Comparison of Glucocorticoid Receptors Liganded with Dexamethasone or Progesterone (43318)

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Abstract. **The initial goal of this work was to examine directly the properties of glucocorticoid receptors bound with antagonists. Cortexolone, progesterone, and R-5020 were the antagonists studied. The tritiated agonists, dexamethasone and triamcinolone acetonide, served as controls. Although the three antiglucocorticoids interfered with agonist binding to the glucocorticoid receptor, direct binding of the tritiated antagonists could not be reproducibility demonstrated using either a charcoal assay or rapid techniques like high performance liquid chromatography or vertical tube rotor ultracentrifugation. Ultraviolet radiation was used to attach covalently tritiated steroid to the receptor. This technique allowed the identification of species that bound agonist or antagonist. That the two classes of steroids bound to the same receptor was shown using a monoclonal antibody directed against the glucocorticoid receptor. These labeled species had the same physical properties upon ultracentrifugation, DEAE cellulose chromatography, and high performance liquid chromatography. It is concluded that although the interaction of antiglucocorticoids like progesterone with the glucocorticoid receptor may be fleeting, antagonists do interact with the glucocorticoid receptor and form complexes with grossly similar properties as those derived from an interaction with agonists.** [P.S.E.B.M. 1991, Vol 198]

Elucidation of the structure and function of the glucocorticoid receptor has required synthetic steroids. The naturally occurring agonists, cortisol and corticosterone, dissociate so rapidly from the receptor that their interaction is difficult to monitor directly. Only recently has our laboratory been able to apply rapid techniques like high performance liquid chromatography (HPLC) and vertical tube rotor ultracentrifugation to characterize the size and shape of the corticosterone-glucocorticoid receptor complex (1). On the other hand, the synthetic steroids, dexamethasone and triamcinolone acetonide, have a high affinity for the receptor and remain bound long enough for studies of the complex (2).

The properties of glucocorticoid receptor complexes formed with antiglucocorticoids have been studied almost exclusively using synthetic steroids. Work has involved steroids like dexamethasone mesylate (3) and RU 486 (4). In general, results with these steroids lead to the conclusion that antiglucocorticoids bind to the same receptor as agonists and, in general, form similar complexes.

Although this conforms nicely to the current dogma of steroid hormone action, there are still reasons to question whether these results accurately predict the interaction of potential "natural" antiglucocorticoids. Dexamethasone mesylate, for example, is a nonreversible probe that labels covalently the receptor (3). It is possible that this property restricts its interaction with the receptor at sites other than the agonist site. Indeed, in some cases agonist steroids when modified with mesylate can act as either agonists or antagonists (5).

Currently, RU 486 is the most widely studied antiglucocorticoid. This compound, however, is also an antiprogestin (6). Progesterone, is the most widely recognized naturally occurring steroid that has antiglucocorticoid hormone action (7). Whether antiprogestins and progestins interact identically with the glucocorticoid receptor is not established, even though it is known that both may act as antiglucocorticoids. Our laboratory has published evidence that progestins and antiproges-

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tins may not interact with the glucocorticoid receptor in the same way (8); progestins enhance the rate of agonist dissociation from the glucocorticoid receptor while RU 486 does not (8–10). We have interpreted this data as indicating these two classes of compounds interact with different portions of the glucocorticoid receptor (11). We have proposed that "progestin-like" antiglucocorticoids have a different binding site on the glucocorticoid receptor than agonists and antagonists like RU 486.

To form a more complete understanding of antiglucocorticoid hormone action, we decided to investigate more fully the interaction of progestins with the glucocorticoid receptor. In this article we report our studies on the binding of tritiated progesterone to the glucocorticoid receptor and the structure of that resulting complex.

Materials and Methods

Already reported are the techniques used for growing and harvesting AtT-20 cells (12), preparing particle-free cytosol (13), diluting steroids (14), steroid incubation (15), the charcoal-dextran assay (15), intact cell incubation (16), DEAE cellulose chromatography (17), sucrose gradient ultracentrifugation (18, 19), the definition of displaceable binding (15), high performance liquid chromatography (1), and vertical tube ultracentrifugation (1).

