Inhibition of Thymidine Kinase by Cortisone[1] (34445)

TAKEO KANEKO AND G. A. LEPAGE

Life Sciences Research, Stanford Research Institute, Menlo Park, California 94025

Various effects of glucocorticoids on normal and neoplastic cells have been reported. The growth inhibitory effects of glucocorticoids on lymphocytic cells have been well established (1–3). Fibroblasts cultured *in vitro* were also found to be inhibited by glucocorticoids. The possible inhibition by corticocosteroids of DNA polymerase or their effects on the permeability of cell membrances were suggested (4, 5). In bone marrow cells, a marked reduction of the mitotic index was observed at certain times after treatment with high doses of cortisone (6). However, no inhibitory effects on neoplastic cells other than lymphocytic or fibroblastic cells have been reported (7–11). Only an inhibitory effect on respiration of Ehrlich ascites cells by high doses of prednisolone was explored (12). This paper reports some studies on the nature and the site of action of cortisone on the synthesis of DNA in Ehrlich ascites cells.

Materials and Methods. Female Swiss mice were each implanted with 6.5×10^5 Ehrlich ascites cells and used 5 days later for *in vivo* and *in vitro* studies. Each mouse received either saline or a sterile suspension of Upjohn cortisone acetate, 50 mg/kg sc, at designated times before the metabolic studies. For *in vivo* studies, radioactive precursors were injected ip and metabolic utilization was allowed to occur for 1 hr.

For *in vitro* studies with Ehrlich ascites cell suspensions, the cells were harvested, washed 3 times in isotonic saline, and centrifuged at 1470*g* for 2 min. A 0.25-ml portion of the packed cell mass was incubated in Robinson's medium (13) of total volume 10 ml. Incubations were carried out for 30 min at 37° in 50-ml Ehrlenmeyer flasks in a metabolic shaker in 95% O_2–5% CO_2. The reaction was stopped by chilling and adding 0.8 ml of cold 26% perchloric acid (PCA).

The fractionation and preparation of the ascites cell DNA were as follows. The cells were washed 3 times with 5 vol of cold 2% PCA and 3 times with cold 95% ethanol. The precipitate was partially dried *in vacuo*, suspended in 10% NaCl, neutralized with NaOH to pH 7, and heated at 100° for 1 hr. To the extracts, 3 vol of cold 95% ethanol were added. The precipitated nucleic acids were collected by centrifugation, dissolved in 2 ml of 0.1 *M* NaOH and incubated at 37° for 18 hr. After chilling, 0.1 ml of 2 *M* HCl was added to the solution. The precipitated DNA was washed with cold 95% ethanol, redissolved in 0.1 *M* NaOH, precipitated by adding 2 *M* HCl, washed with cold 95% ethanol, and dissolved in 1.0 ml of 0.1 *M* NaOH. Aliquots were counted in a Nuclear Chicago scintillation counter and the DNA content was determined by absorption at 260 mμ, with pure DNA derived from calf thymus as a standard.

The enzyme extract was prepared as follows: 5-day implants of Ehrlich ascites cells were suspended in saline and centrifuged for 2 min at 1470*g*. The cells were diluted 1:3 with ice-cold distilled water and subjected to osmotic shock for 2 min. The swollen cells were homogenized with a high speed Virtis 23 blender for 2 min. The homogenate was centrifuged at 4° for 90 min at 16,000*g*. The supernatant fluid was decanted and used as the enzyme extract. The protein concentration varied from 9 to 11 mg/ml, as determined by the method of Lowry *et al.* (14).

The incubation tubes for DNA polymerase each contained TTP-2-^{14}C, 40 mμmoles (2.5 μCi/μmole); Tris buffer, pH 7.8, 55 μmoles; $MgCl_2$, 7.5 μmoles; creatine phosphate, 6.25 μmoles; creatine phosphokinase, 100 μg; dGTP, dATP, and dCTP, 25 mμmoles each;

[1] This work was supported by USPH Grant No. CA 10938 from the National Cancer Institute.

