Glucocorticoid Receptors and Mechanism of Resistance in the Cortisol-sensitive and -resistant Lines of Lymphosarcoma P1798

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SUMMARY

The physicochemical properties of glucocorticoid receptors were studied in cortisol-sensitive and -resistant lines of mouse lymphosarcoma P1798. Steroid-receptor complexes formed when cell suspensions from these tumors were incubated with triamcinolone acetonide-3H (TA-3H) were assayed in a 27,000 x g supernatant fraction by means of sucrose density gradient analysis. After incubation of cells at 0° for 30 min, most of the bound radioactivity was associated with the 27,000 x g supernatant obtained by homogenization of cells in a buffer containing a low concentration of salt. The sedimentation coefficient of the steroid-receptor complex observed was 7 to 8 S. At 0°, the buffer containing high salt (0.15 M KCl) extracted TA-3H-receptor complex sedimenting at 4 to 5 S. When incubated at 37°, the high-salt buffer extracted bound radioactivity that sedimented at ~4 S. There was some residual bound radioactivity sedimenting as a broad peak at 5 to 8 S in the low-salt extract after 30 min of incubation at 37°; however, additional bound radioactivity which sedimented at ~4 S could be obtained from this preparation by extraction of the 27,000 x g pellet with the 0.15 M KCl buffer. When compared with the sensitive tumor, the resistant tumor showed 20 to 50% reduction in TA-binding sites and slight but significant changes in certain sedimentation coefficients, but no difference in affinity toward TA. Pretreatment in vivo with cortisol acetate decreased the number of glucocorticoid-binding sites to 10 to 30% of those observed in the sensitive tumor. These results suggest that the solid resistant tumor is composed of a mixed population of cells and that the resistant cells are characterized by a significant reduction in specific glucocorticoid-binding sites.

INTRODUCTION

It is well established that the administration of glucocorticoids results in a rapid fall in the number of circulating lymphocytes as well as in regression and dissolution of certain normal and malignant lymphoid tissues (4, 15). These effects constitute the basis for their use in the therapy of certain cancers and some other diseases of the lymphoid system. However, the clinical use of these steroids is often hampered by the rapid development of resistance to hormone therapy. Studies in our laboratory have used thymus gland and cortisol-sensitive and -resistant lines of mouse lymphosarcoma P1798 as experimental model systems for investigating the mechanism of the lymphocytolytic action of glucocorticoids and of resistance to these hormones. Current experimental evidence suggests that, in both the sensitive P1798 tumor and rat thymus, inhibition of glucose uptake (17, 20), decreased incorporation of specific precursors into nucleic acids and proteins (14, 21, 22) and decreased oxidation of free fatty acids (26), play a role in the cytosis caused by glucocorticoids. The interaction of steroid hormones with specific receptors in target cells was shown to be an early essential step in the sequence of events leading to biological response (19). We recently identified glucocorticoid receptors in the P1798 tumor (10, 11) and characterized glucocorticoid-binding macromolecules in rat and mouse thymocytes (9). In the present study, we used the potent synthetic glucocorticoid TA for studying certain characteristics of the specific glucocorticoid-binding components of mouse lymphosarcoma P1798 and their relationship to glucocorticoid-induced cytosis and to the development of insensitivity to glucocorticoids. Some of these data have appeared in a preliminary report (8).

MATERIALS AND METHODS

Chemicals. TA-1,2,4-3H (9.5 and 10.7 Ci/m mole) and epicortisol were purchased from Schwarz/Mann, Orangeburg, N. Y. Unlabeled TA was a gift from Squibb, New Brunswick, N. J. The purity of the radioactive TA was checked by thin-layer chromatography, with a solvent system of chloroform:dioxane:water (2:1:1), or chloroform:methanol:acetic acid (90:10:2), as previously described (9). Roswell Park Memorial Institute 1640 culture medium (16) was purchased from Grand Island Biological Company, Grand Island, N. Y. Stock solutions of steroids were prepared in absolute ethanol and stored at 4°.

Animals and Tumors. The DBA/2 × BALB/c F1, mice

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used in these studies were bred in our laboratory. Both sexes were used and tumors were implanted into mice 6 to 7 weeks of age as previously described (22). Both cortisol-sensitive and -resistant lines of lymphosarcoma P1798 were used in the experiments. Tumors used in the various studies were 14 to 16 days old and were routinely tested for sensitivity by the injection of a test dose (75 mg/kg/day) of cortisol acetate for 3 days into animals bearing either of these tumor lines. The cortisol-sensitive tumor underwent almost complete regression during steroid administration, while the cortisol-resistant tumor continued to grow.

