Properties of Glucocorticoid Receptors in Epstein-Barr Virus-transformed Lymphocytes from Patients with Familial Cortisol Resistance

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Abstract

In a previous report of two patients with familial glucocorticoid resistance due to reduced numbers of glucocorticoid receptors (GR), we have shown decreased numbers of GR in peripheral mononuclear cells and cultured fibroblasts but normal affinity of GR in both patients. In this study, peripheral lymphocytes from these patients, one patient's son and daughter, and normal subjects were transformed with Epstein-Barr virus. Reduced numbers and normal affinity of GR were found in the Epstein-Barr virus-transformed lymphocytes from both patients while the son and daughter had normal numbers and affinity of GR. The thermal stability of GR and thermal activation of cytosolic receptors in both patients were found to be normal. Although the percentages of nuclear bound GR were similar in both patients and normal controls, the absolute amounts of nuclear bound GR of the patients were about one-half that of normal controls. These abnormal properties of GR (reduced numbers of GR) were preserved in the transformed cells from the patients.

Introduction

We have previously reported two patients in the same family with primary cortisol resistance accompanied by a decrease in numbers of GR in peripheral mononuclear cells (1). These patients, patient I (35-year-old male) and patient II (mother), had sustained hypercortisolism, but they presented no manifestation of Cushing's syndrome. Although there was a slight elevation of plasma deoxycorticosterone levels in patient I and corticosterone in patient II, both patients exhibited mild hypertension without hypokalemia. Both patients were partially resistant to Dex adrenal suppression. Reduced numbers but normal affinity of GR were found in the peripheral mononuclear cells from both patients and in the cultured skin fibroblasts from patient I (2). Tritiated thymidine uptake in the cultured fibroblasts from patient I was not inhibited to the extent observed with fibroblasts from normal individuals, indicating a resistance of the patient's cells to Dex (3). The aim of this study was to examine the properties of GR after transformation by EBV in lymphocytes obtained from the patients and other family members.

Materials and Methods

[3H]Dexamethasone (49 Ci/mmol) was purchased from New England Nuclear (Boston, MA); Dex, dithiothreitol, and EDTA from Sigma Chemical Co. (St. Louis, MO); DNA-cellulose was from Bio-Rad Laboratories, Inc. (Sydney, Australia).

Transformation of Lymphocytes with EBV

Sterile heparinized blood (20 ml) was collected from the patients, patient I's son and daughter, and ten normal subjects and diluted 2-fold with saline. The mononuclear cell fraction was separated by Ficoll-Hypaque density gradient centrifugation. The mononuclear cell suspensions (5 x 10⁶ cells/ml) were mixed with 1 volume of 10% sheep RBC suspensions in saline treated with neuraminidase and 1 volume of FCS and then incubated on ice for 2 h. After Ficoll-Hypaque density gradient centrifugation, the B-lymphocytes were separated from the T-lymphocytes which formed rosettes. The B-lymphocytes were washed twice with PBS, pH 7.4, and suspended at a density of 5 x 10⁶ cells/ml in RPMI 1640 supplemented with 20% FCS. Aliquots (0.2 ml) of B-lymphocyte suspensions were mixed with 0.1 ml of medium containing EBV-derived B95-8 cells (2.5-5.0 x 10⁶ cells/ml) in 24-well plastic culture plates. After incubation at 37°C for 1 h in an atmosphere of 5% CO₂-95% humid air, 0.8 ml RPMI 1640 supplemented with 20% FCS was added to each well. Four days later, the same volume of fresh medium was added. After 2 to 3 weeks, the transformed cells began to proliferate and were then transferred to 100-mm tissue culture plates and cultured in RPMI 1640 with 10% FCS.

GR Assay in Whole Cell

Whole cell binding studies were performed as previously described (1). Transformed cells were cultured in serum free RPMI 1640 for 24 h prior to assay and were judged 90% viable by trypan blue exclusion assay. Aliquots of these cells were incubated in RPMI 1640 with seven concentrations of [3H]Dex (0.31–20 nM) for 6 h at 24°C in the presence or absence of a 100-fold molar excess of unlabeled Dex. After incubation, the cells were washed with 3 ml ice-cold PBS three times to remove free steroid and centrifuged at 400 x g for 10 min. After the final wash, the pellets were counted for radioactivity. Specific binding was calculated by subtracting nonspecific binding from total binding and these data were analyzed according to the method of Scatchard (4).

