an effect on the activity of the transaminase reaction would seem to be sufficient to explain alterations in intracellular amino acid concentrations, without postulating a steroid effect on cellular uptake of amino acids.

In summary, a new bioassay for anti-inflammatory steroids is described, which involves the growth-inhibitory effect of these steroids on a mouse lymphoma cell line growing *in vitro*. Several synthetic compounds are many times as active as hydrocortisone, while other steroids are devoid of activity. A drug-resistant variant of this cell line has been isolated *in vitro*, and it has been shown that the resistant variant is cross-resistant to all other active steroids. We have not been able to describe definitively the mechanism of action of the steroid-induced growth-inhibition, but we have at least shown that there is very early inhibition of protein synthesis upon steroid treatment, and this is apparently accompanied by early effect on transaminase enzymes within the cell, leading to increased levels of intra-cellular alanine, and decreased levels of aspartic and glutamic acid. We do not know in just

what way, if any, this is related to the growth-inhibitory effect of these steroids.

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