Nuclear Binding of Steroid-Receptor Complex to Lymphosarcoma P1798 Resistant and Sensitive Cells and Effect of Concanavalin A on Receptor Levels

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SUMMARY

Glucocorticoid-resistant P1798 cell lines have been found to contain levels of glucocorticoid receptor comparable to receptor levels in glucocorticoid-sensitive P1798 cells. Previously, most of the P1798 resistant cells examined were found to contain low levels of glucocorticoid receptor, and this was thought to account for the resistance of these cells to glucocorticoid treatment. Resistant cells with high receptor levels exhibited 10 to 50% lower levels of nuclear binding than did sensitive cells. In addition, 90% of the glucocorticoid-receptor complex could be extracted from resistant nuclei with 0.2 M NaCl, while only 55% of the complex could be extracted from sensitive nuclei, indicating that the affinity of the hormone-receptor complex for resistant nuclei may be weaker than the affinity of the hormone-receptor complex for sensitive nuclei. The effect of concanavalin A was also examined in P1798 sensitive and resistant cells. Concanavalin A effectively lowered glucocorticoid receptor levels in the sensitive cells by 45%, while receptor levels of the resistant cells were only slightly lowered. The effect of concanavalin A was both temperature-dependent (effective at 37° but not 0°) and time-dependent. Thus glucocorticoid resistance of P1798 cells appears to have a more complex mechanism than previously proposed.

INTRODUCTION

It has been proposed previously by this laboratory that the resistance of P1798 lymphosarcoma cells to glucocorticoids may be attributed to a low level of cellular glucocorticoid receptor sites in the resistant cells (4). The receptor levels of the resistant line were 20 to 50% lower than the level of receptor found in the sensitive line (4). Indeed, lower receptor levels have been shown to account for steroid resistance by others (9, 10). However, some recent transplants of the P1798 resistant tumor have been found that contain levels of glucocorticoid receptor comparable with the level of receptor found in the sensitive tumor. Despite this increase in receptor content, the new line was still resistant to glucocorticoid treatment. It was of interest, therefore, to determine whether resistance may be at the level of the nuclear binding sites. Intact nuclei were isolated under very gentle conditions following the method of Wira and Munck (11). With this technique, the temperature-dep-endent uptake of the glucocorticoid-receptor complex by the nucleus could be measured in both tumor lines.

Recent reports (2, 3) indicated that concanavalin A lowered the number of glucocorticoid receptor sites in isolated thymocytes. Since P1798 sensitive cells and thymocytes react similarly when treated with glucocorticoids, we decided to examine the effect of concanavalin A on both the sensitive and the new resistant lines of P1798 cells. Perhaps the cellular membranes of the sensitive and resistant cells differ with respect to concanavalin A-binding sites. Behrens et al. (1) have already shown that sensitive and resistant cells differ with respect to their cell surface charge and to their membrane glycoprotein chromatographic behavior.

MATERIALS AND METHODS

Chemicals. [1,2,4-3H]Dexamethasone (9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) was purchased from New England Nuclear, Boston, Mass. (specific activity, 27 to 29 Ci/mmmole). Unlabeled dexamethasone and concanavalin A were purchased from Sigma Chemical Co., St. Louis, Mo. RPMI 1640 media (5) was purchased from Grand Island Biological Co., Grand Island, N. Y. Stock solutions of steroids were stored at 4° in absolute ethanol. Concanavalin A was dissolved in RPMI 1640 media. ACS scintillator was purchased from Amersham/Searle, Arlington Heights, Ill.

Animals and Tumors. P1798 resistant and sensitive tumors were implanted into DBA/2 x BALB/c F1 mice as previously described (8). Mice were sacrificed by cervical dislocation 11 to 16 days following tumor implantation, and the tumors were removed. Suspensions of tumor cells were prepared from 4 to 6 pooled tumors in RPMI 1640 media by the method of Rosen et al. (7). Cells were counted in a hematocrit and brought to a final concentration of 106 cells/ml with RPMI 1640 media, pH 7.2.

Measurement of Total Cellular Binding (11). Two ml of cells were usually incubated with 2 µl of [3H]dexamethasone...
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(7 \times 10^{-8} \text{ M}) for 1 hr at 0° or 37° under 95% air-5% CO₂. Following this initial incubation, high-affinity [3H]-dexamethasone binding was measured by adding a 0.2-ml aliquot of cells to 10 ml of media (50-fold dilution) and incubating the diluted cells for an additional 30 min. High-affinity dexamethasone binding remains unaffected in the diluted cells incubated in media at 0°, whereas complete dissociation of the steroid-receptor complex occurs in the cells incubated at 37°. Nonspecific binding is [3H]-dexamethasone bound at 37° that is not dissociable even after 1 hr. Duplicate 4-ml aliquots of the diluted cells were centrifuged in 5-ml conical centrifuge tubes twice for 2 min at 1000 x g, with the cells being kept chilled at all times. The media were removed by aspiration, and fresh media (4 ml) were added. The cells were centrifuged 2 additional times for 2 min, again with the cells chilled. The supernatant was removed with a Pasteur pipet, and the radioactivity of the cell pellet was measured in 10 ml of ACS scintillator. Efficiency of measuring radioactivity was 30%.

