ALKALINE PHOSPHATASE INDUCTION IN HELA CELLS BY PREDNISOLONE: REVERSAL BY CORTEXOLONE

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During the last few years, work originating from several laboratories has revealed remarkable correlation between the antiinflammatory effects of several steroids and their ability to inhibit growth of cells in culture. Most frequently such *in vitro* effects are measured in terms of toxicity to cultured fibroblast lines (Pratt and Aronow, 1966) or to various cells of lymphatic origin (Cardoso, 1961; Burton *et al*. 1967). Another potentially valuable system for studying glucocorticoid activity *in vitro* is the induction of alkaline phosphatase in several heteroploid cell lines (Cox and McLeod, 1962). Its greatest advantage is the apparent lack of gross toxicity of steroids to the cells, making possible detailed investigations of several biochemical and morphological parameters affected by this group of hormones (Melnykovych and Bishop, 1967, Melnykovych *et al*. 1967).

Structural requirements for the induction of alkaline phosphatase in HeLa cells include, among others, the presence of the OH group in position 11 of the steroid molecule. Thus, cortisone and its synthetic 11-keto analogs (e.g. prednisone) are inactive (Melnykovych, 1962). This is understandable in view of the limited ability of the established cell lines to reduce the 11-keto position to 11-hydroxy (Ruhmann and Berliner, 1965).

In his search for a specific cellular site of action of cortisol, Munck (1965) has found that the specific effect of a steroid on isolated thymic

cells depends on the structural integrity of the cell preparation. For example, deoxycorticosterone which shows negligible in vivo glucocorticoid activity is similarly inactive toward intact thymic cells. However, in thymic preparations damaged during isolation, the activity of deoxycorticosterone in inhibiting glucose uptake is comparable to that of cortisol. It should be noted that deoxycorticosterone has no inducing activity on alkaline phosphatase because it lacks hydroxyl groups in the 11- and 17-positions, both of which are necessary for this type of activity (Melnykovych, 1962).

Munck's recent report (1968) indicating possible antagonism between cortisol and cortexolone (11-deoxycortisol, Reichstein's substance "S") caused us to examine the effects of this compound on the induction of alkaline phosphatase. We have found that cortexolone, when added simultaneously with prednisolone, partially prevents the increase in the level of alkaline phosphatase. The specificity of this effect is indicated by similar antagonism between cortexolone and other steroids which induce alkaline phosphatase in HeLa cells.

Materials and Methods

The HeLa-S3 line was kindly furnished to us by Dr. M. J. Griffin from the Department of Biochemistry at the University of Oklahoma. The cells were grown in Eagle's MEM medium supplemented with 10 per cent calf serum. In the induction experiments prednisolone and other steroids were added to the cultures at the time of inoculation. The cultures were incubated for 3 days at 37°. Cell monolayers were then washed with balanced salt solution and the cells were lysed with 1 per cent sodium deoxycholate. The methods for alkaline phosphatase and protein determinations have been previously described (Melnykovych and Sylber, 1966).

Results and Discussion

In Table 1 are shown the results of an experiment performed in test tube cultures. At the highest level of cortexolone (29.5×10^{-6} M) the inducible alkaline phosphatase was inhibited by about 72 per cent, while no effect was

Table 1

Cortexolone ($M \times 10^{-6}$)	Prednisolone (1.4×10^{-6} M)		Control	
	Protein (µg/culture)	Alk. Phosphatase (Sp. Activity)	Protein (µg/culture)	Alk. Phosphatase (Sp. Activity)
29.5	204	0.31	203	0.20
5.8	251	0.80	219	0.21
1.2	242	1.03	211	0.21
0	234	1.13	159	0.22

Table 1. Suppression of alkaline phosphatase induction by cortexolone. The values represent averages of triplicate cultures inoculated with 100×10^3 cells per tube and incubated for 3 days. Alkaline phosphatase activity determined as described by Melnykovych and Sylber (1966).

seen on the basal level of the enzyme in the control series of tubes. A similar reversing effect was evident in the flask culture experiment where, in addition to prednisolone, two other steroids were used to induce alkaline phosphatase. They were 9α-fluoroprednisolone which is a stronger inducer than prednisolone and cortisol which has considerably lower inducing potency. The results (Table 2) show that cortexolone is much less effective with more potent inducers than with the weaker ones. Thus in the 9α-fluoroprednisolone series at 0.28×10^{-6} M concentration, activity in the cortexolone treated culture was almost three times that of the basal level while at the comparable concentration much less activity was present in the prednisolone and cortisol series. At the lowest concentrations of inducing steroids (0.06×10^{-6} M), the enzyme activity in the cortisol series was reduced by cortexolone almost to the basal level, while considerable activity was evident in both the prednisolone and

