The Dexamethasone Receptor in the Novikoff Hepatoma

CHARACTERIZATION AND CHANGES IN CONCENTRATION AND NUCLEAR UPTAKE DURING GLUCOCORTICOID THERAPY

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Novikoff hepatoma ascites cells, grown intraperitoneally in rats, are shown to possess a high-affinity, low-capacity dexamethasone-binding protein. The receptor protein has an intracellular localization and concentration, association constant ($K_a$), glucocorticoid specificity and nuclear uptake in the presence of dexamethasone comparable with those of the G-protein of rat liver. During therapy of the tumour-bearing animal with cortisol, marked cyclic variations were observed in the concentration and nuclear uptake of the putative G-protein in the tumour cells; more transient variations were also observed in the $K_a$ value of the receptor protein.

Previous studies from this laboratory (Webb & Wozney, 1968; Raab & Webb, 1969; Rizzo et al., 1971; Dorman & Webb, 1974) demonstrate the reversible inhibitory effect of the glucocorticoids on DNA synthesis and mitosis in regenerating rat liver induced by partial hepatectomy. Thus a low dose of cortisol administered 19h after operation, although suppressing the initial burst of DNA synthesis normally occurring between 20 and 30h, released DNA synthesis in a highly synchronous peak centred at 35h. Loeb et al. (1973) have demonstrated the suppression by glucocorticoids of DNA synthesis in cultured hepatoma cells derived from slow-growing rat hepatoma 5123D. A dexamethasone-binding protein has been identified in a rat hepatoma in tissue culture (HTC cells) although this particular line is insensitive to glucocorticoids (Higgins et al., 1973; Singer et al., 1973).

The present paper describes the characteristics of the dexamethasone-binding system in the rapidly growing Novikoff hepatoma cells and the temporal changes therein, during therapy of the tumour-bearing rats with glucocorticoids. This study was patterned after comparable studies (Keefer et al., 1974, 1975) that established a correspondence between fluctuations in uridine kinase isoenzymes and the development in vivo of tumour-cell resistance to 5-azacytidine.

Materials and Methods

Ascites-tumour cells

Novikoff ascites-tumour cells with a transplant generation time of 7 days were carried in the intraperitoneal cavity of 140-160g female rats of the Sprague-Dawley strain (Laboratory Supplies, Indianapolis, Ind., U.S.A.). The aspirated tumour cells were washed in cold balanced-salt solution (0.13 m-NaCl–5.0mM-KCl–8.0mM-MgCl$_2$) until free of erythrocytes, before reinoculation or experimental use. The tumour line was maintained by inoculating with 2.0ml of a 1:3 (v/v) dilution of washed cells pelleted at 2000g for 5min. Since the cells used in each experiment were derived from several animals the tumour-cell suspension used for reinoculation was also derived from these animals to ensure that the tumour-cell line was representative of the cells used in each experiment.

Before assay of the glucocorticoid-receptor proteins, the washed cells were incubated at 37°C in Eagle's Minimal Essential Medium (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.) fortified with 2.2g of NaHCO$_3$, 0.1mg of streptomycin sulphate and 10000 units of penicillin G/litre, to deplete the cells of all endogenous corticosteroid. After washing in the balanced-salt solution, the cells were resuspended in 1.0vol. of homogenization buffer consisting of 50mm-Tris–HCl, pH7.8, 1.0mm-dithiothreitol, 1.0mm-MgCl$_2$ and 2.0mm-CaCl$_2$.

Preparation of cytosol and nuclei

The cytosol fraction was prepared from a slurry of the ascites cells in homogenization buffer by first permitting the cells to swell for 10min at 0°C, shearing them with 25 strokes of a tight-fitting Dounce homogenizer, then centrifuging the homogenate at 105000g for 60min. The 105000g supernatant or cytosol fraction was assayed immediately for glucocorticoid-binding activity. For the preparation of nuclei, 1vol. of washed cells was resuspended in medium A (0.3m-sucrose–2.0mm-magnesium acetate–3.0mm-CaCl$_2$–0.5mm-dithiothreitol–0.1% Triton X-100–10.0mm-Tris–HCl, pH8.0), then homogenized with 20 strokes in a Dounce homogenizer. After
dilution with 1 vol. of medium B (2.0 mM-sucrose–3.0 mM-magnesium acetate–2.0 mM-CaCl₂–0.5 mM-dithiothreitol–10.0 mM-Tris–HCl, pH 8.0), a portion of the homogenate was layered over 3.0 ml of medium B and the discontinuous gradient was centrifuged for 1 h at 34900g. The nuclei were suspended in 1.0 mM-sucrose (complete medium B diluted 1:1 with water), then collected by centrifugation for 5 min at 3020g. After suspension of the nuclei in 1.0 mM-sucrose, their concentration was estimated by counting in a haemocytometer. The DNA recovery was 65% (corrected for mitochondrial DNA) based on DNA analysis of the homogenate and nuclei, or 70%, based on the number of nuclei recovered and number of cells from which they were derived.

