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Glucocorticoid-Resistant Glioma Cells That Contain Cytosolic Receptors (40854)[1]

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Abstract. The relationship between cytosolic glucocorticoid receptors and two different physiological responses produced by dexamethasone (DEX) was investigated in steroid-sensitive C6 glioma cells and newly-derived, steroid-resistant C6H cells. Cell density measurements showed that cell proliferation was suppressed in 7.6 μM DEX-treated C6 cultures relative to controls. Scanning electron microscopy revealed that DEX-treated C6 cells and their processes were flattened compared to control C6 cells. In contrast, DEX failed to produce a growth-inhibitory response and morphological alterations in C6H cultures. Whole cell assays for glucocorticoid receptors indicated that specific binding (cpm/mg protein) of [^{3}H]DEX and [^{3}H]triamcinolone acetonide was similar in C6 and C6H cells. Assays employing cell-free preparations also showed that C6 and C6H cells contained similar amounts of steroid binding to cytosolic receptor sites. Thus, the loss of two physiological responses to DEX action in C6H cells is due to some metabolic defect other than the loss of cytosolic glucocorticoid receptors.

The synthetic glucocorticoid dexamethasone (DEX) is employed routinely in the treatment of patients with malignant gliomas. Although the drug is used primarily to reduce cerebral edema associated with these neoplasms, there is also clinical evidence to suggest that these steroids may exert direct antitumor effects (1). Antitumor responses produced by glucocorticoids have also been observed in brain tumor cell cultures. For example, DEX suppresses cell proliferation in a dose-related manner in the C6 glioma model system *in vitro* (2). The mechanism underlying this growth-inhibitory response is not known. The generally accepted model for steroid action contends that the binding of the steroid to specific cytosolic receptor molecules is a necessary event for glucocorticoids to elicit physiological responses.

Certain target cells may develop resistance to glucocorticoid action. Murine fibroblast L cells, which are growth-inhibited by glucocorticoids, can develop resistance to the growth-suppressive effects of these steroids; this resistance has been shown to be associated with reduced cytosolic receptor binding (3). Similar observations have been reported for resistant leukemia cells (4, 5).

There is little information regarding the relationship between glucocorticoid receptors and physiological effects produced by these steroids in glioma systems. During studies designed to examine the relationship between glucocorticoid receptor binding and the growth-inhibitory response in C6 cells, we found a resistant strain (designated C6H) which fails to be growth-inhibited after 3 days exposure to 7.6 μM DEX. Therefore, the present investigation was initiated to characterize further this cell strain with respect to the growth-inhibitory and morphological responses to glucocorticoid treatment, and to compare amounts of glucocorticoid receptor binding in resistant C6H cells to those in sensitive C6 cells.

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Materials and methods.[3] *Cell cultivation.* Rat glioma C6 cells (6) (American Type Culture Collection, Rockville, Md.) have been subcultured in our laboratory for several years. The C6H strain was derived from the C6 line. C6H cells were subcultured in another laboratory under different nutritional conditions for many months prior to these studies. The number of metaphase chromosomes, as determined previously (7), in C6 and C6H cells was identical ($N = 42$). All C6 and C6H cultures were grown as monolayers at 37° in plastic tissue culture flasks containing Ham's F10 medium (8) (Pacific Biological Co., Richmond, Calif.) as described (9) except that the medium contained 1 μg/ml amphotericin B (North American Biologicals, Miami, Fla.) and 50 μg/ml gentamicin (Schering Corp., Kenilworth, N.J.) rather than penicillin–streptomycin.

Assay for growth inhibition. The cell proliferation experiments were initiated by seeding cells at densities of $\sim 10^5/cm^2$. On the following day (designated Day 0), the cultures were divided into groups. One group each of C6 and C6H cultures served as the controls and received fresh medium. The remaining groups of cultures (one each of C6 and C6H) were treated with fresh medium plus 7.6 μ*M* DEX (disodium phosphate; Merck Sharp & Dohme Research Laboratories, West Point, Penn.). DEX stock solutions and medium containing the steroid were prepared immediately prior to use (10).

At daily intervals, the culture medium was discarded from triplicate cultures from each group and the monolayers were washed five times with phosphate-buffered saline (PBS, pH 7.4). The cells were detached from the flask with 0.5 m*M* ethylenediamine tetraacetic acid (EDTA) and were counted with a hemacytometer. Mean cell densities ± S.E./cm^2 of flask area were calculated. The Student's *t* test (two tailed) was employed to determine if mean values obtained from the treated cultures differed significantly ($p < 0.050$) from control values.

