Biochemical Pharmacology, 1965, Vol. 14, pp. 1065–1072. Pergamon Press Ltd., Printed in Great Britain.

EFFECT OF GLUCOCORTICOID STEROIDS ON CELLS IN TISSUE CULTURE*

ALEXANDER PIHL and PER EKER†

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway

(*Received* 22 *January* 1965; *accepted* 11 *March* 1965)

Abstract—Prednisolone inhibited growth in monolayer cultures of human liver cells (Chang), mouse fibroblasts (L cells) and human cancer cells (HeLa). In the case of the Chang cells and the L cells the inhibition was proportional with the logarithm to the concentration of prednisolone as well as of the mineralocorticoid, aldosterone, which was included for comparison.

At the lowest concentration tested (0·25 μM) the Chang cells were found to be the most sensitive cell type. The inhibition by prednisolone was exerted primarily during the first 10 hr of incubation and was associated with a marked temporary enhancement of glucose utilization and lactate production. No significant changes occurred in cell morphology or in the cellular content of DNA, RNA and protein.

After exposure for 24 hr to 25 μM of prednisolone the liver cells appeared to be insensitive to the growth inhibiting action of higher prednisolone concentrations. However, a renewed response in glucose metabolism was observed with high prednisolone concentrations.

GLUCOCORTICOID hormones exert a growth inhibiting effect on most mammalian cells in tissue culture.[1–8] Limited information is available on their biochemical effects on isolated cells growing in the absence of influence from other tissues, and the underlying mechanism of the growth inhibiting action of glucocorticoid steroids is poorly understood.[9]

In attempts to throw light on the mechanism of action of glucocorticoid hormones and on the possible role of steroids in the control of tissue growth, studies have been initiated to determine the effect of such hormones on mammalian cells in tissue culture. This paper reports experiments on the effect of prednisolone on growth and metabolism of Chang liver cells. Also data on the growth inhibiting effect of prednisolone and aldosterone on mouse fibroblasts (L cells) and on human cancer cells (HeLa) are reported. Part of this work has previously been reported in a preliminary communication.[10]

MATERIALS AND METHODS

Cultivation of cells

The experiments described were carried out with exponentially growing monolayer cultures of mouse fibroblasts (strain L, clone 929), human cancer cells (HeLa), and human liver cells (Chang), grown on Eagle's medium, supplemented with 15%

* Supported by Stang's Foundation.

† Fellow of The Norwegian Cancer Society.

horse serum. The methods for preparations of medium and for propagation and maintenance of stock cultures have been described previously.[11, 12]

Experimental procedure

Cells from stock cultures obtained by trypsinization were suspended in medium and the cell density adjusted. Aliquots were pipetted into glass flasks which were incubated at 37°. After 24 hr the old medium was replaced by fresh medium containing different concentrations of the hormones. These were dissolved in the medium immediately before use, and the solutions were sterilized by filtration. Pure medium was added to the control cultures. The whole procedure was carried out at 37°.

Analytical

The cell number was determined with an automatic cell counter. Protein was determined by the Lowry method,[13] as modified for tissue culture by Oyama and Eagle,[14] using crystalline bovine albumin as standard. After extraction of cells, as described by Whitfield *et al.*,[15] DNA was determined by the indole method of Ceriotti,[16] and RNA by the orcinol method,[17] using Schwartz preparations of DNA and RNA as standards. Glucose was determined by the glucose oxidase method[18] and lactate by the method of Barker and Summerson, as modified by Ström.[19] All data reported represent the average of several independent experiments, in which each value was based on analyses of 3 different flasks.

Reagents

Prednisolone (Δ^1-hydrocortisone) was obtained as the water soluble phosphate ester, sodium *bis*(prednisolone-21)-phosphate (Glucortin) from Fredriksberg Chem. Fabr. A/S, Copenhagen, Denmark. Dexamethasone (9α-fluoro-16α-methyl prednisolone) was obtained as the water soluble sodium sulphate ester (Dexa-Scheroson) from Schering A. G., Berlin. Water soluble aldosterone (Aldocorten) was obtained from Ciba.

Glucose oxidase was obtained from Boehringer & Soehne GmbH., Mannheim, Germany.

All other chemicals used were commercial products of the highest purity.