ATP, 1.25 μmoles; denatured DNA, 125 μg; Ehrlich enzyme extract, 1.8 - 2.4 mg of protein in a final volume of 1.00 ml. Incubation was carried out for 30 min in air at 37°. The reaction was stopped by adding 0.08 ml of cold 26% PCA. The precipitates were washed 3 times with 2% PCA and 3 times with cold 95% ethanol. The acid-insoluble product, presumably DNA, was dissolved in 0.9 ml of 2% PCA, heated for 10 min at 100°, cooled, and centrifuged. The supernatant was neutralized with concentrated NH_4OH and an aliquot was counted in a scintillation counter.

Nucleoside kinase activity was determined by the following procedure. The enzyme reaction mixture containing Tris buffer, 10.5 μmoles; ATP, 7.5 μmoles; creatine phosphate, 24 μmoles; creatine phosphokinase, 100 μg; precursors, 0.1 ml; NaF, 25 μmoles; and cell-free extract, 2.7–3.6 mg of protein in 1.0 ml final volume, was incubated for 30 min in air at 37°. The reaction was terminated by immersion of the tubes in a boiling water bath for 2 min. After cooling, the reaction mixtures were centrifuged for 5 min at 1470*g*. The supernatant fluids were chromatographed on Whatman No. 3 MM paper in isobutyric acid–NH_4OH–H_2O (66:1:33; v/v/v). The chromatograms were cut into 1-cm strips and counted directly in a toluene phosphor solution.

For the assay of thymidylate kinase, the same procedure was followed as above with the following exceptions: (a) $MgCl_2$, 7.5 μmoles, was added to the assay mixture, and (b) TMP-2-^{14}C was used as the substrate in place of labeled nucleoside.

Results. Studies in vivo. The *in vivo* incorporation of thymidine-$_3$H into DNA of Ehrlich ascites cells treated with 50 mg/kg of cortisone was significantly lower than that in the controls (Table I). The lowest value was observed after 24 hr. At 48 hr, the incorporation almost returned to the control level. The data are not listed, but 5 or 0.5 mg/kg of cortisone caused no marked reduction in the incorporation, as compared with the control.

The incorporations of the other precursors into DNA were not affected by the cortisone (50 mg/kg) at 24 hr, as shown in Table II.

TABLE I. Effect of Cortisone on Incorporation of Thymidine-^{3}H into DNA of Ehrlich Ascites Cells *in Vivo*.[a]

Time after cortisone (hr)	Incorporation (cpm/mg DNA)	% Control
—	45,600	100
6	31,600	69.5
15	24,300	53.3
24	14,400	21.6
48	44,600	97.8

[a] Female Swiss mice bearing 5-day implants of Ehrlich ascites tumor were each given cortisone, 50 mg/kg sc, or saline. At designated times each mouse received thymidine-^{3}H, 0.28 μmole (20 μCi), and metabolic utilization was allowed to occur for 1 hr. Each value is the average of three separate determinations.

Studies in vitro. The *in vitro* incorporations of these precursors into DNA of Ehrlich ascites cells pretreated with 50 mg/kg of cortisone were also investigated (Table III). The incorporation of thymidine-^{3}H was depressed to about half that of the control, though in this system the inhibition was not

TABLE II. Effect of Cortisone Pretreatment on Incorporation of Glycine-2-^{14}C, Adenine-8-^{14}C, Orotic Acid-6-^{14}C, and Uridine-2-^{14}C into DNA of Ehrlich Ascites Cells *in Vivo*.[a]

Precursor	Cortisone pretreatment	Incorporation (cpm/mg DNA)	% Control
Glycine-2-^{14}C	(−)	1274	100
	(+)	1311	102.9
Adenine-8-^{14}C	(−)	3505	100
	(+)	3441	98.2
Orotic acid-6-^{14}C	(−)	131	100
	(+)	140	106.9
Uridine-2-^{14}C	(−)	1061	100
	(+)	988	93.1

[a] Female Swiss mice bearing 5-day implants of the Ehrlich ascites tumor were each given cortisone, 50 mg/kg sc, or saline. Twenty-four hr later each mouse received glycine-2-^{14}C, 1 μmole (1 μCi); adenine-8-^{14}C, 30 μg (1.84 μCi); orotic acid-6-^{14}C, 0.5 μmole (1.5 μCi); or uridine-2-^{14}C, 150 μg (2.5 μCi), and metabolic utilization was allowed to occur for 1 hr. Each value is the average of three separate determinations.