The amount of [3H]-dexamethasone bound in diluted cells at 37° was subtracted from the amount of [3H]-dexamethasone at 0° to obtain the specific binding of dexamethasone. The media wash did not have any effect upon the specific binding of dexamethasone to the cells but did lower the radioactive background values.

Siliconized glassware was used in all cell experiments.

**Nuclear Binding.** The procedure described above for cellular binding was carried out exactly as described above, except that 1.5 mM MgCl₂ was used instead of media when cells are diluted. Thus, total cellular binding and nuclear binding can be measured by pipetting aliquots from the same cell incubation into RPMI 1640 media or 1.5 mM MgCl₂.

**Salt Extraction of Nuclei.** After cells have been diluted 50-fold in 1.5 mM MgCl₂, the cytoplasmic membrane breaks open leaving intact nuclei (11). The nuclei were incubated at 0° (total binding) and 37° (nonspecific binding) for 30 min. A number of replicate 4-ml aliquots of the diluted nuclei from the 0°- and 37°-incubated nuclear samples were placed into individual 5-ml conical centrifuge tubes that were chilled on ice. The nuclear pellets were collected by centrifugation as described above and washed with 4 ml of 1.5 mM MgCl₂ containing various concentrations of NaCl and with a Pasteur pipet to disperse the pellet throughout the salt-containing MgCl₂ solution. The nuclei were pelleted again, the supernatant was removed, and the pellets counted in 10 ml of ACS scintillator. Specific binding was determined by subtracting the [3H]-dexamethasone bound to nuclei diluted at 37° from the [3H]-dexamethasone bound to nuclei diluted at 0° in 1.5 mM MgCl₂.

**RESULTS**

To examine the binding of glucocorticoid receptor to nuclei, one must first establish that nuclear binding is temperature dependent. Therefore, P1798 sensitive cells were incubated with [3H]-dexamethasone for 1 hr at 0° and 37° before total cellular binding and nuclear binding were measured as described in "Materials and Methods." Total cellular binding (Table 1) was enhanced slightly when the cells were incubated at 37° rather than at 0°. However, nuclear binding was found to be completely absent when the cells were incubated with [3H]-dexamethasone at 0°. On the other hand, more than 60% of the [3H]-dexamethasone was bound to nuclei when the cells were incubated at 37°, clearly indicating that glucocorticoid-receptor translocation into the nucleus was temperature dependent. The number of receptor sites per sensitive cell was calculated and was almost twice the number of sites calculated by others for binding of [3H]-dexamethasone to thymocytes (11). Binding was linear with cell concentrations of 8 \times 10⁴ to 2 \times 10⁸ cells/ml.

[3H]-Dexamethasone binding was then examined in P1798 sensitive and resistant cells as a function of increasing steroid concentration at 37°. A comparison of total cellular binding (Chart 1) and nuclear binding, (Chart 2) indicated that both cell types had the same number of receptor sites per cell but that the nuclei of sensitive cells had a greater capacity to bind receptor complex than did resistant nuclei. In 4 separate experiments with cells from different weekly transplants of resistant tumor, the differences observed in nuclear binding were always observed, but the differences ranged from 10 to 50%. Some of these differences may be due to the fact that the tumor cells were isolated from intact animals. In 1 set of experiments in which adrenalectomized mice were used, the difference in nuclear binding between sensitive and resistant cells was 37% (Chart 2A).

For further investigation of the specificity of glucocorticoid receptor binding to nuclei, the isolated nuclear-bound

$\text{Table 1}$

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Dilution</th>
<th>Binding</th>
<th>Specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>dpm/ml</td>
</tr>
<tr>
<td>0°</td>
<td>Media</td>
<td>Total cellular</td>
<td>70,500 ± 2,050*</td>
</tr>
<tr>
<td>37°</td>
<td>Media</td>
<td>Total cellular</td>
<td>79,400 ± 1,414*</td>
</tr>
<tr>
<td>0°</td>
<td>1.5 mM MgCl₂</td>
<td>Nuclear</td>
<td>0</td>
</tr>
<tr>
<td>37°</td>
<td>1.5 mM MgCl₂</td>
<td>Nuclear</td>
<td>49,800 ± 9,095</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

