IN VITRO BINDING OF CORTISOL-1,2-^{3}H BY A SUBSTANCE IN THE SUPERNATANT FRACTION OF P1798 MOUSE LYMPHOSARCOMA*

N. Hollander and Y. W. Chiu

Research Institute for Skeletomuscular Diseases, Hospital for Joint Diseases & Medical Center, New York, N. Y.

Received September 27, 1966

The binding of cortisol by subcellular fractions of liver, muscle, and thymus has been reported by several investigators (Brunkhorst 1964, Bellamy 1963, Litwak 1965, DeVenuto 1965, Morris 1966). The uptake of cortisol by rat liver exceeds that which is explainable by diffusion and suggests that intracellular binding is responsible for the higher intracellular concentration (Bellamy 1962). Similar findings have been reported for P1798 lymphosarcoma (Hollander 1966). Cortisol appears to bind to all subcellular fractions in the tissues studied, although Litwak (1965) found in the rat liver greater binding in the supernatant fraction and separated the free from the bound cortisol by means of gel filtration.

The present report describes some of the properties of a cortisol-binding substance in the supernatant fraction of P1798 lymphosarcoma, the specificity of the cortisol binding, the effect of other steroids on the binding of cortisol, and the

* This investigation was supported in part by Grant No. AM 07982 from the National Institutes of Health.

possible physiological significance of the cortisol-binding substance in this tissue.

Experimental

Corticoid-sensitive and corticoid-resistant strains of P1798 lymphosarcoma were carried in BALB/c mice. When adrenalectomized mice were used, the procedure was done in tumor-bearing animals four days prior to sacrifice. Tumors were removed and placed in iced 0.25 M sucrose in 0.1 M Tris pH 7.4. All subsequent procedures were done at 0 - 4°. The tumor was minced, rinsed twice in sucrose-Tris buffer, and a 30% homogenate was prepared in a Potter all-glass homogenizer, using 5 strokes. The homogenate was centrifuged sequentially at 2000 x g, 15,000 x g, and then in the ultracentrifuge at 105,000 x g for one hour. The particle-free supernatant was removed and either used immediately or stored at -60°, where it retained its binding capacity for at least three weeks.

0.5 ml aliquots of supernatant were added to 3.5×10^{-4} ug, 35,800 dpm of cortisol-1,2-^{3}H,* and allowed to stand at 4° with stirring for varying periods of time. The mixture was then passed through a column of Sephadex G-25 and fractions collected of the protein and post-protein bands. Recovery of total radioactivity from the columns was 99 - 100%. The amount of protein

* Cortisol-1,2-^{3}H, specific activity 16.3 C/mM, was obtained through the courtesy of the National Institutes of Health, Endocrinology Study Section. The specific activity of cortisol was determined after thin-layer chromatography in ethyl acetate. In previous experiments (Hollander and Chiu 1966) precursor cortisol gave a single peak of radioactivity in several chromatographic systems.

was determined either by absorption at 280 mu or by the Lowry procedure. The two procedures correlated within 4%. The protein fraction was extracted in individual tubes or pooled before extraction with two volumes of ethyl acetate 4 times, once with added cortisol carrier. The dried extract was chromatographed on Whatman #1 paper in benzene:methanol:water (2:1:1, by volume) with equilibration time 4½ hr, development time 1½ hr. The uv absorbing spot was eluted and the eluate counted in a Packard scintillation counter. The radioactivity bound to protein could be accounted for as 95% cortisol and 5% cortisone. No other metabolites were found. Final results were expressed as dpm/mg protein.

In order to characterize the cortisol-binding substance, aliquots of the supernatant were preincubated with various hydrolytic enzymes (trypsin, pancreatic ribonuclease, α-amylase, and papain) at appropriate pH and temperature before testing for cortisol-binding activity.

Starch block electrophoresis of the tumor supernatant was done in barbital buffer at pH 8.2. Sections of the block were eluted and the cortisol-binding capacity determined. The electrophoretic pattern was compared to that of normal mouse serum.