**Steroid-binding and nuclear-uptake studies**

The modified steroid-binding assay (Beato & Feigelson, 1972) contained 1.0–16.0 pmol of labelled steroid (sp. radioactivity 17000 c.p.m./pmol) with or without unlabelled steroid and 100 µl of cytosol, in a total volume of 200 µl. After incubation for 2 h at 0°C, the protein-bound steroid was assayed by use of DEAE-cellulose filter discs (DE-81 filter; Whatman Co., Freehold, N.J., U.S.A.) presoaked for 1 h in 0.02 M-Tris–HCl buffer, pH 7.9 at 4°C, containing 1.5 mM-EDTA (Santi et al., 1973). Specific binding was estimated by subtracting the non-specific binding occurring in the presence of a 500-fold excess of unlabelled dexamethasone in 15% (v/v) ethanol. The latter was dissolved in 15% ethanol, the concentration in the assay being less than 2%; this ethanol did not alter the steroid-binding reaction (Baxter & Tomkins, 1971; S. Liu, unpublished observations).

The nuclear uptake of receptor-bound labelled glucocorticoid was estimated (Kalimi et al., 1973) by incubating the unfractinated cytosol receptor proteins at 0°C for 2 h in a 60-nM solution of the labelled glucocorticoid. The free steroid was removed by use of dextran-coated charcoal, then the glucocorticoid-charged cytosol was incubated for 30 min at 0°C (controls), or at 20°C (experimental) with samples of the nuclei. The volume of the reaction mixture was adjusted to 1.0 ml with 50 mM-Tris–HCl buffer, pH 7.8, containing 1.0 mM-dithiothreitol, 1.0 mM-MgCl₂ and 2.0 mM-CaCl₂. After incubation, the nuclei were washed with 2×5.0 ml of medium B diluted 1:1 with water, then dissolved in solubilizer before radioassay in liquid scintillant (Isolab, Akron, Ohio, U.S.A.).

**Protocol for the development and assay of resistance (cf. Aronow & Gabourel, 1962)**

To develop resistance to glucocorticoids, rats bearing the Novikoff ascites-tumour cells received a subtherapeutic intraperitoneal dose of cortisone acetate (25 mg/kg) on days 2, 4 and 6 after the intraperitoneal inoculation of the tumour cells, each transplant generation (i.e. each 7 days). Alternatively, to test for glucocorticoid resistance in any one transplant generation, the tumour-bearing animals received 0.85% NaCl (controls) or 75 mg of cortisone acetate/kg on days 4, 5 and 6 after inoculation of the cells, then on day 7 cells were removed for assay of the rate of DNA synthesis. The latter parameter was measured by incubating the washed Novikoff ascites-tumour cells in Eagle's Minimal Essential Medium containing 5 µCi of [³H]thymidine for 20 min at 37°C. After incubation the DNA was extracted from the washed cells for the determination of its specific radioactivity, as described by Rizzo et al. (1971). The rate of DNA synthesis is expressed as c.p.m. incorporated per 1×10⁷ cells. The DNA content of nuclei purified from the Novikoff hepatoma cells was measured with diphenylamine (Rizzo et al., 1971).

**Results**

Novikoff ascites cells exhibited only minimal dexamethasone-binding activity when assayed immediately after their removal from the intraperitoneal cavity, since the animals were not adrenalectomized. However, the relevant receptor protein is readily detectable in the cytosol fraction after a 2 h incubation of the cells in vitro at 37°C in Eagle's Minimum Essential Medium to deplete the cells of endogenous glucocorticoids. Since no further change occurred in the binding capacity of the cytosol of cells derived from untreated or glucocorticoid-treated rats on further incubation, a preincubation period of 2 h was selected for all subsequent experiments.