Preparation and Incubation of Cell Suspensions. All the experimental procedures were carried out at 0 to 4° unless otherwise specified. Suspensions of tumor cells were prepared from 5 to 7 pooled tumors in protein-free Roswell Park Memorial Institute 1640 medium, by means of the technique described by Rosen et al. (20). Cell suspensions at a concentration of ~4 x 10^7 cells/ml were incubated in open flasks for 30 min with 2.2 to 2.7 x 10^-8 M TA-^3H, at either 0° or 37°, in a 95% air:5% CO_2 atmosphere and with constant shaking. At the end of the incubation period, cells were cooled in ice and separated from the medium by centrifugation at 120 x g for 3 min. The labeled cells were washed 3 times with medium to remove nearly all the free and weakly bound steroid (25). After the 3rd wash, cells were separated from the medium by centrifugation at 270 x g for 5 min and were used for the isolation of subcellular fractions containing the steroid-receptor complex.

Preparation of Cell Extracts. Cell extracts were prepared by homogenization of cell pellets in Tris-HCl buffer with the use of Kontes all-glass Potter-Elvehjem tissue grinders. The low-salt extract was obtained by homogenization in 1.1 volumes of 0.02 M Tris-HCl, pH 7.5, 0.4 mM disodium EDTA. The high-salt extract was obtained by homogenization in 1.0 volume of 0.01 M Tris-HCl, pH 7.5, 0.4 mM disodium EDTA, to which 0.1 volume of 0.11 M Tris-HCl, pH 7.5, containing 1.65 mM KCl was added to give a final concentration of 0.15 mM KCl. The cell homogenates were centrifuged at 27,000 x g, and the supernatants were used for sucrose gradient analysis as previously described (9).

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RESULTS

Sedimentation Analysis of Glucocorticoid Receptors in Mouse Lymphosarcoma P1798. Both the cortisol-sensitive (Chart 1) and -resistant tumors (Chart 2) show similar sedimentation profiles in 5 to 20% sucrose gradients. A single peak of bound radioactivity is observed under low-salt conditions following incubation of cell suspensions with TA-^3H at 0°. The sedimentation coefficient of the complexes identified under these experimental conditions is 7 to 8 S when determined in reference to bovine serum albumin (4.6 S) as a marker. When assayed under high-salt conditions, bound radioactivity sedimented at 4 to 5 S. Results similar to these were previously reported for glucocorticoid receptors in mouse thymocytes (9) from which the P1798 tumor was originally derived (12). After incubation at 37°, the high-salt extract from both tumors contained a large

![Sedimentation Analysis of Glucocorticoid Receptors in Mouse Lymphosarcoma P1798](chart1.png)
amount of bound radioactivity sedimenting at ~4 S. There was some bound radioactivity in the low-salt extract sedimenting as a broad peak in the 5 to 8 S region of the gradient. However, additional bound radioactivity with a sedimentation coefficient of ~4 S could be extracted from the 27,000 × g pellet of this low-salt preparation by reextraction with high-salt (0.15 M KC1). The 27,000 × g pellet contains most of the cellular DNA (> 95%). The amount of bound radioactivity extractable from the pellet with 0.15 M KC1 buffer could be increased about 2-fold by the use of a 0.4 M KC1 buffer.

Table 1 summarizes and compares the sedimentation coefficients determined for glucocorticoid receptors in the 2 tumor lines under various experimental conditions. It would appear from the data that under the conditions used here, there are small (although statistically significant) differences in sedimentation coefficients between the sensitive and the resistant tumors.

Binding of TA by Soluble Fractions from the Sensitive and Resistant P1798 Tumors: Scatchard Plot Analysis. Earlier studies had revealed a 20 to 50% decrease in the number of TA binding sites per mg protein in the resistant P1798 tumor, compared with the steroid-sensitive line (10). Also, no major differences could be observed in steroid uptake or metabolism between the 2 tumor lines (2, 6). In view of the marked differences in the biological response to glucocorticoids between the sensitive and the resistant P1798 tumor, causes other than the above were sought as a basis for resistance.

Chart 3 shows the amount of TA-3H specifically bound to receptors in the tumors after incubation of soluble fractions with various steroid concentrations for 6 hr. These studies were carried out in the presence of either nonradioactive epicortisol or TA. It is expected that both of these steroids would compete almost equally for nonspecific binding sites in the tumors, but since only TA has glucocorticoid activity, only this steroid would compete also for glucocorticoid-specific binding sites. Thus the difference in displacement of TA-3H by epicortisol and TA is taken as a measure of the amount of specifically bound TA-3H. As can be seen in Chart 3, there is a limited number of specific binding sites in both sensitive and resistant lines of the P1798 tumor. The binding curves reach a plateau at a steroid concentration of approximately 10^-7 M, indicating that at this concentration most of the specific binding sites are saturated.