Morphological studies. Control and DEX-treated C6 and C6H cells were prepared for scanning electron microscopy (SEM) by fixation *in situ* with 3% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) for 2 hr at 37°. The cells were postfixed in 1% osmium tetroxide for 30 min followed by dehydration through a graded series of water–ethanol solutions to 100% ethanol. The specimens were critical point dried, coated with gold, and examined with a Zeiss Novascan 30 microscope.

Glucocorticoid receptor binding assays. Assays employing whole cell preparations and cell-free extracts described by Hackney *et al.* (3) were used to measure glucocorticoid receptor binding in C6 and C6H cells.

For whole cell assays, packed cells (0.1–0.2 ml) were resuspended in 2 ml Earle's balanced salt solution (BSS, pH 7.4) supplemented with 0.1% glucose. These preparations also contained 0.01 μ*M* [^{3}H]triamcinolone acetonide ([^{3}H]TA, 33.7 Ci/mmole; New England Nuclear, Boston, Mass.) or 0.1 μ*M* [^{3}H]DEX (35.4 Ci/mmole; Amersham/Searle Corp., Arlington Heights Ill.) with or without a 100-fold excess of the respective unlabeled steroid. In some experiments, a 100-fold excess of epicortisol (Research Plus Steroid Laboratories, Inc., Denville, N.J.), the inactive stereoisomer of cortisol, was substituted for unlabeled TA or DEX. These whole cell preparations were incubated for 30 min at 37° with gentle agitation after which they were centrifuged at 600*g* at 4°. All subsequent steps were performed at 0–4°. The cell pellets were washed of excess radioactivity by resuspension in BSS followed by low-speed centrifugation. The sedimented cells were resuspended in 0.2 ml hypotonic buffer (10 m*M* *tris* (hydroxymethyl) aminomethane–HCl, 0.4 m*M* EDTA, pH 7.5). Cell disruption was accomplished with a Dounce homogenizer (20 strokes with the tighter fitting pestle). The nuclei and cellular debris were pelleted by centrifugation (1000*g*) and the supernatant

[3] Dexamethasone is 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione. Triamcinolone acetonide is 9α-fluoro-11β,16α,17α,-21-tetrahydroxypregna-1,4-diene-3,20 dione cyclic 16, 17-acetal. Epicortisol is 11α,17α,21-trihydroxy-4-pregnene-3,20-dione.

fluids were centrifuged at 105,000*g* for 60 min. The high speed supernatant fluids were chromatographed on Sephadex G-25 columns (0.7 × 10 cm) to separate protein-bound glucocorticoid from free steroid. Fractions obtained in this manner were assayed for radioactivity with a Packard liquid scintillation spectrometer (Model 3390) after 0.1 ml from each eluate was added to 5 ml Aquasol (New England Nuclear, Boston, Mass.). Fractions containing bound steroid were assayed for protein content by the method of Lowry *et al.* (11). Binding was calculated as cpm/mg protein. Specific binding is defined as the difference in binding measured between samples incubated with the labeled steroid alone and those samples which were incubated with labeled steroid plus excess unlabeled steroid.

The measurement of specific binding with cell-free preparations was carried out as described for the whole cell preparations except for the following modifications. All operations were performed at 0–4°. Cells packed by centrifugation were resuspended in hypotonic buffer to 2.5 times the packed cell volumes. After cells were disrupted with a Dounce homogenizer, supernatant fluids were obtained by centrifugation at 105,000*g* for 60 min. Aliquots of the supernatant fluids were incubated with 0.1 μM [^{3}H]DEX with or without a 100-fold excess of the unlabeled steroid. Samples were incubated for 4 hr. Protein-bound and free steroid were separated by chromatography on Sephadex G-25 columns, and specific binding values were calculated as indicated above.

Results. Our preliminary observations showed that DEX failed to suppress cell proliferation in C6H cultures treated for 3 days. Therefore, this effect was examined further by performing daily cell density measurements in control and 7.6 μM DEX-treated C6 and C6H cultures over a 5-day period. These measurements are presented in Fig. 1. Cell densities in the treated C6 cultures were 50% lower relative to controls between Days 4 and 5 (Fig. 1A). The kinetics of cell proliferation in control C6H populations were similar to those observed in control C6 cell populations (Fig. 1B). In contrast to the treated C6 cultures,