Table 1 indicates that after a 1 h incubation of the ascites-tumour cells in medium containing [³H]dexamethasone, approx. 50% of the radioactivity is located in the nucleus, whereas very little is present in the cytoplasm in a bound form. These data, which are consistent with results on steroid-hormone-responsive tissues (Jackson & Chalkey, 1974; Wira & Munck, 1974), are explained by the presently accepted mechanism of intracellular glucocorticoid transport (Kalimi et al., 1973). According to this mechanism (Thompson & Lipmann, 1974) the nuclear uptake of the glucocorticoid depends on its binding to a transport or receptor protein which is present in limited concentration in the cytoplasm. Thus after 1 h incubation most of the dexamethasone–receptor complex has translocated to the nucleus, leaving very little (0.7%) bound steroid in the cytoplasm. Wira & Munck (1974) also found a rapid transfer of most of the bound cortisol in the cytoplasm of rat thymus cells to a nuclear form when the temperature...
was raised from 3° to 37°C. These initial results strongly supported the presence of a functional receptor system for dexamethasone in the cytoplasm.

Subsequent studies were directed toward the characterization of the receptor protein. The time-course of the binding of dexamethasone with the protein and the saturation kinetics with dexamethasone are presented in Fig. 1. Binding was completed within 120 min of incubation at 0°C, and near saturation of the receptor was attained at a dexamethasone concentration of 80 nM. An analysis of the data by the method of Scatchard (1949) gave an association constant (K_a) of 1.3 x 10^8 M^-1 with dexamethasone; the concentration of the receptor sites was estimated to be 1.7 x 10^-9 M.

Table 2 shows that these values are very comparable with those reported for normal rat liver and for hepatoma tissue-culture cells.

The data in Table 3 compare the ability of various non-radioactive steroids to compete with [3H]dexamethasone for the putative G-protein receptor in the Novikoff cells. The profile of the dexamethasone-binding protein in the Novikoff cells is very similar to that reported for the dexamethasone receptor in rat hepatoma (HTC) cells in culture (Baxter & Tomkins, 1970), lactating mammary glands (Shyamala, 1973) and leukaemic blast cells (Lipmann et al., 1973). The specificity of the binding protein appears, therefore, to be very similar to that of the dexamethasone-binding protein characterized in several other tissues.

Fig. 2 indicates that the dexamethasone-receptor protein in the cytosol of Novikoff hepatoma cells is

Table 1. Subcellular distribution of dexamethasone in Novikoff ascites cells

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Radioactivity from 1 x 10^6 cells (c.p.m.)</th>
<th>Radioactivity (% of total in homogenate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total homogenate</td>
<td>5134</td>
<td>100</td>
</tr>
<tr>
<td>Nuclei</td>
<td>2670</td>
<td>52.0</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>329</td>
<td>6.4</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>585</td>
<td>11.4</td>
</tr>
<tr>
<td>Cytosol-protein bound</td>
<td>36</td>
<td>0.7</td>
</tr>
<tr>
<td>Cytosol-free</td>
<td>1514</td>
<td>29.5</td>
</tr>
</tbody>
</table>

Fig. 1. Kinetic properties of the dexamethasone-binding protein of Novikoff ascites cells

All cells were preincubated for 2 h at 37°C in Eagle's Minimal Essential Medium before assay. (a) The cytosol fraction was incubated for various times with 50 nM-[3H]dexamethasone before analysis. (b) The cytosol fraction was incubated with various concentrations of [3H]dexamethasone for 2 h at 0°C before analysis; insert is Scatchard (1949) analysis of the data. All values represent duplicate analysis and have been corrected for non-specific binding. The cytosol protein was 20 mg/ml.
Table 2. Comparison of kinetic parameters of dexamethasone receptors

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Hepatic tissue</th>
<th>Liver*</th>
<th>HTC cells†</th>
<th>Novikoff‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Association constant (M⁻¹)</td>
<td>2.5x10⁶</td>
<td>3.3x10⁸</td>
<td>2.6x10⁻⁹</td>
<td>1.3x10⁸</td>
</tr>
<tr>
<td>Receptor concentration (m)</td>
<td>1.2x10⁻⁸</td>
<td>1.7x10⁻⁹</td>
<td>1.7x10⁻⁹</td>
<td></td>
</tr>
</tbody>
</table>

* Data from Beato & Feigelson (1972).
† Data from Baxter & Tomkins (1971).
‡ S.E.M. values for this data are shown in Fig. 3.

Table 3. Effect of various unlabelled steroids on the binding of [³H]dexamethasone to cytoplasmic proteins

The data represent the average of three determinations ± S.E. The [³H]dexamethasone was present in the binding assays at a concentration of 100 nm.