Chart 4 illustrates a Scatchard plot analysis (24) of the specific binding data from Chart 3. By using this method of analysis we observed a single straight line with identical slopes for each tumor. This indicates that only a single class of specific binding sites was detected by the assay. Since the

<table>
<thead>
<tr>
<th>Sedimentation coefficients*</th>
<th>0°</th>
<th>37°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>High-salt</td>
<td>Low-salt</td>
</tr>
<tr>
<td>P1798/S</td>
<td>4.6 ± 0.08 (6)*</td>
<td>7.1 ± 0.06 (6)</td>
</tr>
<tr>
<td>P1798/R</td>
<td>4.7 ± 0.10 (6)*</td>
<td>7.4 ± 0.04 (6)</td>
</tr>
</tbody>
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* Mean ± S.E.
* Numbers in parentheses, number of experiments.
* Differences between P1798/R and P1798/S are significant (p < 0.01).
sensitive and resistant tumors contained 4.20 and 4.26 mg protein per ml soluble fraction, respectively, the equilibrium dissociation constant for the reaction, TA + receptor = TA-receptor complex, was calculated to be approximately $1.5 \times 10^{-8}$ M (0.7 to 1.9 $\times 10^{-8}$ M in other experiments) for each tumor. It can be calculated, from the intercept on the abscissa, that there were 0.39 pmole of TA bound per mg protein in the resistant P1798 tumor compared with 0.53 pmole of bound steroid in the sensitive line, a decrease of about 25% in specific binding sites in this particular experiment. Since binding to TA protects the binding component from degradation (10), these calculations are predicated on the assumption that there is no loss of bound receptor during the experiment.

**Effect of Glucocorticoid Treatment on the Number of Binding Sites in Lymphosarcoma P1798.** The failure to observe a substantial difference in the number of binding sites and the lack of difference in affinity toward TA between the sensitive and resistant tumors, in addition to the observation of changes in the number of binding sites in tumors from different transplants, suggested the possible existence of a mixed cell population in the P1798 tumor. For a test of this hypothesis, tumor-bearing animals were given injections of 75 mg cortisol acetate per kg for 2 days, and the tumors were allowed to grow for another 3 days before being assayed for binding sites in vitro. In a preliminary experiment (Table 2), we tested tumors for residual TA-binding sites at various time intervals after cortisol acetate injection. The sensitive tumor showed a gradual increase to control levels in 3 days, while the number of binding sites in the resistant tumor remained low. Since in previous studies no significant difference could be observed in steroid uptake or metabolism between the 2 tumor lines (2, 6), the results suggested that the 3-day lag between in vivo steroid treatment and in vitro determination of binding sites is sufficient for elimination of exogenous steroid and thus was routinely used in our studies. We reasoned that glucocorticoids in vivo would kill many of the sensitive cells and consequently increase the ratio of resistant to sensitive cells in the solid tumor. If the resistant cells contain significantly specific glucocorticoid-binding macromolecules than the sensitive cells, the change in the cell ratio following cortisol treatment should result in a marked decrease in the number of binding sites in the treated tumor, compared with untreated controls, when assayed in vitro with TA-3H.

Table 3 summarizes the results of 10 experiments. In each of the individual experiments, each group contained at least 5 tumor-bearing animals. There was no significant difference in the number of TA-binding sites in sensitive tumors treated with the glucocorticoid in vivo, compared with untreated controls. However, treated resistant tumors showed an additional decrease in the number of TA-binding sites, compared with resistant tumors obtained from untreated mice. While the untreated resistant tumors had about 60% of the number of binding sites observed in the sensitive tumors, the glucocorticoid-treated resistant tumors had only about 20% of the number of binding sites compared with the sensitive control line.

**DISCUSSION**

Four possible explanations for the failure of the resistant tumor to respond to glucocorticoids were examined in this study. These include (a) a significant reduction in the number of specific binding sites, (b) a reduced affinity of the steroid hormone for the receptor molecule, (c) failure of the

<table>
<thead>
<tr>
<th>Time after injection (hr)</th>
<th>P1798/S (% of control)</th>
<th>P1798/R (% of control)</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>61</td>
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</tr>
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<td>49</td>
<td>70</td>
<td></td>
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<tr>
<td>73</td>
<td>93</td>
<td>30</td>
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</tbody>
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**Table 3**

**Effect of glucocorticoid treatment on tumor-binding capacity**

Tumor-bearing animals were given injections of 75 mg cortisol acetate per kg for 2 days starting at Day 11 after tumor transplant. The mice were killed on Day 15 and the TA-3H-binding capacity of pooled tumors in each group was assayed in vitro by means of the charcoal adsorption technique.

