EFFECT OF CORTICOSTEROIDS ON THE FORMATION OF ALKALINE PHOSPHATASE IN HELA CELLS

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Studies of the enzymatic make-up in established lines of mammalian cells have revealed remarkable similarity among cells of diverse origins[1]. Alkaline phosphatase was found to constitute an exception since it varied in activity not only from one strain to another but also within the same strain of cells, depending upon the clone being studied[2,3]. Although little is known about the factors controlling the levels of this enzyme in cell cultures, a recent study of Cox and MacLeod[3] has shown that prednisolone increased the level of alkaline phosphatase in HeLa cells. The work reported in this communication primarily involved a study of the relationship between the steroid structure and its ability to stimulate alkaline phosphatase.

Experimental

HeLa cells were grown in tubes for six days at 37° in lactalplus medium[4] supplemented with 10 per cent calf serum and various levels of steroid. The cultures were started from an inoculum of 10^5 cells in one ml per tube. After three days of incubation the medium was replaced and the cells were incubated for another three days. At the end of the incubation period the medium was withdrawn by suction, the glass-adherent cells were washed three times with 5 ml of balanced salt solution and drained. To each tube was added 2.0 ml of 0.5M glycine buffer (pH 9.1), 0.5 ml. of 0.05M $MgCl_2$ and 0.4 ml of distilled water. The tubes were shaken on a reciprocal shaker at 37° for 30 minutes, and 0.5 ml of the substrate solution containing 1.0 mg of p-nitrophenylphosphate

(California Corporation for Biochem. Res.) was added to each tube. Shaking was continued for another 30 minutes and the reaction was stopped by adding 10 ml of 2 per cent Na_2CO_3 in 0.1N NaOH to each tube. The concentration of the liberated p-nitrophenol was determined colorimetrically at 410 mμ. Protein was determined in duplicate cultures by the method of Oyama and Eagle[5]. Alkaline phosphatase activity was expressed in terms of arbitrary units, each unit being equal to the number of μmoles of p-nitrophenol liberated in 2 hrs. per mg of cell protein. No differences in activity were observed when, instead of cells adherent to glass, sonicated cells or cell suspensions were used as the source of enzyme.

A list of steroids tested for the stimulation of alkaline phosphatase is shown in Table I. All steroids were tested at levels ranging from 10.0 to

Table I

Steroids Tested for Stimulation of Alkaline Phosphatase in HeLa Cells

Stimulatory	9α-Fluorohydrocortisone Dexamethasone Medrol 6α-Fluoroprednisolone Hydrocortisone 9α-Fluoro-16α-hydroxyhydrocortisone-16α,17α-acetonide Prednisolone 9α-Fluoro-16α-hydroxyhydrocortisone 1,2-Dehydro-9α-fluoro-16α-hydroxycorticosterone Hydrocortisone-21-acetate 2α-Methylhydrocortisone-21-acetate Triamcinolone 14α-Hydroxyhydrocortisone
Slightly stimulatory	9α-Fluorocorticosterone
Nonstimulatory	11ξ-Hydroxyhydrocortisone 9α-Fluoro-6β,16α-dihydroxyhydrocortisone-16α,17α-acetonide 9α-Fluoro-11β-hydroxyprogesterone 9α-Fluorocortisone 11-Desoxycorticosterone Corticosterone Cortisone Prednisone Progesterone Reichstein's Substance S 21-Deoxy-9α-fluoro-16α-hydroxyhydrocortisone 6α-Hydroxyhydrocortisone 11-Epi-hydrocortisone 20β-Dihydro-9α-fluorohydrocortisone 6β-Hydroxyhydrocortisone Testosterone

0.01 μg/ml. Since only ten-fold dilutions were used, definite peaks for optimal activity were not obtained. Steroids that stimulated alkaline phosphatase by 300 per cent or more were classified as "positive". Only one compound (9α-fluorocorticosterone) gave stimulation of an intermediate order (230 per cent). While all of the "positive" steroids are known to have high glucocorticoid activity, those classified as "negative" are either weakly active as glucocorticoids or are inactive. Cortisone and its derivatives that lack hydroxyl in the 11 position constitute an exception since they did not stimulate alkaline phosphatase, in spite of their glucocorticoid properties *in vivo*. Since hydrocortisone is reported to be the active form of cortisone[6], HeLa cells probably do not have the capacity to reduce cortisone to hydrocortisone. The nonsteroid anti-inflammatory agents (acetylsalicylic acid and phenyl butazone) were tested in conjunction with the steroids and were found to be inactive.