<table>
<thead>
<tr>
<th>Non-radioactive steroid (100-fold excess)</th>
<th>[³H]Dexamethasone bound (% of control value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>99±4</td>
</tr>
<tr>
<td>4-Androsten-3,17-dione</td>
<td>113±7</td>
</tr>
<tr>
<td>β-Oestradiol</td>
<td>92±6</td>
</tr>
<tr>
<td>Diethylstilboestrol</td>
<td>80±5</td>
</tr>
<tr>
<td>Oestrial</td>
<td>76±27</td>
</tr>
<tr>
<td>11α-Hydroxyprogesterone</td>
<td>74±21</td>
</tr>
<tr>
<td>Testosterone</td>
<td>60±13</td>
</tr>
<tr>
<td>17α-Methyltestosterone</td>
<td>58±14</td>
</tr>
<tr>
<td>Progesterone</td>
<td>22±9</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>13±5</td>
</tr>
<tr>
<td>Cortisol</td>
<td>11±4</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>11±2</td>
</tr>
<tr>
<td>Triamcinolone</td>
<td>10±4</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>8±2</td>
</tr>
<tr>
<td>Dexamethanone</td>
<td>5±3</td>
</tr>
</tbody>
</table>

Table 4. Comparison of kinetic parameters of dexamethasone receptors

lable. Thus approx. 50% of the binding activity is lost after a 10 min incubation at 35°C and after storage for 24 h at 4°C. The stability of the receptor is, however, enhanced by the presence of 60 nm-dexamethasone. In terms of both kinetic and stability characteristics the dexamethasone-binding protein in these hepatoma cells appears to be identical with that present in normal rat liver (Beato & Feigelson, 1972).

Of particular interest is the fluctuation in several parameters of the receptor protein in the ascites-tumour cells during treatment of the tumour-bearing animal with glucocorticoids, since the latter are commonly used as cancer chemotherapeutic agents. The results summarized in Fig. 3 show that changes occur in (a) the concentration of the receptor sites, i.e. nmol of dexamethasone bound/mg of cytosol protein, (b) the concentration of putative nuclear binding sites, i.e. pmol of dexamethasone-receptor complex/1x10⁷ nuclei, and (c) the association constant (Kₐ) for the dexamethasone-receptor complex during therapy over a period of 14 transplants with cortisone acetate. (The latter glucocorticoid was selected as the chemotherapeutic agent, since it has prolonged action in vivo and protocols are available for the induction of tumour-cell resistance in animal systems.) Although these experiments have extended over a period of 14 weeks, they have been duplicated. In a second series of experiments the initial peak in nuclear binding was centred at generation 2, rather than generation 1. Aside from this minor exception, the time-dependence and the magnitude of the cyclic changes were identical (i.e. duplicated to within 15%). The standard errors, shown for the control (untreated) animals, are based on four to six rats.

By generation 12 the receptor-protein concentration appears to have at least temporarily stabilized at approx. 50% of the control value. The values shown represent the saturation values from the dexamethasone concentration-dependence curve (cf. Fig. 1b) and were consistently 10–12% lower than the number of binding sites (n) calculated from the Scatchard (1949) plot. Marked cyclic increases occurred in the nuclear binding (uptake) parameter, and in some cases the cycle extended over approximately four transplant generations. A transient but reproducible increase also was observed in the Kₐ of the dexamethasone receptor protein in generations 1 and 6.

The data in Table 4 suggest that after 7 or 12 generations of treatment in vivo with low doses of cortisone acetate, the tumour cells exhibit significant resistance to therapeutic doses (75 mg/kg) of the glucocorticoid, in contrast with the sensitive line carried in the untreated animals. Concurrent measurements (results not shown) ruled out the possibility that this loss of sensitivity in the resistant line was due to decreased uptake of the steroid by the cells, the kinetics and capacity for cellular uptake being identical in the sensitive and resistant lines. The ratios of [³H]thymidine incorporation into DNA of control and cortisone-treated tumour cells was confirmed by mitotic counts. Thus the [³H]-thymidine incorporation into the sensitive line was inhibited by 54%, whereas mitosis was inhibited by 62% (cf. legend to Table 4).