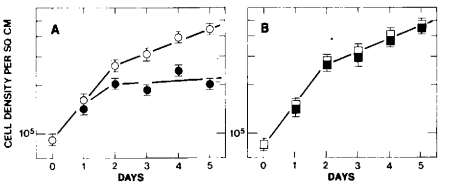
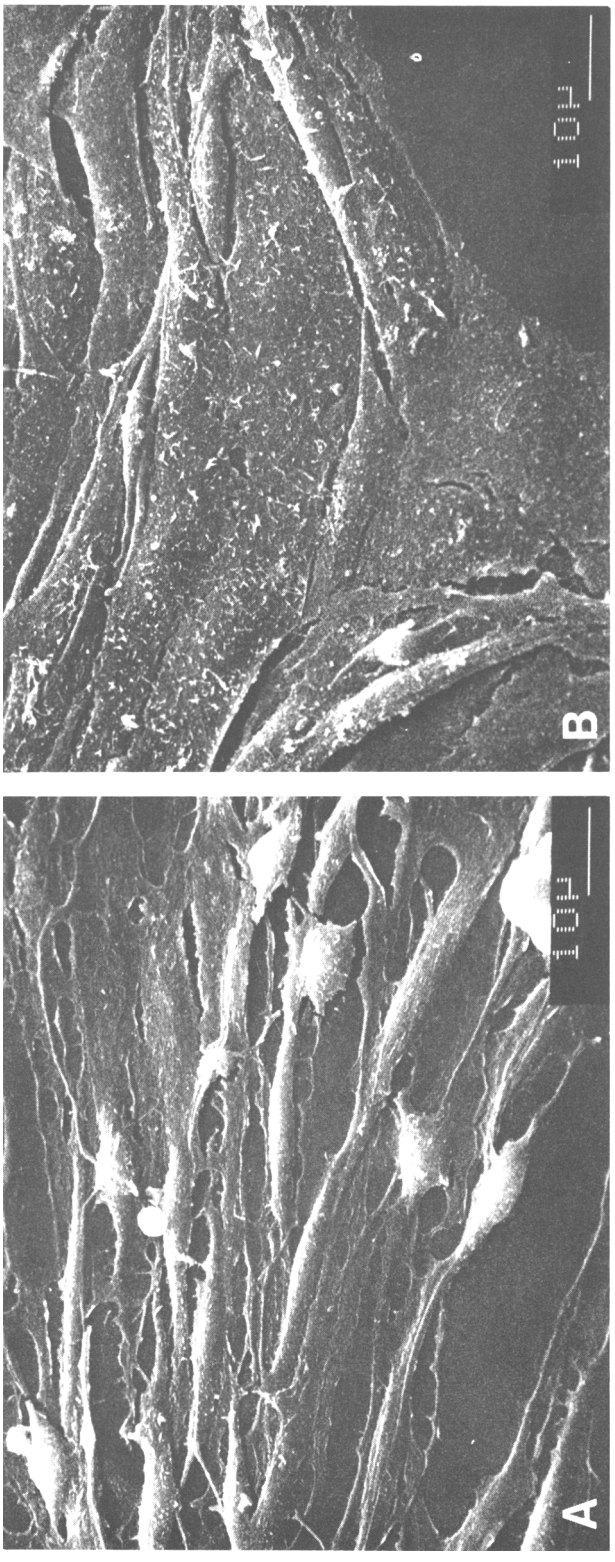


FIG. 1. Effect of dexamethasone (DEX) on cell proliferation in C6 and C6H cultures. The symbols represent mean values ± S.E. (A) Control C6 cells (○); 7.6 μM DEX-treated C6 cells (●; $p < 0.05$ on Days 2–5). (B) Control C6H cells (□); 7.6 μM DEX-treated C6H cells (■).

cell densities in DEX-treated C6H cultures did not differ significantly from densities measured in control C6H cultures. Thus, DEX fails to elicit a growth-inhibitory response in C6H cultures over the 5-day period.

SEM studies by Berliner *et al.* (12) indicate that the morphology of glucocorticoid-treated C6 cells is altered relative to control cells. These alterations include flattening of the cells and their processes over the flask surface. We therefore examined DEX-treated C6H cells to determine whether they would respond in the same manner. Accordingly, control and DEX-treated C6 and C6H cells were seeded at 10^4/cm^2 and were incubated for 4 days. SEM investigations revealed that control C6 cells appeared rounded or spindle-shaped with thin elongated processes (Fig. 2A). In contrast, the treated C6 cells and their processes appeared flattened after exposure to the glucocorticoid for 4 days (Fig. 2B). The morphology of control C6H cells (Fig. 2C) did not appear to differ from the morphology of control C6 cells. When C6H cells were exposed to DEX for 4 days, cell flattening did not occur; their morphology was similar to both control C6 and control C6H cells (Fig. 2D). Thus, DEX fails to elicit the morphological alterations in C6H cultures which occur in glucocorticoid-treated C6 cultures.