TABLE III. Effect of Cortisone Pretreatment on Incorporation of Glycine-2-^{14}C, Adenine-8-^{14}C, Orotic Acid-6-^{14}C, Uridine-2-^{14}C, and Thymidine-^{3}H into DNA of Ehrlich Ascites Cells *in Vitro*.[a]

Precursor	Cortisone	Incorporation (cpm/mg DNA)	% Control
Glycine-2-^{14}C	(−)	991	100
	(+)	1247	125.8
Adenine-8-^{14}C	(−)	3564	100
	(+)	3230	90.6
Orotic acid-6-^{14}C	(−)	98	100
	(+)	107	109.2
Uridine-2-^{14}C	(−)	6784	100
	(+)	7854	115.8
Thymidine-^{3}H	(−)	29,000	100
	(+)	15,573	53.7

[a] Female Swiss mice bearing Ehrlich ascites tumor were each injected with saline or cortisone, 50 mg/kg sc. After 24 hr, cells were drawn out and washed three times. The cells were packed at 1470*g* for 2 min. The cells were incubated, 0.25-ml aliquots in 10 ml of Robinson's medium in 25-ml Erlenmeyer flasks for 30 min, with the following precursors: glycine-2-^{14}C, 10 μmoles (10 μCi); adenine-8-^{14}C, 150 μg (9.2 μCi); orotic acid-6-^{14}C, 300 μg (7.7 μCi); uridine-1-^{14}C, 375 μg (6.25 μCi). Each value is the average of three separate determinations.

as marked as that of the *in vivo* experiment. Incorporations of precursors other than thymidine-^{3}H were not significantly inhibited in this system.

DNA polymerase. Table IV shows various parameters affecting the Ehrlich ascites cell DNA polymerase. The primer DNA was essential, and deletion of dGTP, dATP, and dCTP decreased the enzyme activity to about 35% of that for the complete system. The polymerase activity of ascites cells treated with cortisone was depressed to 93.9% of the control. But this inhibition would not be sufficient to explain the results of the *in vivo* and *in vitro* experiments.

Thymidylate kinase: Thymidylate kinase activity of Ehrlich ascites cells pretreated with cortisone was also compared with the control. The result obtained is illustrated in Table V. Thymidylate kinase was depressed to 85.5% of the control, in extracts of cortisone-treated cells.

Thymidine kinase and other nucleoside kinases. Table VI shows various parameters affecting the thymidine kinase of Ehrlich ascites cells. Deletion of creatine phosphate and creatine phosphokinase decreased the enzyme activity to 10%. The effect of cortisone on thymidine kinase activity was illustrated in Fig. 1. The formation of TMP was

TABLE IV. DNA Polymerase Activity in Enzyme Extracts of the Ehrlich Ascites Cells.[a]

System	Cortisone pretreatment	Incubation time (min)	Activity (cpm/mg of protein)	% Control
Complete	(−)	0	0	
	(−)	15	960	
	(−)	30	1449	100
	(−)	45	1878	129.6
½ Enzyme level	(−)	30	730	50.4
Minus dGTP, dATP	(−)	30	502	34.6
Minus primer	(−)	30	0	0
Complete	(+)	30	1361	93.9

[a] The complete system contained TTP-2-^{14}C, 40 μmoles; Tris buffer, pH 7.8, 55 μmoles; $MgCl_2$, 7.5 μmoles; creatine phosphate, 6.25 μmoles; creatine phosphokinase, 100 μg; dGTP, dATP, and dCTP, 25 μmoles each; ATP, 1.25 μmoles; denatured DNA, 125 μg; and Ehrlich enzyme extract, 1.8–2.4 mg of protein, in a final volume of 1.00 ml. Incubation was carried out for 30 min at 37°. Each value is the average of duplicate determinations in two experiments. Abbreviations used: dGTP, deoxyguanosine-5′-triphosphate; dATP, deoxyadenosine-5′-triphosphate; dCTP, deoxycytidine-5′-triphosphate; TTP, thymidine-5′-triphosphate; ATP, adenosine-5′-triphosphate.