Nuclear Binding of [3H]Dex

Transformed lymphocytes were incubated with four concentrations of [3H]Dex (0.31–2.5 nM) in the presence or absence of a 100-fold molar excess of unlabeled Dex at 37°C for 30 min. After incubation, the cells were washed three times with ice-cold PBS. One-fifth were used to determine whole cell binding and the remainder to determine nuclear binding. To obtain the nuclear fraction, cell pellets were suspended in 3 ml ice-cold hypotonic buffer (pH 8.0) containing 20 mM N-tris(hydroxymethyl)methylglycine, 2 mM CaCl₂, and 1 mM MgCl₂ and immediately frozen in ethanol-dry ice (5). After room temperature thawing, the lysed cells were centrifuged at 250 x g for 5 min at 4°C. The nuclear pellets were washed twice with hypotonic buffer and counted for the radioactivity. The DNA content was measured by a fluorometric method reported by Labarca and Paigen (6).

Preparation of Low-Speed Cytosol

Transformed lymphocytes were washed three times with room temperature PBS. Pellets were resuspended in ice-cold ETB buffer in an all-glass Dounce homogenizer using 20 manual strokes. These homogenates were centrifuged at 11,000 x g for 10 min. The supernatants represented the low-speed cytosol (2 mg protein/ml cytosol) (7). Protein content was measured by Bio-Rad protein assay.

DNA-Cellulose Chromatography

The low-speed cytosol was incubated with 50 nM [3H]Dex at 0°C for 4 h in the presence or absence of 100-fold molar excess of unlabeled Dex. Dextran-coated charcoal (final concentration, charcoal 1% (w/v), dextran 0.1% (w/v)) was added to the cytosol and centrifuged to remove unbound steroid. Activation was accomplished by incubating the cytosol at 25°C for 30 min, followed by an ice bath, and loaded onto a 1-ml 4% DNA-cellulose column that had been equilibrated with ETB buffer. After 15 min incubation, the column was washed with six column volumes of ETB buffer (8). The sample was eluted with ETB buffer containing stepwise addition of KCl (0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 M). Fractions (0.5 ml) were collected and their radioactivity was determined.

Thermostability of GR

Transformed lymphocytes were washed twice with room temperature PBS and preincubated in RPMI 1640 at 30°C, 37°C, and 42°C for 60 min; then 50 nM [3H]Dex in the presence or absence of 100-fold excess unlabeled Dex was added for 60 min. After this incubation at the indicated temperatures, cells were washed three times...
times with PBS and centrifuged, and the pellets were counted for radioactivity (8).

Results

Dex Binding in Whole Cells. The binding capacity of GR in the transformed cells from patients I and II were about one-half those of normal subjects (Figs. 1 and 2; Table 1). The apparent dissociation constant ($K_d$) was normal for both patients. The binding capacity and $K_a$ of GR in cells of the son and daughter of patient I were normal.

![Image of Fig. 1](https://example.com/f1.png)

**Fig. 1.** Specific dexamethasone binding to EBV-transformed lymphocytes. Cells obtained from patient I, patient II, patient I’s son and daughter, and then normal subjects were incubated concurrently for 6 h at 24°C. Shaded area, range of the ten normal subjects. Values were expressed as the mean ± SD (bars) of three experiments.

![Image of Fig. 2](https://example.com/f2.png)

**Fig. 2.** Scatchard analysis of dexamethasone binding to GR of EBV-transformed lymphocytes in patient I, patient II, patient I’s son and daughter, and ten normal subjects (shaded area). The number of receptors is indicated at the intercept of the x-axis, while apparent affinity is demonstrated by the slope of the line (slope = $1/K_a$).

![Image of Fig. 3](https://example.com/f3.png)

**Fig. 3.** DNA-cellulose chromatography GR-[3H]DEX complexes obtained from EBV-transformed lymphocytes of patient I, patient II, and five normal subjects after heat activation. Low-speed cytosol (1.0 ml; 2 mg protein/ml) was preincubated with 50 nM [3H]Dex for 4 h at 0°C. Activation of GR-[3H]Dex complexes was produced by a 30-min incubation at 25°C.

![Image of Table 4](https://example.com/t4.png)

**Table 4 GR-[3H]Dex complexes bound to DNA-cellulose column**

The percentages of nuclear receptors were calculated as counts for radioactivity of nuclear fraction divided by counts for radioactivity of whole cell binding, then multiplied by 100.

<table>
<thead>
<tr>
<th>Concentration of added [3H]Dex (nM)</th>
<th>% of nuclear receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.31</td>
<td>Patient I 15.2, 16.7</td>
</tr>
<tr>
<td>0.63</td>
<td>Patient II 17.3, 16.3</td>
</tr>
<tr>
<td>1.25</td>
<td>17.0, 19.6</td>
</tr>
<tr>
<td>2.50</td>
<td>17.3, 14.1</td>
</tr>
<tr>
<td>4.00</td>
<td>17.9 ± 2.1*</td>
</tr>
<tr>
<td>8.00</td>
<td>18.0 ± 2.4*</td>
</tr>
<tr>
<td>16.00</td>
<td>19.6 ± 1.6*</td>
</tr>
</tbody>
</table>

* Mean ± SD, $n = 8$.