In order to examine the long-term effects of steroids in tissue culture, the following experiment was set up: Three cultures of HeLa cells from the same inoculum were planted in bottles. Two cultures (B and C) were grown in the presence of 1 μg/ml of prednisolone, the third culture (A) was kept as a control. Transfers were made after three days of incubation, at which time alkaline phosphatase and total cell protein were determined. One fourth of the total harvest was used as inoculum for the next culture. After the third consecutive transfer, prednisolone was omitted from culture B. As shown in Figure 1, the initial high level of alkaline phosphatase which resulted after the first exposure of the cells to the steroid declined rapidly on successive transfers. Omission of prednisolone (culture B) slightly accelerated this progressive decline. Possible explanations, based on accumulation of prednisolone to a toxic level, steroid inactivation, and selection of steroid-insensitive cells, are being explored.

In order to determine whether prednisolone affects enzyme activity, alkaline phosphatase was determined in cells assayed in the presence of this

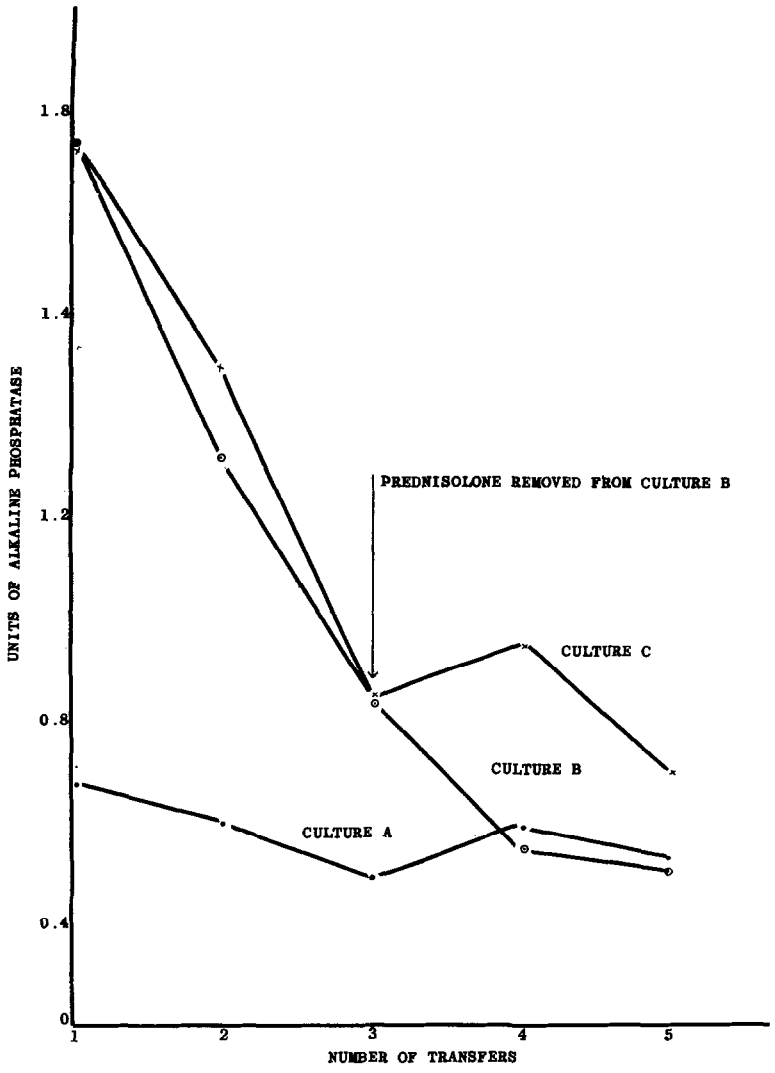

Figure 1

EFFECT OF PROLONGED CULTIVATION IN THE PRESENCE OF PREDNISOLONE ON ALKALINE PHOSPHATASE ACTIVITY IN HeLa CELLS

steroid. As may be seen in Table II, the presence of steroid was essential during cell growth. No stimulation was observed if prednisolone was added to the reaction mixture during the enzyme assay.