The knowledge that glucocorticoid resistance is often associated with diminished receptor binding suggested to us that C6H cells might display altered receptor binding compared to C6 cells. In order to determine whether both cell types contain receptors,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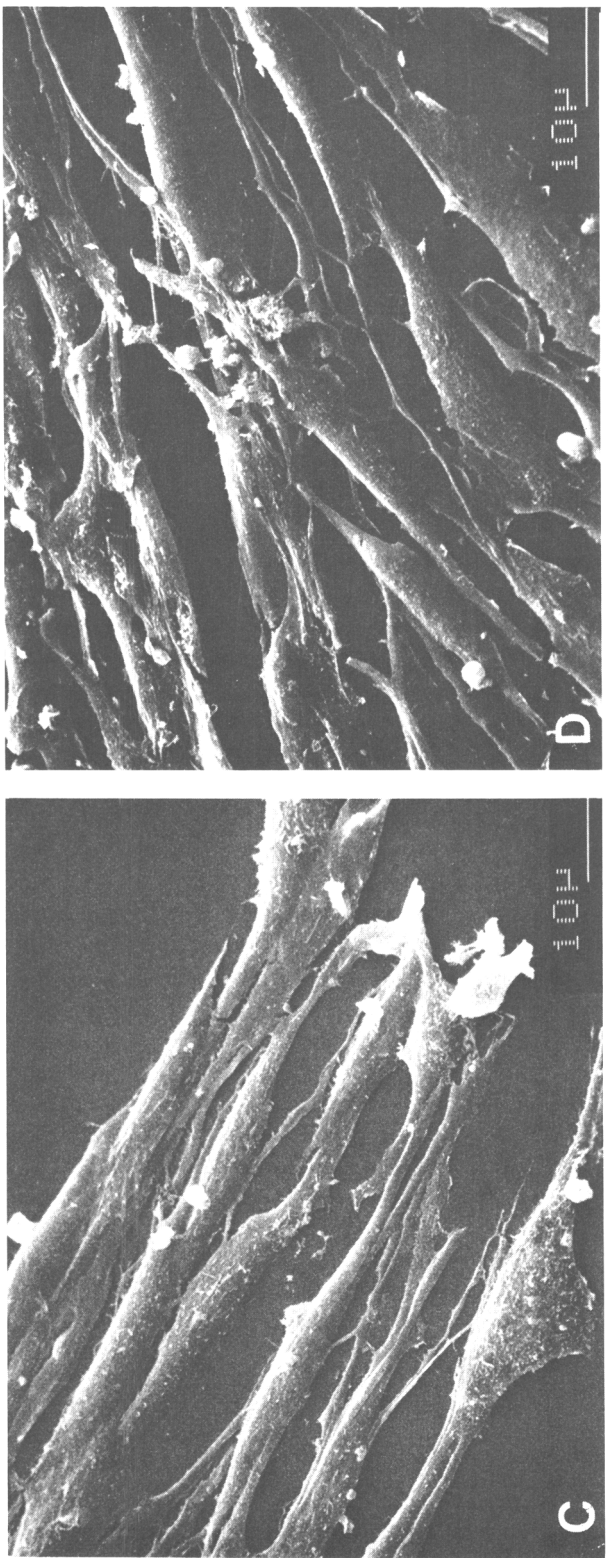

FIG. 2. Effect of dexamethasone (DEX) on the morphology of C6 and C6H cells. Cells were grown at low densities for 4 days in the absence or presence of 7.6 μM DEX prior to *in situ* preparation for SEM. The magnifications are $\times 1200$ in each case. (A) Control C6 cells. (B) 7.6 μM DEX-treated C6 cells. (C) Control C6H cells (D) 7.6 μM DEX-treated C6H cells.