TABLE V. Effect of Cortisone Pretreatment on Thymidylate Kinase Activity of Ehrlich Ascites Cells.[a]

Cortisone pretreatment	Incubation time (min)	Activity (cpm/mg of protein)	% Control
(−)	30	3119	100
(+)	30	2667	85.5

[a] The reaction mixture consisted of Tris buffer, pH 7.8, 10.5 μmoles; $MgCl_2$, 7.5 μmoles; ATP, 7.5 μmoles; creatine phosphate, 24 μmoles; creatine phosphokinase, 100 μg; TMP-2-^{14}C, 4 μmoles (0.25 μCi); NaF, 25 μmoles; and cell-free extract equivalent to approximately 3 mg of protein in a final volume of 1.00 ml. Incubation was carried out for 30 min at 37°. Each value is the average of duplicate determinations in two experiments. Abbreviations used: TMP, thymidine-5′-monophosphate.

depressed by cortisone to about 17% of the control. The effect of cortisone on some other kinases was investigated. The results obtained are illustrated in Table VII. Adenosine

TABLE VI. Thymidine Kinase Activity in Enzyme Extracts of the Ehrlich Ascites Cells.[a]

System	Cortisone pretreatment	Activity (cpm/mg of protein)	% Control
Complete	(−)	1093	100
Minus cell-free extract	(−)	0	0
Minus ATP	(−)	575	52.7
Minus CP or CPK	(−)	112	10.2
Complete	(+)	190	17.4

[a] The complete system contained thymidine-^{3}H, 28 mμmoles (2 μCi); Tris buffer, pH 7.8, 10.5 μmoles; ATP, 7.5 μmoles; creatine phosphate (CP), 24 μmoles; creatine phosphokinase (CPK), 100 μg; NaF, 25 μmoles; and cell-free extract equivalent to 2.7–3.6 mg of protein in a final volume of 1.00 ml. Incubation was carried out for 30 min at 37°. Each value is the average value of two separate determinations in two experiments.

kinase and uridine kinase were not inhibited by pretreatment with cortisone, but deoxyuridine kinase was inhibited to 25.0% of control, which correlates with the inhibition of thymidine kinase.

Discussion. Pratt and Aronow suggested, in a study of cultured fibroblasts, that the inhibition of thymidine incorporation by cortisone might result from the inhibition of DNA polymerase by the drug (5). But in our experiments in Ehrlich ascites cells, only slight inhibition of DNA polymerase was obtained. This is to be expected from the results of the *in vivo* and *in vitro* experiments; that is, the site of inhibition by cortisone should be on a path in which incorporations of glycine-2-^{14}C, adenine-8-^{14}C, orotic acid-6-^{14}C, or uridine-2-^{14}C were not affected. The inhibition of thymidine kinase by cor-

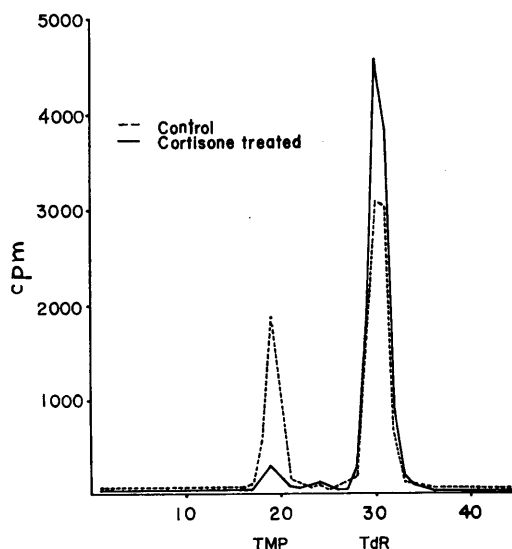

FIG. 1. Effect of pretreatment with cortisone on thymidine kinase activity in extracts of Ehrlich ascites cells. Successive 1-cm strips from a chromatogram were counted directly in a scintillation system with a toluene phosphor. Counts per minute (cpm) are plotted for successive strips.

tisone correlated well with the results on whole cells. This result is of interest in relation to the observation that thymidine kinase has been observed to increase in rapidly proliferating cells (15–19).