It is hoped that inhibitors might offer some information concerning the mechanism of alkaline phosphatase formation. Among several studied, only iodoacetate and epinephrine were found to be active at levels which were nontoxic to cell growth (Figure 2). At the levels indicated in the figure neither of these two inhibitors had any effect on alkaline phosphatase activity.

Table II

Effect of Prednisolone on the Activity of Alkaline Phosphatase in HeLa Cell Sonicates

Steroid present in the medium during growth (1 μg/ml)	Units of Alkaline Phosphatase	
	Control	1 μg prednisolone/ml during assay
Cortisone	0.51	0.53
Hydrocortisone	1.68	1.68
Prednisolone	1.73	1.73
Control	0.50	0.50

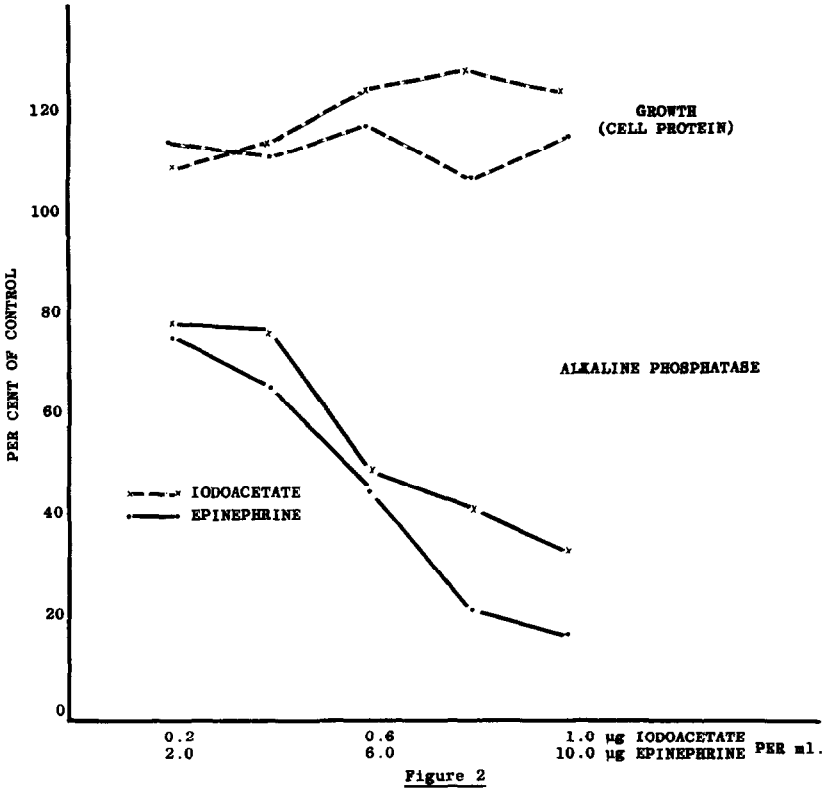

Figure 2

EFFECTS OF IODOACETATE AND EPINEPHRINE ON PREDNISOLONE STIMULATION OF ALKALINE PHOSPHATASE IN HeLa CELLS

In addition to increased alkaline phosphatase levels in HeLa cells grown in the presence of glucocorticoids, these cells were larger, had a tendency to form cell aggregates after trypsinization, and did not lower the pH of the medium

as rapidly as did control cells. At present, interpretation of these observations is not possible. It is not known whether the stimulation results from an increased de novo synthesis, is caused by an activation of an inactive enzyme precursor, or is the result of prevention of the continuous leakage of the enzyme into the surrounding medium from the surface layers of the cell.

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