**Total cellular binding at 37° is significantly higher than total cellular binding at 0°.**

$\text{p} < 0.05.$
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Chart 1. Specific cellular binding of [3H]dexamethasone ([3H]Dex) to sensitive and resistant P1798 cells. P1798 sensitive (0.7 x 10^6 cells/ml) (•) and (0.69 x 10^6 cells/ml) (O) resistant cells from adrenalectomized mice were incubated with various concentrations of [3H]dexamethasone for 1 hr at 37° before specific total cellular binding was measured. Ordinate, specific dpm of [3H]dexamethasone bound per 10^6 cells in thousands of counts.

Chart 2. Specific nuclear binding of [3H]dexamethasone ([3H]Dex) to sensitive and resistant P1798 cells. In A, P1798 sensitive (•) and resistant (O) cells from Chart 1 were incubated for 1 hr at 37° before an aliquot of the cells was diluted 50-fold into 1.5 mM MgCl₂ as described in “Materials and Methods.” In B, in a separate experiment sensitive cells (1.5 x 10^6 cells/ml) (•) and resistant cells (1.54 x 10^6 cells/ml) (O) from intact mice were incubated for 1 hr at 37° with various concentrations of [3H]dexamethasone. An aliquot of the cells was diluted 50-fold into 1.5 mM MgCl₂ to determine specific binding to nuclei. Ordinates, specific dpm of [3H]dexamethasone bound per 10^6 nuclei in thousands of counts.

Chart 3. Extraction of nuclear-bound [3H]dexamethasone receptor with various concentrations of NaCl. P1798 sensitive (1.26 x 10^6 cells/ml) (•) and resistant (1.4 x 10^6 cells/ml) (O) cells were incubated with [3H]dexamethasone for 1 hr at 37°. Aliquots of the cells were then diluted 50-fold in 1.5 mM MgCl₂ at 0° and 37°. Four-ml aliquots of the nuclei were then extracted with 1.5 mM MgCl₂ containing various concentrations of NaCl at either 0° or 37°. The nuclei were then washed and pelleted as described in “Materials and Methods.”

Table 2

Inhibition of glucocorticoid binding in resistant and sensitive P1798 cells following concanavalin A treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Concana-valin A (mg/ml)</th>
<th>Specific cellular binding (dpm/10^6 cells)</th>
<th>% of control (av.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>None</td>
<td>180,000</td>
<td>100</td>
</tr>
<tr>
<td>Sensitive</td>
<td>0.50</td>
<td>116,000</td>
<td>65 ± 2.6*</td>
</tr>
<tr>
<td>Resistant</td>
<td>None</td>
<td>186,000</td>
<td>100</td>
</tr>
<tr>
<td>Resistant</td>
<td>0.05</td>
<td>163,000</td>
<td>89 ± 6.8*</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

hormone receptor was extracted with different concentrations of salt. Resistance to salt extraction may indicate a greater affinity of nuclei for the [3H]dexamethasone receptor. A typical result is shown in Chart 3. Nuclei isolated from sensitive cells form a complex with the [3H]dexamethasone receptor that is more resistant to salt extraction than is the nuclear-receptor complex isolated from resistant cells. Greater than 90% of the labeled receptor was extracted from resistant nuclei with 0.2 M NaCl, while 40% of the receptor complex remained bound to nuclei of sensitive cells. Similar results were obtained with 3 preparations of resistant and sensitive cells from different weekly transplants.

During the course of the investigation, an additional resistant tumor line was found that did not exhibit a difference in nuclear steroid binding or cytoplasmic binding when compared with sensitive cells. However, the salt extraction of hormone-receptor complex from nuclei was always more complete and at a lower salt concentration with resistant nuclei than was the extraction of receptor complex from nuclei isolated from sensitive cells.

Effects of Concanavalin A. As discussed earlier, the incubation of thymocytes in the presence of concanavalin A has been shown to cause a diminution of glucocorticoid receptor. The significant drop in receptor level, it was postulated, could account for the fact that concanavalin A can reverse the inhibitory effect that glucocorticoids have on glucose uptake in thymocytes. The effect of concanavalin A on P1798 sensitive and resistant cells was examined to determine whether glucocorticoid receptor levels could also be lowered in these tumors.

The isolated cells were incubated with 0.5 mg of concanavalin A for 45 min at 37° before [3H]dexamethasone binding was measured at 0° (Table 2). The results indicate that
specific cellular glucocorticoid receptor levels of the sensitive cells drop significantly following concanavalin A treatment, while receptor levels of resistant cells drop only slightly. The maximal effect of concanavalin A on sensitive cells was observed when cells were incubated for 30 min at 37° (Chart 4).