Table 2

Inducing Steroid	Concentration ($M \times 10^{-6}$)	Alkaline Phosphatase (Sp. Activity)	
		Cortexolone (29.5×10^{-6} M)	Control
9α-Fluoroprednisolone	1.40	1.42	3.27
	0.28	1.17	2.74
	0.06	0.71	2.21
Prednisolone	1.40	0.75	2.40
	0.28	0.72	2.34
	0.06	0.72	1.13
Cortisol	1.40	0.76	2.07
	0.28	0.73	1.26
	0.06	0.53	0.76
None	0.0	0.46	0.43

Table 2. Effects of cortexolone on the alkaline phosphatase induction by three steroids with different degrees of glucocorticoid activity. Experimental conditions similar to those described in Table 1, except that T-30 flasks inoculated with 0.5×10^6 cells were used instead of test tubes.

9α-fluoroprednisolone series. These results seem to support the assumption that the biological activity of glucocorticoids reflects their affinity for the binding sites on the cell. Similarly it may be postulated that the inhibitory effect of cortexolone is based on its ability to compete for those sites.

We have reported previously that prednisolone induces alkaline phosphatase only when added to the culture during the early phases of growth (Melnykovych, 1966). In order to test whether a similar relationship may be shown with the

reversing activity of cortexolone, the following experiment was done. Duplicate flask cultures were set up either with or without prednisolone. Cortexolone (29.5×10^{-6} M) was added to two flasks in each of the two series at the beginning of incubation, after one day, after two days, or on the third day immediately preceeding harvest. The average number of units of alkaline phosphatase in each pair of cultures was plotted as a function of time of exposure to cortexolone. As may be seen in Figure 1, the effect of cortexolone on alkaline phosphatase was less pronounced when it was added after one day of incubation. Essentially no effect was observed when cortexolone was added one day preceeding the harvest (i.e. after two days of incubation) or immediately before the experiment was terminated. Similarly, in subsequent experiments, no effect of cortexolone was demonstrated when the inhibitor was added to the alkaline phosphatase assay mixture.

The structural specificity of the cortexolone effect is now being investigated in detail. Thus far, all of the C21, 11-deoxycorticosteroids tested,

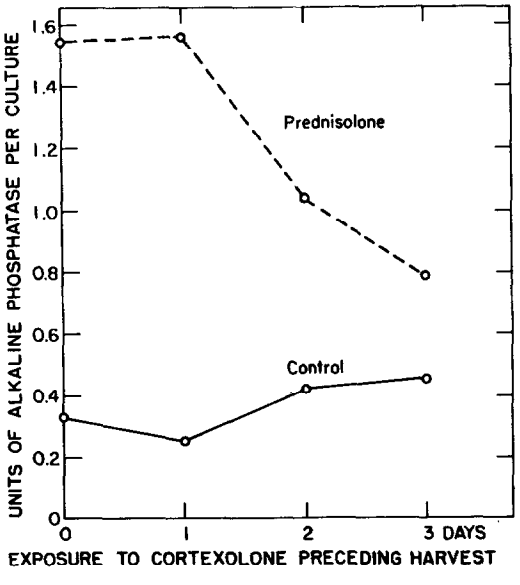

Figure 1. Effect of time of exposure to cortexolone on the inhibition of alkaline phosphatase induction in HeLa cells. For details of this experiment see text.

including progesterone, have been shown to have some reversing activity on prednisolone stimulated alkaline phosphatase. Similarly active were those 11-OH steroids which because of the lack of a hydroxyl in position 17 do not induce alkaline phosphatase (e.g. corticosterone). In contrast, 11-keto steroids, such as prednisone and cortisone were inactive. These preliminary findings support Munck's contention that the specific binding of the glucocorticoid requires 11β-OH or 11-deoxy structure.

The implications of this finding are manifold. As an immediate prospect they offer encouragement to the search for specific glucocorticoid-binding sites by providing another criterion for study of the binding specificity as related to biological activity. This is particularly important in view of the observation that both the inducing effects of prednisolone and the reversal of the induction by cortexolone are somehow related to the growth cycle. The more distant promise is the potential contribution of this study to the understanding of the emergence of steroid resistant clones in originally susceptible populations, both *in vitro* and *in vivo*. It seems that effective analogs of glucocorticoids may arise from several hydroxylation and dehydrogenation reactions leading to interference with the chemotherapeutic effects of these compounds.

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