Summary. In vivo and *in vitro* studies revealed that incorporation of thymidine-^{3}H into DNA was inhibited in Ehrlich ascites cells treated with cortisone 24 hr before. Incorporations of other DNA precursors were not inhibited. Thymidine kinase was markedly inhibited by pretreatment with cortisone.

TABLE VII. Effect of Pretreatment with Cortisone on Some Nucleoside Kinase Activities of Ehrlich Ascites Cells.[a]

Precursors	Cortisone pretreatment	Activity (cpm/mg of protein)	% Control
Adenosine-8-^{14}C	(−)	23,638	100
	(+)	25,852	109.4
Uridine-2-^{14}C	(−)	2050	100
	(+)	2339	114.1
Deoxyuridine-2-^{14}C	(−)	472	100
	(+)	121	25.

[a] The reaction mixture consisted of Tris buffer, pH 7.7, 10.5 μmoles; ATP, 7.5 μmoles; creatine phosphate, 24 μmoles; creatine phosphokinase, 100 μg; precursors [adenosine-8-^{14}C, 0.03 μmole (1 μCi), uridine-2-^{14}C, 15 μg (0.25 μCi) or deoxyuridine, 2.3 mμmoles (0.1 μCi)]; NaF, 25 μmoles; and cell-free extract equivalent to 2.7–3.6 mg of protein in a final volume of 1.00 ml. Incubation was carried out for 30 min at 37°. Each value is the average of two separate determinations in two experiments.

Other nucleoside kinases (except deoxyuridine kinase), thymidylate kinase, and DNA polymerase were not appreciably affected by the cortisone treatment.

1. Gabourel, G. D. and Aronow, L., J. Pharmacol. Exptl. Therap. **136**, 213 (1962).

2. Cardoso, S., Biochem. Pharmacol. **11**, 1 (1962).

3. Jaffe, J. J., Fischer, G. A., and Welch, A. D., Biochem. Pharmacol. **12**, 1081 (1963).

4. Berliner, D. L., Cancer Res. **25**, 1085 (1965).

5. Pratt, W. B. and Aronow, L., J. Biol. Chem. **241**, 5244 (1966).

6. Cardinali, G., Cardinali, G., Decaw, B. M., Handler, A. H., and Aboul-Enein, M., Cancer Res. **24**, 969 (1964).

7. Mass, R. E., Cancer Chemotherapy Rept. **16**, 257 (1962).

8. Pitot, H. C. and Morris, H. P., Cancer Res. **21**, 1009 (1961).

9. Rosen, F., Cancer Res. **23**, 1447 (1963).

10. Morita, Y. and Munck, A., Biochim. Biophys. Acta **93**, 150 (1964).

11. Cho, Y. S., Pitot, H. C., and Morris, H. P., Cancer Res. **24**, 52 (1964).

12. Saito, T. and Kaneko, T., Tohoku J. Exptl. Med. **95**, 87 (1968).

13. Robinson, J. R., Biochem. J. **45**, 68 (1949).

14. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

15. Weissman, S. M., Smellie, R. M. S., and Paul, J., Biochim. Biophys. Acta **45**, 101 (1960).

16. Bianchi, P. A., Butler, J. A. V., Crathorn, A. R., and Shooter, K. V., Biochim. Biophys. Acta **53**, 123 (1961).

17. Bianchi, P. A., Crathorn, A. R., and Shooter, K. V., Biochim. Biophys. Acta **61**, 728 (1962).

18. Bukonsky, G. and Roth, J. S., Cancer Res. **25**, 358 (1965).

19. Canellakis, E. S., and Jaffe, J. J., J. Biol. Chem. **234**, 2096 (1959).

Received June 11, 1969, P.S.E.B.M., 1970, Vol. 133.