Covalent labeling of the glucocorticoid receptor with tritiated agonist or antagonist was carried out by an adaptation of the technique described by Ilenchuk and Walters (20). AtT-20 cells from 8 liters of cell culture were gathered and washed once with Buffer I. After this all steps were carried out at 0 to 4°C. Approximately 10 ml of packed cells were obtained and lysed using a metal dounce with 20 ml of Buffer III. Particle-free cytosol was prepared and divided into two 10-ml fractions. One was made 40 n*M* in tritiated dexamethasone or triamcinolone acetonide and the other 40 n*M* in tritiated progesterone or R-5020. After an overnight incubation, the samples were placed atop a Spectroline model TR-302 transilluminator (302-nm ultraviolet; Spectronics Corporation, Westbury, NY) for 20 min. The samples were in 12- × 75-mm polycarbonate tubes kept 3 cm above the box in an ice bath. Charcoal-dextran was used to removed free steroid. The usual procedure (15) was modified, in that the charcoal was pelleted in the tube before addition of the cytosol. The cytosol was shaken for 10 min and repelleted by centrifugation in a Beckman model TJ-6 refrigerated centrifuge at 2000 rpm for 20 min. Each sample was partially purified by sequential DEAE cellulose chromatography, ammonium sulfate precipitation, and HPLC. The DEAE column had a 20-ml bed, was washed with 50 ml of Buffer III, and then eluted with a 160-ml linear gradient of 0 to 0.4 *M* KCl in Buffer III. Four-milliliter fractions were collected and 200-μl aliquots assayed for radioactivity. The tubes in the peak of radioactivity were combined and made 40% in ammonium sulfate. The precipitate formed was collected using a Beckman 13.1 rotor at 13,000 rpm for 30 min. The precipitates were redissolved in 2 ml of Buffer III and placed on an HPLC setup that was comprised of TSK 3000 and TSK 4000 columns and eluted as described (1). Each fraction contained 1.5 ml, of which 25 μl was removed for radioactivity determination. The peak was collected. Some of these pooled samples of the peak were analyzed by sucrose gradient ultracentrifugation (Fig. 6), DEAE cellulose chromatography (Fig. 7) or HPLC (Fig. 7). The DEAE cellulose chromatography utilized a 0.5-ml sample and a 2-ml bed of resin. It was eluted with 80 ml of 0 to 0.4 *M* KCl linear gradient in Buffer III. The HPLC also used a 0.5-ml sample and tandem TSK 2000 and TSK 3000 SW columns with Buffer IV run at 0.5 ml/min with 0.5-ml collections.

Buffer ingredients were as follows: Buffer I—10 m*M* Tris-HCl, 154 m*M* NaCl (pH 7.4); Buffer II—50 m*M* Tris-HCl, 1 m*M* EDTA, 6 m*M* monothioglycerol (pH 7.4); Buffer III—Buffer II made 5 m*M* in Na_2MoO_4; and Buffer IV—Buffer II without monothioglycerol.

Results

Most of these studies involve the cloned AtT-20 mouse pituitary tumor cell. This cell line secretes ACTH in a glucocorticoid-responsive manner (14) and contains glucocorticoid receptors that have been extensively characterized (10). Antiglucocorticoid interactions in this system are monitored by competition of agonist binding to this receptor (9, 15).

The results shown in Figure 1 demonstrate that the antagonists progesterone, R-5020 (a synthetic progestin), and cortexolone (11-deoxycortisol) all inhibit intact cell binding of tritiated dexamethasone in a manner similar to that demonstrated by nonradioactive competing agonists (dexamethasone, triamcinolone acetonide, and corticosterone). Their order of competitive potency suggests that these antiglucocorticoids interact with the receptor with less affinity than any of the agonists. Progesterone may have an affinity similar to that of corticosterone, an agonist whose glucocorticoid receptor complexes we were recently able to identify (1).