RESULTS

Growth studies

Previous studies on the growth inhibiting effect of glucocorticoid hormones on cells in tissue culture[1–8] have given variable and in part conflicting results. Presumably, the effects observed depend not only on the cell type studied, but also on the experimental conditions used. While Nowell[4] observed logarithmic dose-effect relationships the data of other investigators[5] indicate that the growth inhibition was directly proportional with the steroid concentration. Whether the extent of the growth inhibition increases with the length of the exposure or reaches maximum shortly after the addition of the hormone, does not seem to have been established.

The effect of increasing concentrations of prednisolone and aldosterone on the rate of multiplication of three different cell lines, is shown in Fig. 1. With L cells and Chang cells the inhibition was found to be proportional with the logarithm to the concentration of the hormones, in principle agreement with the findings of Nowell[4] on human

leukocytes. In the case of the HeLa cells the dose-effect relationships appeared to be more complex. At low hormone concentrations the liver cell was found to be the most sensitive cell type, a definite inhibition being observed with 0·25 μM. Moreover, the curves suggest that inhibition might have been observed with even lower concentrations.

In the case of the Chang cells, experiments were also carried out with the fluorinated steroid, dexamethasone, which was found to inhibit growth to a somewhat lesser extent than prednisolone (Fig. 1). Previously, Perlman *et al.*[8] found L cells to be much

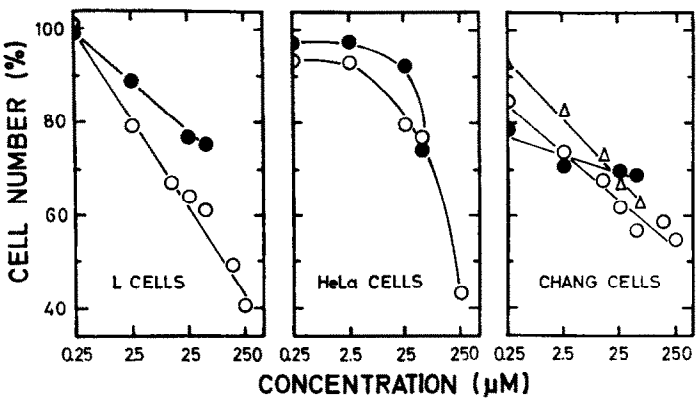

FIG. 1. Effect of concentration of steroids on rate of cell multiplication. 2-ml samples (2×10^5 cells) in Carrel flasks were flushed with 5% CO_2 in air, and incubated at 37°. After 24 hr the medium was replaced by fresh medium containing increasing concentrations of the hormones. After further incubation for 48 hr the total number of cells per culture was counted. The data are expressed as the number of cells in per cent of that found in the untreated control cultures. The data represent the average of 2 independent experiments. ○ — ○, prednisolone; ● — ●, aldosterone; △ — △, dexamethasone.

more sensitive to fluorinated steroids than to the non-fluorinated parent compounds, while Gey's adrenal cells proved to be quite insensitive to the fluorinated derivatives.

The effect of prednisolone (25 μM) on the multiplication of Chang cells, as a function of time, is shown in Fig. 2. It appears (Fig. 2A) that the inhibition was particularly prominent during the first 24 hr of incubation. The more detailed studies in Fig. 2B indicate that prednisolone actually exerted a strong inhibiting action only during the first 10 hr. Subsequently, the treated cells seemed to grow exponentially at a rate that was only moderately reduced compared to that of the control cells. Nowell[4] similarly reported that prednisolone exerted most of its effect on human leukocytes during the first 24 hr. However, Cox *et al.*[7] found with L cells and with various human epithelial cells lines that the growth inhibition by prednisolone was maximal after 40 or more hr of incubation.

The shape of the growth curves (Fig. 2) might conceivably be accounted for by a rapid inactivation of prednisolone. However, this possibility seems to be ruled out by the finding that when the medium was replaced after 24 hr by fresh medium containing different concentrations of prednisolone (25, 125, and 250 μM) the subsequent growth rate was the same as that found when no replacement was made. This is shown for 250 μM of prednisolone in Fig. 2A. Figure 1 suggests that when Chang liver cells are

exposed to a certain concentration of prednisolone they subsequently become insensitive to the growth inhibiting effect of higher hormone concentrations.