Results and Discussion

The cortisol-binding fraction from tumor supernatant traveled coincidentally with the protein peak on Sephadex-75 whereas the cortisol-binding fraction from mouse serum, presumably transcortin, was considerably retarded on a Sephadex-75 column. The macromolec-

ular substance of interest was tentatively identified as a protein. Its cortisol-binding ability can be destroyed by trypsin and papain but not by ribonuclease or amylase. The decrease in cortisol-binding capacity which occurred after treatment with proteolytic enzymes was proportional to the decrease in protein concentration.

In the electrophoretic system used, the cortisol-binding protein traveled as an α-1 globulin.

Under the conditions used, the binding of cortisol was found at all times examined to be greater in corticoid-sensitive than corticoid-resistant tumors. (Fig. 1)

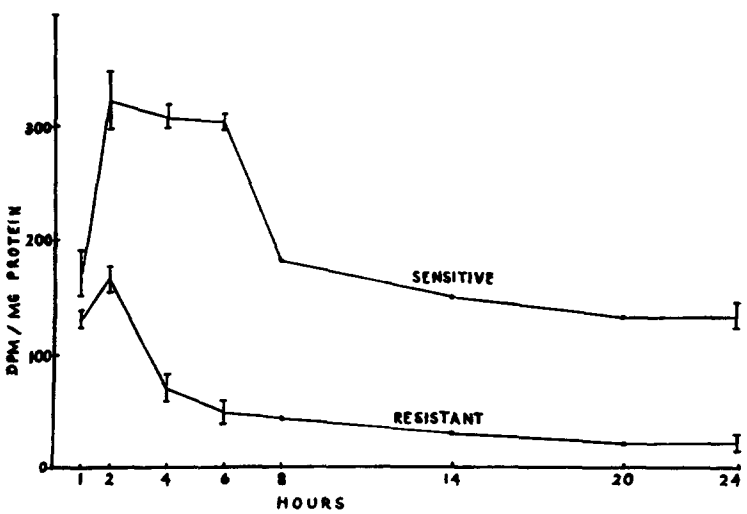

Fig. 1. Specific activity of bound cortisol after incubation at 4° for the indicated hours. Where indicated, the points represent results obtained from 2 homogenates prepared at different times.

In previous experiments (Hollander and Chiu 1966) no differences were detected in either total uptake or metabolism of cortisol on the basis of corticoid sensitivity of the tumor. However, the amount of free cortisol which enters tissue slices by diffu-

sion exceeds by 100 or 1000 fold the bound cortisol found in the present experiments.

Because of the low level of radioactivity, it was necessary to demonstrate that the binding was not an artifact. This evidence came from the study of the effects of adrenalectomy and of binding inhibitors. Tumors from adrenalectomized mice bound considerably more cortisol than those from intact animals. (Fig. 2)

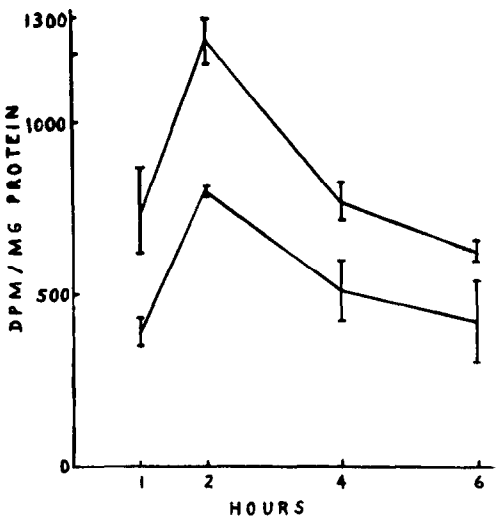

Fig. 2. Specific activity of bound cortisol in tumors from adrenalectomized animals. Incubation was at 4° for the indicated hours. Upper curve = corticoid-sensitive tumor; lower curve= corticoid-resistant tumor. Each point represents an average of 2 samples prepared at different times. Samples from intact (Fig. 1) and adrenalectomized animals were run at the same time.