The temperature dependence of concanavalin A treatment was then examined. Sensitive cells were incubated for 45 min at 0° and 37° before the cells were incubated with [3H]dexamethasone. In 2 separate experiments (Table 3), concanavalin A was found to be effective in reducing glucocorticoid receptor levels only when the cells were incubated at 37°.

The effect of concanavalin A treatment was also examined after [3H]dexamethasone was bound to the sensitive and resistant cells (Table 4). The results were very similar to experiments conducted when concanavalin A was preincubated with the cells. The level of glucocorticoid receptor was decreased by almost 90% following concanavalin A treatment in sensitive cells, while the level of receptor decreased only slightly in resistant cells.

One problem in all of these experiments was that concanavalin A caused both sensitive and resistant cells to clump. Thus the apparent glucocorticoid receptor difference may only be a reflection of clumping of the cells. However, in all of the above experiments, both cell types respond to clumping in a similar way. The percentages of unclumped cells remaining after sensitive and resistant cells were incubated with 0.5 mg of concanavalin A per ml were 17 and 21%, respectively. An attempt was made to analyze the unclumped cells alone by filtering out the clumped cells. The results have thus far been inconclusive. Improved methods of filtration will have to be developed to separate the clumped cells from the unclumped cells so that [3H]dexamethasone binding can be measured in the separated cell populations.

**DISCUSSION**

In light of more recent data, the mechanism for glucocorticoid resistance of P1798 resistant cells does not appear to be as simplistic as previously postulated (4). Originally, resistance was attributed to low levels of glucocorticoid receptor in the resistant cells. However, resistant P1798 cell lines have now been found with high glucocorticoid receptor levels. These resistant cells have a defect in the nuclear binding of the glucocorticoid receptor. The fact that over 90% of the glucocorticoid receptor can be extracted from resistant nuclei with 0.2 M NaCl indicates a weak affinity of the resistant nuclei for the receptor complex. On the other hand, the receptor complex appears to bind with high affinity to the sensitive nuclei. Sensitive nuclei also have a greater number of receptor-binding sites than do resistant nuclei. It is not yet known whether resistant nuclei have defective binding sites or whether the glucocorticoid receptors of resistant cells have an altered affinity for dexamethasone.
conformation with a lower affinity for the nuclear binding sites. This question can probably now be answered with the nuclear isolation conditions described in the "Materials and Methods" and by Wira and Munck (11) to perform mixing experiments.

Additional differences between glucocorticoid-resistant and sensitive cells have now been found with respect to their response to concanavalin A. The incubation of P1798 sensitive cells with concanavalin A caused a drop in glucocorticoid receptor levels. This phenomenon has been observed previously with thymocytes (2, 3). The mechanism for the decrease in receptor levels is not known, but the process has been shown by us to be both temperature and time dependent. Concanavalin A probably does not affect the uptake of dexamethasone, because glucocorticoid binding was still decreased when cells were treated with dexamethasone prior to concanavalin A treatment. Cidlowski and Munck (3) have preliminary data indicating that receptor levels of thymocytes can also be lowered by treating cells with cyclic 3':5'-GMP. Perhaps the action of concanavalin A is mediated through cyclic 3':5'-GMP. They also indicate that receptor levels may also decrease due to the activation of an ATPase by concanavalin A that would reduce intracellular ATP, ultimately causing a reduction in receptor levels. The fact that glucocorticoid receptor levels were not significantly lowered when P1798 resistant cells were treated with concanavalin A may indicate that regulation of the glucocorticoid receptors by concanavalin A has been lost in the resistant cell line. This defect may be linked to glucocorticoid resistance.

The failure of P1798 resistant cells to respond to concanavalin A treatment was not surprising since it has been shown that the sensitive and resistant cells have different cell surface charge properties (1). In addition, it has recently been shown that P1798 resistant cells have a lower number of insulin receptors than do sensitive cells (6). Perhaps the number of concanavalin A-binding sites in sensitive cells and resistant cells also differ. This possibility is open to future study. However, both resistant and sensitive cells respond similarly to concanavalin A with respect to clumping. If clumping is related to the number of concanavalin A-binding sites, then both cell types may have similar numbers of concanavalin A-binding sites. Of course, this is still speculation.

Thus, P1798 sensitive and resistant cells present a complex picture. A single mechanism for resistance may be difficult to formulate, inasmuch as additional differences between the 2 lines become more apparent. It may be that it is through the cooperative action of several relatively small defects that the resistant P1798 tumor fails to respond to glucocorticoid treatment rather than via a single mechanism.

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REFERENCES

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