Metabolic studies

Several authors have reported that the growth inhibiting effect of glucocorticoid hormones *in vitro* is associated with effects on cell size,[2, 5, 7] and on the cellular content of DNA,[7] RNA[7] and protein.[5, 7] In the present studies phase contrast microscopy revealed no signs of morphological abnormalities in the treated cells. In particular,

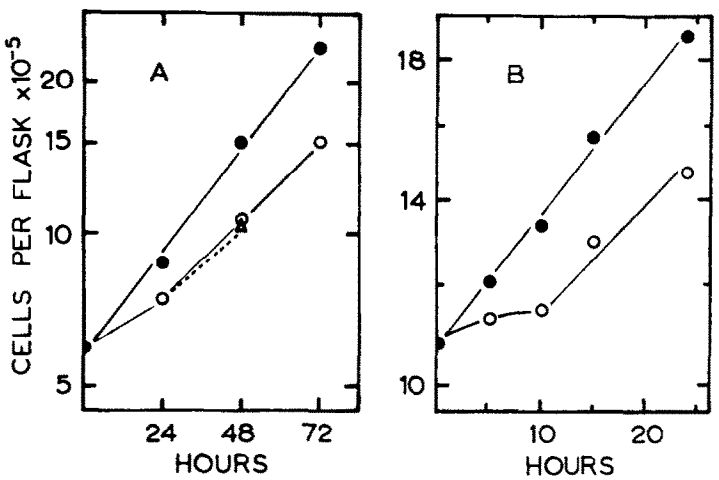

FIG. 2. Multiplication of Chang liver cells in the presence of prednisolone, as function of time. The cells were incubated with 25 μM of prednisolone under conditions described in legend to Fig. 1, and cell counts were performed at the times indicated. Each point represents the average of 2 independent experiments. ○ — ○, control; ● — ●, prednisolone; △ — △, medium replaced after 24 hr with fresh medium containing 250 μM of prednisolone.

no evidence of giant cell formation was found. The DNA, RNA and protein analyses showed that in the untreated, as well as in the prednisolone treated cultures, the total content of these constituents increased in proportion to the cell number. Thus, the content per cell of DNA, RNA and protein remained constant throughout the experimental period, as shown in Fig. 3. Likewise, the amount of protein extracted during the isolation of the nucleic acids (P_1 – P_2) was not influenced by the hormone treatment.

The results in Fig. 4 demonstrate that the growth inhibiting action of prednisolone on the liver cells was associated with pronounced effects on their glucose metabolism. Thus, prednisolone in a concentration of 25 μM increased markedly the glucose consumption per cell per unit time during the first 24 hr. The concomitant lactate production was also increased, although to a lesser extent. With dexamathasone similar, but smaller effects were observed. During the subsequent periods the glucose utilization and lactate production were the same in the treated and untreated cultures. It is apparent (Fig. 4) that the values per cell per hour decreased with time in all cultures, presumably due to the fact that the glucose concentration of the medium

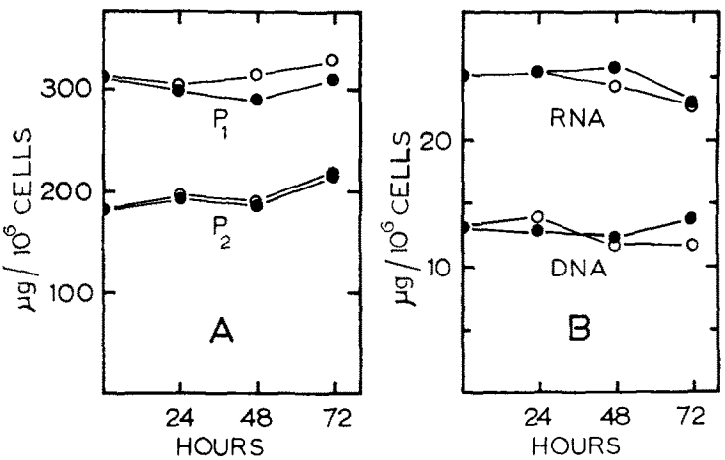

FIG. 3. Effect of prednisolone on the content of protein, DNA, and RNA of Chang liver cells. The cells were incubated with 25 μM of prednisolone as described in legend to Fig. 1. The total protein (P_1), the residual protein after extraction of the nucleic acids (P_2), and the DNA and RNA contents of the cultures were determined after 24, 48 and 72 hr. The results are expressed in μg per 10^6 cells. The data represent the average of two independent experiments. ●—●, control; ○—○, prednisolone.