Nuclear Binding. Table 2 shows the nuclear retention of GR-[3H]Dex complexes. The percentages of nuclear bound receptors of both patients were approximately those of normal subjects. Absolute amounts of nuclear receptors per 10 ng of DNA of both patients were reduced to about one-half those of normal subjects (Table 3).

![Image of Table 3](https://example.com/t3.png)

**Table 3 Absolute amounts of GR-[3H]Dex complexes bound to nuclei**

Absolute amounts of GR-[3H]Dex complexes bound to nuclei were calculated when cells were incubated with 2.5 nM [3H]Dex (Table 2).

<table>
<thead>
<tr>
<th>GR complexes (fmol/10 μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient I 2.8, 2.4</td>
</tr>
<tr>
<td>Patient II 3.5, 3.0</td>
</tr>
<tr>
<td>Control 5.8 ± 0.7*</td>
</tr>
</tbody>
</table>

* Mean ± SD, $n = 8$.

Nuclear Binding. Table 2 shows the nuclear retention of GR-[3H]Dex complexes. The percentages of nuclear bound receptors of both patients were approximately those of normal subjects. Absolute amounts of nuclear receptors per 10 μg of DNA of both patients were reduced to about one-half those of normal subjects (Table 3).

DNA-Cellulose Chromatography. GR-[3H]Dex complexes eluted in two peaks with ETG buffer containing 0.1 and 0.15 M KCl (Fig. 3). The elution pattern in both patients was similar to those of normal subjects. Table 4 summarizes the ratio of GR-[3H]Dex complexes retained on the DNA-cellulose column.
The binding capacity of GR in EBV-transformed lymphocytes from normal subjects was increased 7-fold over that of peripheral mononuclear cells. On the other hand, the binding affinity of GR in transformed lymphocytes from normal subjects remained the same as that of peripheral mononuclear cells. These results correspond to those of Tomita et al. (9) who reported that viral transformation of peripheral lymphocytes was associated with increased numbers of GR whereas other qualitative characteristics of the receptor remained similar. The binding capacity of GR in transformed cells of patients I and II was also increased 6- to 7-fold that of peripheral mononuclear cells, while the binding affinity of GR was the same as that of peripheral mononuclear cells. Therefore, the number of GR in these patients' cells was decreased to about one-half those of normal subjects. The affinity of GR in cells from both patients was about one-half those of normal subjects.

Discussion

The binding capacity of GR in EBV-transformed lymphocytes from normal subjects at various incubation temperatures (30°C, 37°C, and 42°C). The binding capacity of GR in cells from normal subjects was decreased with elevated temperature. At any temperature, the binding capacity of GR in cells from both patients was about one-half those of normal subjects.

versus retained plus flowthrough GR-[3H]Dex complexes in each subject. No significant difference was found in the DNA-cellulose binding of the activated GR from both patients as compared to those from normal subjects.

Thermotability of GR. Fig. 4 shows the specific binding capacity of GR in transformed lymphocytes at various incubation temperatures (30°C, 37°C, and 42°C). The binding capacity of GR in cells from normal subjects was decreased with elevated temperature. At any temperature, the binding capacity of GR in cells from both patients was about one-half those of normal subjects.

The percentages of GR-[3H]Dex complexes bound to nuclei in both patients were the same as normal ones. These results suggested that the transfer of GR-[3H]Dex complexes to nuclei in both patients was normal. Since the elution pattern in DNA-cellulose chromatography of GR-[3H]Dex complexes in both patients was similar to those in normal subjects, the affinity of GR in both patients' cells to DNA was normal. The ratio of GR-[3H]Dex complexes retained to the DNA column against the retained plus flowthrough in both patients was also normal. Therefore, heat activation of GR complexes in both patients appears to be accomplished normally. Although elevated temperature caused decreases in [3H]Dex binding to cells in both normal subjects and patients, at each incubation temperature, [3H]Dex binding to cells in patients was reduced to about one-half that in normal ones. Therefore, thermolability of GR in both patients was demonstrated to be normal.

Decreased numbers with normal affinity of GR in transformed lymphocytes from our patients with familial cortisol resistance were demonstrated as shown in peripheral mononuclear cells. No abnormalities of the patients' GR in nuclear binding, DNA binding, and thermolability were found. Since the abnormal properties of GR in these patients also remained in transformed lymphocytes, these cells appear to be a useful source for analyzing gene abnormalities.

Acknowledgments

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References

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