The binding could be inhibited by addition of 1 ug of unlabeled cortisol, corticosterone, or progesterone, to some extent cortisone, but not by testosterone, 11 β-hydroxyandrostenedione, estradiol-17β, or Δ1,4-androstadiene-3,17-dione. (Table 1)

Table 1

Binding of Cortisol in the Presence of Other Steroids

Added Steroid	dpm/mg Protein
none	1400
cortisol	0
corticosterone	130
progesterone	150
cortisone	860
11 β-hydroxyandrostenedione	1330
testosterone	1100
estradiol-17β	1380
Δ 1,4 androstadiene-3,17-dione	1300

The system contained 0.5 ml sensitive tumor supernatant from adrenalectomized mice, 3.5×10^{-4} ug tritiated cortisol; incubated at 4° for 2 hours, then passed through Sephadex-25. The steroids when added were at a level of 1 ug per 0.5 ml of incubation mixture. All steroids were added to the test tube in methanol solution and evaporated prior to the addition of supernatant.

Addition of 9 α-fluoroprednisolone, which is a potent corticoid *in vivo*, was expected to inhibit the binding of cortisol. Instead, however, it increased the binding of cortisol, especially after prolonged incubation at 4°, and markedly so in the case of sensitive tumor supernatant. (Fig. 3) It is possible that 9α-fluoroprednisolone protects the cortisol-binding site of a corticoid-responsive tissue.

The results of the present experiments suggest that the binding of cortisol in the P1798 tumor supernatant may have physiological significance because it is greater in the corticoid-sensitive tumor supernatant; it is increased after adrenalectomy, and it is decreased in the presence of unlabeled cortisol, corticosterone, and cortisone. Possibly the inhibition of cortisol binding by

progesterone is related to the cortinlike activity of progesterone noted under certain circumstances (Noble 1950).

Purification of the binding protein in the P1798 tumor supernatant is now in progress.

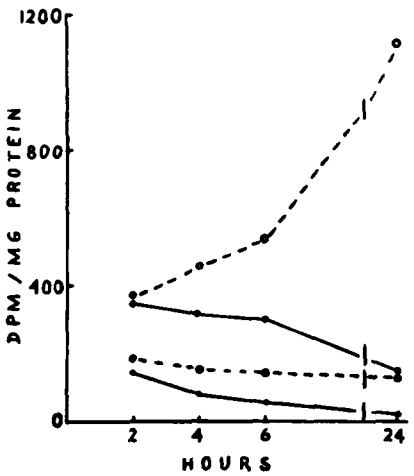

Fig. 3. Specific activity of bound cortisol in tumor supernatant from intact mice. Each sample, which contained 3.5 x 10^{-4} ug of tritiated cortisol per 0.5 ml of supernatant, was incubated for the indicated times in the presence (dash line) or absence (solid line) of 1 ug 9α-fluoroprednisolone. The upper 2 curves = corticoid-sensitive tumor; the lower 2 curves = corticoid-resistant tumor.

References

Bellamy, D. Biochem. J. 87, 334 (1963).

Bellamy, D.; Phillips, J.G.; Jones, I.C. and Leonard, R.A. Biochem. J. 85, 537 (1962).

Brunkhorst, W.K. and Hess, E.L. Biochim. Biophys. Acta 82, 385, (1964).

DeVenuto, F. and Chader, G. Report #639 U.S. Army, Med. Res. Lab. (1965).

Hollander, N. (abstract) 2nd Internatl. Congress on Hormonal Steroids, Milan, Italy (1966).

Hollander, N. and Chiu, Y.W. Endocrinol. 79, 168 (1966).

Litwak, G.; Fiala, E.S. and Filosa, R.J. Biochim. Biophys. Acta 111, 569 (1965).

Morris, D.J. and Barnes F.W., Jr. Fed. Proc. 25, 281 (1966).

Noble, R.L. in The Hormones ed. by Pincus, G. and Thimann, K.V. Vol. II p. 156, Acad. Press, N.Y. (1950).