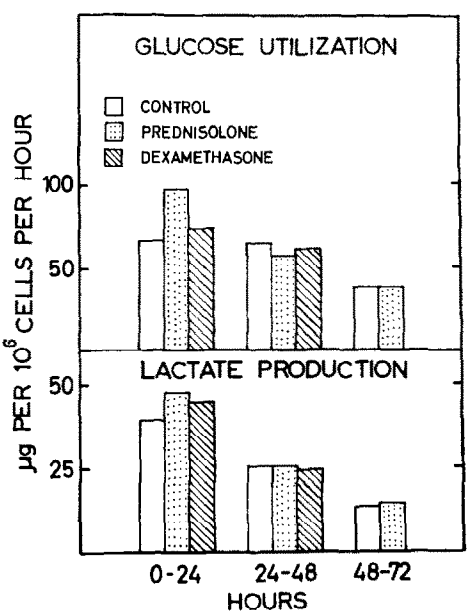

FIG. 4. Effect of prednisolone (25 μM) and dexamethasone (25 μM) on glucose utilization and lactate production of Chang liver cells. Glucose and lactate were determined on aliquots of the medium. The values reported (the average of two independent experiments) were obtained by dividing the total glucose utilization or lactate production for the respective periods by the mean number of cells per hour, obtained by integrating the growth function between the time limits.

declined continously throughout the experiments. When the medium was replaced after 24 hr with fresh medium, the glucose utilization and lactate production of the control cultures were raised to the level found during the first 24 hr (Fig. 5).

Since the transient effect of prednisolone on glucose metabolism appeared to coincide with the transient effect on cell division, it was of interest to see whether glucose metabolism, like growth, became insensitive to the action of prednisolone.

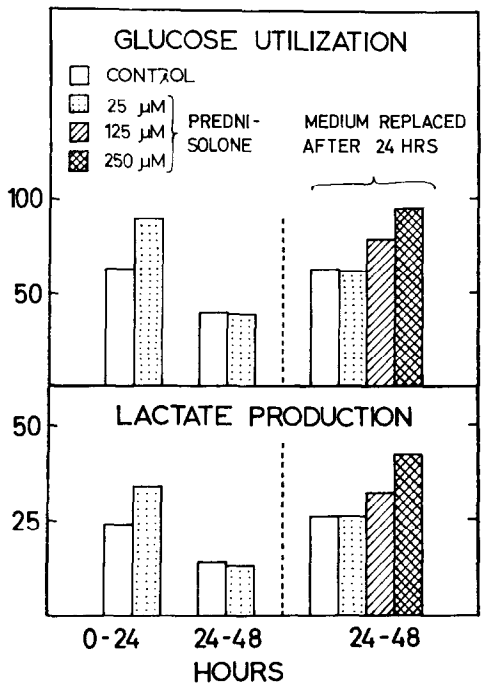

FIG. 5. Effect of prednisolone on glucose utilization and lactate production of Chang liver cells, preincubated for 24 hr with 25 μM of prednisolone. Chang cells were incubated with 25 μM of prednisolone and the glucose utilization and lactate production were determined after 24 and 48 hr. In some cultures the medium was renewed after 24 hr of incubation with medium containing increasing concentrations of prednisolone. In the controls the medium was replaced by fresh medium. The glucose utilization and lactate production during the subsequent 24 hr were determined as described in legend to Fig. 4.

The results shown in Fig. 5 demonstrate that renewal of the medium after 24 hr with medium containing the initial prednisolone concentration (25 μM), did not raise the glucose consumption and lactate production over and above the values of the control culture. However, unlike growth, glucose metabolism showed a renewed response when higher hormone concentrations were used.

DISCUSSION

The data presented in this paper confirm the findings of previous authors that different cell lines in tissue culture exhibit different sensitivity to the growth inhibiting effect of adrenal steroids.[6–8] Thus, the cell types studied differ in their sensitivity and dose reponse to both prednisolone and aldosterone.