TABLE I. SPECIFIC BINDING OF DEXAMETHASONE AND TRIAMCINOLONE ACETONIDE IN C6 AND C6H CELLS[a]

Expt	Day	Glucocorticoid	Specific binding (cpm/mg protein)	
			C6	C6H
1	5	DEX	3734 ± 371[b]	4449 ± 189[b]
2	5	TA	4080[c]	4184[c]
3	10	TA	5705[c]	6128[c]

[a] Whole cell assays for glucocorticoid receptor binding were performed as described under Materials and Methods.
[b] Mean ± S.E. of three replicate determinations.
[c] Single determinations.

the specific binding of [^{3}H]DEX and [^{3}H]TA in whole cell preparations was measured at two different times after C6 and C6H cells were established in culture. Receptor binding was examined on Day 5 because the growth-inhibitory response and cell flattening are clearly expressed in C6 cultures by this time. Receptor binding was examined 10 days after cultures were established because induction of glycerol phosphate dehydrogenase and nuclear binding of [^{3}H]cortisol are maximal in C6 cultures at this time (13). The results from three representative experiments employing whole cell preparations are shown in Table I. Expt. 1 confirmed previous findings that C6 cells contain cytosolic glucocorticoid receptors (14). In addition, this experiment demonstrated that C6H cells also contain receptors. The specific binding of [^{3}H]DEX in C6H cells did not differ significantly from that observed in C6 cells. Radiolabeled TA was substituted for [^{3}H]DEX in Expts 2 and 3; this steroid also produces a growth-inhibitory response in C6 cultures (9). The specific binding of [^{3}H]TA in C6H cells was similar to that which occurred in C6 cells on both Days 5 and 10. Cell-free preparations were also employed in specific binding measurements in order to obtain additional evidence that C6H cells contain glucocorticoid receptors. Table II clearly indicates that the specific binding of [^{3}H]DEX to cytosolic receptors extracted from C6H cells was nearly identical to that extracted from C6 cells.

In order to evaluate further the specificity of the glucocorticoid binding receptors, epicortisol was tested for its ability to compete with [^{3}H]DEX and [^{3}H]TA in specific binding experiments employing both whole cell and cell-free preparations. The results (data not shown) demonstrated that a 100-fold excess of epicortisol was unable to compete with the radiolabeled steroids for sites on C6 and C6H glucocorticoid receptors.

Discussion. In contrast to sensitive C6 glioma cells, C6H cells are resistant to the growth-inhibitory response and morphological alterations produced by DEX, even though C6H cells contain cytosolic glucocorticoid receptors.

Specific binding assays with whole cell and cell-free preparations indicate that receptor binding in C6H cells is similar to that which occurs in C6 cells. However, it is possible that this situation may not be true at other glucocorticoid concentrations. Dose–response studies utilizing Scatchard analyses are currently in progress to investigate this possibility. Although the specific binding of radiolabeled glucocorticoids is similar with both cell types, higher specific

TABLE II. SPECIFIC BINDING OF DEXAMETHASONE IN C6 AND C6H CELL-FREE EXTRACTS[a]

Cell type	Specific binding (cpm/mg protein)
C6	23,181 ± 1949[b]
C6H	23,756 ± 1706[b]

[a] Cell-free assays for glucocorticoid receptor binding were performed as described under Materials and Methods 5 days after the cells were established in culture.
[b] Mean ± S.E. of determinations from four separate experiments.

binding values are observed routinely with cell-free preparations compared to whole cell preparations. This discrepancy in the specific binding values which arises by the two different procedures is not surprising because the characteristics of glucocorticoid binding vary markedly, depending upon the method employed (15, 16).

The defect responsible for the refractoriness of C6H cells to DEX action remains to be identified. In several nonglioma tumor cells, the determination of cytosolic receptor binding has been used to predict the clinical effectiveness of glucocorticoid therapy. In general, glucocorticoid-resistant leukemic cells either have fewer receptors or contain receptors of lower binding affinity compared to sensitive cells (4, 5, 17, 18). In contrast, some glucocorticoid-resistant leukemic cell types do contain normal cytosolic receptor levels (19, 20), a situation which appears to be the case with C6H cells. In these leukemic and glioma cells, the alteration or defect responsible for resistance may reside at the translocation process, the binding of the steroid–receptor complex to chromatin, or at a point of regulation as yet undefined (19, 21).

The glucocorticoid-resistant C6H strain could be employed to extend our knowledge of the role of glucocorticoids in experimental brain tumor therapy. For example, glucocorticoid-treated rats bearing intracerebrally-implanted resistant C6H gliomas would be the ideal control for evaluating chemotherapeutic effects produced by various glucocorticoid regimens administered to animals harboring implanted sensitive C6 gliomas. Furthermore, a variety of potential drugs could be tested for their ability to exert direct antiglioma effects in DEX-treated rats bearing C6H tumors. Since glucocorticoids are employed routinely for palliation in brain tumor patients, this C6H *in vivo* model system closely simulates the clinical situation of DEX therapy administered to patients bearing steroid-resistant gliomas.

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