Thus the differences in DNA synthesis as
All cells were preincubated for 2 h at 37°C in Eagle’s Minimal Essential Medium before assay. (a) The cytosol fraction was incubated for 10 min at the indicated temperature followed by the addition of 60 nM-[3H]dexamethasone and subsequent incubation for 2 h at 0°C before analysis. The data are presented as percentages of the binding activity of the zero-time control cytosol. (b) The cytosol fractions were stored at 4°C for the indicated time either with (●) or without (○) 60 nM-[3H]dexamethasone, followed by incubation for 2 h with 60 nM-[3H]dexamethasone before analysis. The data are presented as percentages of the zero-time control which represents the binding activity of freshly prepared cytosol. All data represent duplicate assays.

Rats carrying Novikoff ascites-tumour cells received 25 mg of cortisone acetate/kg intraperitoneally on days 2, 4 and 6 of each transplant generation, the cells being transferred and assayed on day 7. Each value is based on at least three rats, and the standard error was less than 10%.

measured by [3H]thymidine incorporation is not due to fluctuations in the metabolic pools of thymidine in the cells. However, clonal-type selection appears to have occurred during therapy since the DNA content in generations 8 and 13 was 29.0 and 31.5 μg/1 × 10⁶ nuclei respectively, as compared with only 15.8 μg/1 × 10⁶ nuclei in both the sensitive line and generation 4.
Table 4. Effect of glucocorticoid treatment in vivo on DNA synthesis in cells from sensitive and resistant lines

The cortisone acetate was administered for 3 days. Values±s.e.m. (numbers of rats in parentheses) are recorded where three or more rats were used in the measurements. Mitotic values in the sensitive-controls and sensitive-acetate-treated cell lines were 3.4 and 1.3%, respectively.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cortisone acetate (75 mg/kg per day)</th>
<th>10^3 [3H]Thymidine incorporated (c.p.m./1×10^7 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sensitive</td>
<td>-</td>
<td>205±15 (6)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>94±3 (3)</td>
</tr>
<tr>
<td>2. Resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Generation 7</td>
<td>-</td>
<td>214</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>291</td>
</tr>
<tr>
<td>Generation 9</td>
<td>-</td>
<td>454</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>616</td>
</tr>
<tr>
<td>Generation 12</td>
<td>-</td>
<td>130±17 (3)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>100±20 (3)</td>
</tr>
</tbody>
</table>

Discussion

The present results confirm the existence in the rapidly growing Novikoff hepatoma cells of a glucocorticoid-binding protein with properties similar to the G-protein (dexamethasone-binding protein) in normal liver. Despite near-normal concentrations of a putative G-protein in the Novikoff hepatoma cells the enzyme tyrosine transaminase, which is inducible by cortisol in liver, is neither present in the tumour cells in detectable concentrations, nor is it induced by cortisol (Levitan & Webb, 1970; S. Liu, unpublished work).

The present study represents the first attempt to analyse the changes in the glucocorticoid-binding protein (more specifically the G-protein) during the early stages of the development in vivo of tumour-cell resistance to glucocorticoids, though many such studies have been carried out in tissue culture (cf. Thompson & Lipmann, 1974). The principal objective of this study was to select a tumour-cell line that was sensitive to glucocorticoids and to determine the fluctuation in certain parameters of the glucocorticoid-binding protein in response to therapy. By use of the Novikoff ascites cells, which are growth-inhibited but not killed by glucocorticoids, it was possible to follow alterations occurring in vivo, a situation more closely simulating the development of resistance under conditions of cancer chemotherapy.

The results of the present investigation demonstrate that significant changes do occur in the concentration of the dexamethasone-binding protein, its $K_a$ value and nuclear uptake during the development of tumour-cell resistance to glucocorticoids. No simple relationship can be proposed between the fluctuations in the parameters studied and the sensitivity to glucocorticoids. In particular, the significance of the fluctuations in the nuclear uptake of the dexamethasone-receptor complex is not apparent, since little is known about the biochemical significance of this parameter. The occurrence of increased amounts of the putative G-protein in generation 1, yet a standard amount of DNA per nucleus in generation 4, suggests that it is not related to clonal selection of a line that is highly polyploid, unless these changes are transient.

The fluctuations observed in the $K_a$ value are indeed of interest in view of evidence (Lipmann & Thompson, 1973) that more than one molecular species of the G-protein may exist. Differential changes in the relative concentration of two or more binding proteins could account in part for some of the cyclic changes observed in other parameters. Marked changes in the ratio of two isoenzymic forms of uridine kinase during the early phases of the development of resistance to 5-azacytidine was reported (Keefe et al., 1974, 1975). Reversible changes in key activating proteins during chemotherapy may be most relevant to the mechanisms underlying the development of irreversible tumour-cell resistance often due to the loss of the activating protein.

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References


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