The studies with the Chang liver cells seem to reveal several facts of interest. The data indicate that prednisolone exerted most of its action during the early period of incubation. Subsequently, the growth rate of the cells increased although it remained at a somewhat reduced level compared to that of the untreated cells. Interestingly, after the initial period of strong inhibition the cells appeared to be unresponsive to the inhibiting effect of high hormone concentrations. Since the transient nature of the initial growth inhibition could not be accounted for by rapid inactivation of the prednisolone, the results suggest that the cells had somehow adapted themselves to the presence of the steroid. The nature of this "adaptation" is obscure. Cell variants with increased resistance to steroids have previously been selected by cultivation of a mouse lymphoma cell line for several weeks in the presence of hydrocortisone.[20] It seems unlikely that such a mechanism could account for the data observed in our short-term experiments.

The marked temporary enhancement of glucose utilization and lactate production here observed is of interest in view of the fact that glucocorticoid hormones have been found to reduce the uptake and utilization of glucose in various surviving tissues.[21–23] However, in L cells high concentrations of hydrocortisone have previously been found to increase lactate production.[3] The nature of the effect here observed on glucose metabolism in Chang liver cells is currently being studied in more detail.

The growth inhibiting effect of prednisolone on Chang liver cells occurred in the absence of significant changes in morphology and cellular content of DNA, RNA, and protein. The finding that the effect on glucose metabolism coincided with the most pronounced growth inhibition is suggestive of a causal relationship. However, it should be recalled that while after incubation for 24 hr with prednisolone the growth was unaffected by higher concentrations of steroid, a new response in glucose utilization and lactate production was elicited. The possibility obviously exists that the growth inhibition and the effect on glucose metabolism are unrelated aspects of the action of glucocorticoid hormones.

REFERENCES

1. H. Grossfeld and C. Ragan, *Proc. Soc. exp. Biol. Med.* **86**, 63 (1954).
2. M. Holden and L. B. Adams, *Proc. Soc. exp. Biol. Med.* **95**, 364 (1957).
3. H. Grossfeld, *Endocrinology*, **65**, 777 (1959).
4. P. C. Nowell, *Cancer Res.* **21**, 1518 (1961).
5. J. S. Wellington and H. D. Moon, *Proc. Soc. exp. Biol. Med.* **107**, 556 (1961).
6. B. I. Grosser, M. L. Sweat, D. L. Berliner and T. F. Dougherty, *Arch. biochem. Biophys.* **96**, 259 (1962).
7. R. P. Cox and C. M. Macleod, *J. gen. Physiol.* **45**, 439 (1962).
8. D. Perlman, N. A. Giuffre, S. A. Brindle and S. C. Pan, *Proc. Soc. exp. Biol. Med.* **111**, 623 (1962).
9. I. E. Bush, *Brit. med. Bull.* **18**, 141 (1962).
10. A. Pihl and P. Eker, *Scand. J. clin. Lab. Invest.* **15**, 67 (1963).
11. P. Eker and A. Pihl, *Radiation Research* **21**, 165 (1964).
12. P. Eker, *J. biol. Chem.* **240**, 419 (1965).
13. O .H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. V. I. Oyama and H. Eagle, *Proc. Soc. exp. Biol. Med.* **91**, 305 (1956).
15. J. F. Whitfield and R. H. Nixon, *Exp. Cell Res.* **18**, 126 (1959).
16. G. Ceriotti, *J. biol. Chem.* **198**, 297 (1952).
17. W. Mejbaum, *Z. physiol. Chem.* **258**, 117 (1939).
18. A. St. G. Hugett and D. A. Nixon, *Lancet* **2**, 368 (1957).

19. G. STRÖM, *Acta Physiol. Scand.* **17**, 440 (1949).
20. L. ARONOW and J. D. GABOUREL, *Proc. Soc. exp. Biol. Med.* **111**, 348 (1962).
21. B. G. OVERELL, S. E. CONDON, and V. PETROW *J. Pharm., Lond.* **12**, 150 (1960).
22. A. MUNCK, *Biochim. biophys. Acta* **57**, 318 (1962).
23. J. N. FAIN, R. O. SCOW and S. S. SCHERNICK, *J. biol. Chem.* **238**, 54 (1963).