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Treatment</th>
<th>TA-3H-binding*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1798/S</td>
<td>Control</td>
<td>-18.1 ± 11.07</td>
</tr>
<tr>
<td>P1798/S</td>
<td>75 mg cortisol acetate, 2 times</td>
<td>-41.9 ± 9.43³</td>
</tr>
<tr>
<td>P1798/R</td>
<td>Control</td>
<td>-77.7 ± 3.11¹</td>
</tr>
<tr>
<td>P1798/R</td>
<td>75 mg cortisol acetate*</td>
<td></td>
</tr>
</tbody>
</table>

* Results are expressed as percentage change (mean ± S.E.) from P1798/S control.

³p < 0.01, compared with P1798/S control.

¹p < 0.01, compared with P1798/R control.
steroid-receptor complex to undergo a temperature-dependent translocation into the nucleus, and (d) failure of the steroid-receptor complex to bind to an “acceptor” site in the nucleus.

The present studies on glucocorticoid receptors in the sensitive and the resistant lines of the P1798 tumor are in agreement with the general scheme of steroid hormone-receptor interactions. When tumor cells from either the sensitive or resistant tumor were incubated with TA-3H at 0°C and extracted with a high-salt buffer, a complex sedimenting at 4 to 5 S was identified whereas, under low-salt conditions, bound radioactivity sedimented at 7 to 8 S. Since the total amount of bound radioactivity extracted with the high-salt buffer did not differ significantly from that extracted with the low-salt buffer, the 4 to 5 S complex noted under high-salt conditions could represent a subunit of the 7 to 8 S complex, as was previously postulated for the estrogen receptor (7). Following incubation at 37°C and extraction with a high-salt buffer, the TA-3H was bound in an ~4 S complex, but no clearly defined S value could be determined in the low-salt extract, which contained a very broad peak of low radioactivity. However, when the pellet of the low-salt extract was reextracted with a high-salt buffer, a complex sedimenting at ~4 S was observed. The amount of the complex extractable with a 0.15 M KCl buffer from the particulate fraction could be increased over 2-fold by extraction with a 0.4 M KCl buffer. Since the particulate fraction contains most of the cellular DNA, these results suggest that the complex was firmly bound to an intranuclear macromolecule after incubation of tumor cells at 37°C. The latter observation is in agreement with the temperature-dependent translocation of steroid hormone-receptor complexes from the cytoplasm to the nucleus and binding to nuclear “acceptor” sites, as described by others (19). The observation that the amount of extractable complex after 37°C incubation correlated well with the amount of the complex identified following 0°C incubation for both tumors (data not shown) seems to rule out differences in the temperature-dependent translocation mechanism and nuclear binding as causes for hormone resistance. These results, however, do not rule out the possibility that in the resistant line the nuclear translocated complex binds to a site of loss or “biologically unproductive” site in chromatin.

The presence of a modified receptor molecule in the resistant line is suggested by the differences in certain sedimentation coefficients observed in the 2 tumor lines (Table 1). A Scatchard plot analysis (24) designed to investigate whether the small change in the S values is reflected in a change in the affinity toward TA did not reveal differences in the equilibrium dissociation constants for the binding of TA to specific receptors in the 2 tumor lines. Thus, at present, the significance to the mechanism of resistance of the presence of a modified receptor molecule, as suggested by sedimentation analysis is questionable.

We have previously commented (10) on the apparent discrepancy between the marked differences in the sensitivity of the 2 P1798 tumor lines to glucocorticoid treatment and the rather modest differences (20 to 50%) in the level of receptor binding in these 2 tumors. Since that time we have found that the number of glucocorticoid-binding sites in the P1798 tumors varies from one transplant to another, which suggests the presence of a mixed population of cells. If this is the case, it could explain the failure to observe a marked quantitative reduction in the number of binding sites in the resistant tumor as observed in other hormone-refractory tumors (1, 5, 18, 23). Our experiments with glucocorticoid-treated tumors lend some support to the latter hypothesis. Treatment of tumor-bearing animals with cortisol acetate caused a marked decrease in the number of binding sites in the resistant P1798 tumor. Thus, pretreated resistant tumors contained about 20% as many TA-3H receptor-binding sites as were observed in the cortisol-sensitive tumor. In contrast, the untreated resistant tumor had about 60% of the number of binding sites observed in the sensitive neoplasm. It is unlikely that occupation of binding sites in the resistant cortisol-pretreated tumor is the cause for the decreased in vitro binding of TA-3H, since the tumor-bearing animals were killed 3 days after the last injection of the steroid. It is possible to explain our findings on the basis of selective elimination of steroid-sensitive cell populations by glucocorticoid treatment in vivo. Thus our data suggest that the solid resistant tumor is composed of a mixture of cells, and when cortisol-sensitive cells are eliminated, resistance to glucocorticoids is more clearly associated with a marked decrease in glucocorticoid-binding sites. Another possible explanation of our findings, currently under investigation, is that treatment with glucocorticoids may impair the synthesis and/or enhance the degradation of the receptor molecule in the resistant tumor, thus leading to a significant reduction in the number of receptor-binding sites and to resistance.

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