Competition can also be demonstrated using the isolated cytosol glucocorticoid receptor *in vitro*. Figure 2 reveals the relative competitive potencies of dexamethasone and two progestins, progesterone and R-5020, and the antiprogestin RU 486. The latter steroid is clearly the most effective; it binds to the receptor with an affinity even higher than that of dexamethasone. This demonstrates why this unique steroid has

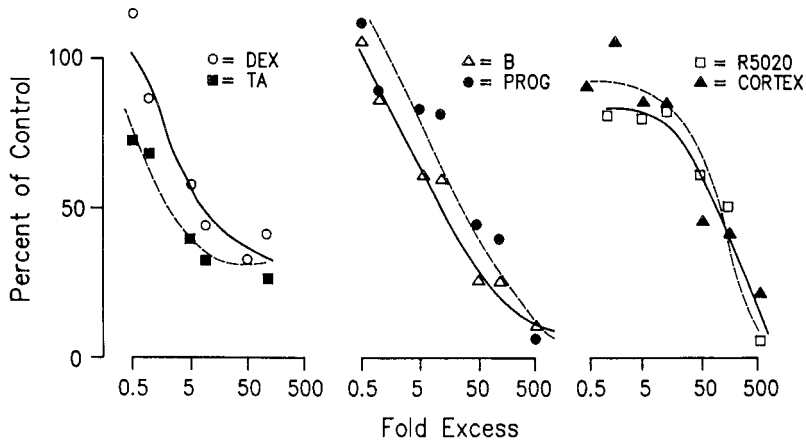

Figure 1. Competition of tritiated dexamethasone binding to intact AtT-20 cells. Cells in media (5×10^6 cell/ml) were exposed to either 10 or 20 n*M* tritiated dexamethasone for 2 hr at 37°C with occasional agitation in the absence or presence of the indicated competing nonradioactive steroids. At that time, duplicate 0.7-ml aliquots were taken and analyzed for binding after washing with Buffer I. In each case the binding by the noncompeted sample was called control and all other samples were expressed as a percentage. The values shown reflect the mean of two to three separate experiments for each steroid. DEX, dexamethasone; TA, triamcinolone acetonide; B, corticosterone; PROG, progesterone; Cortex, cortexolone.

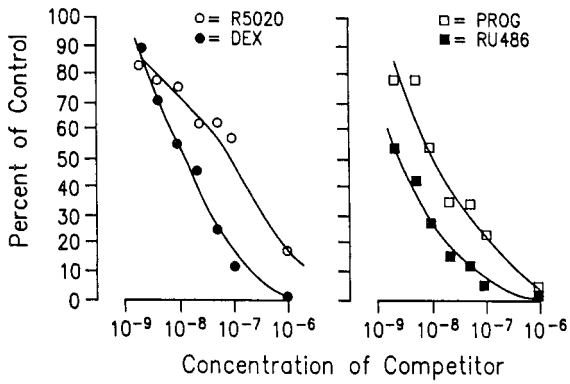

Figure 2. Competition of tritiated dexamethasone binding to the cytosolic glucocorticoid receptor. AtT-20 cytosol was prepared in Buffer II and incubated for 2 hr at 0°C with 10 n*M* tritiated dexamethasone in the presence and absence of the indicated nonradioactive steroids. Duplicate aliquots were removed, treated with charcoal to remove free steroid, and transferred to scintillation vials to determine bound radioactivity. In each case the binding by the noncompeted sample was called control and all other values were expressed as a percentage. The values shown here are the average of four to six separate experiments.

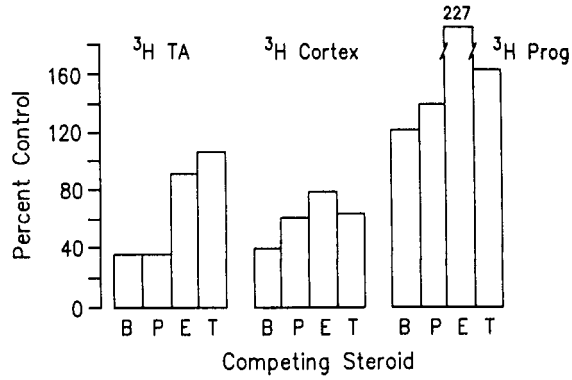

Figure 3. Binding specificity of cytosolic receptor labeled with different tritiated steroids. AtT-20 cytosol was prepared in Buffer III and incubated with 30 n*M* tritiated triamcinolone acetonide, cortexolone, or progesterone. In some cases a 25-fold excess of the indicated nonradioactive steroids was included (B, corticosterone; P, progesterone; E, estradiol; T, testosterone). After 2 hr the samples were treated with charcoal to remove free steroid, centrifuged, and the amount of radioactivity in the supernatant was determined. The values shown are expressed as a percentage of the noncompeted control and are the average of duplicate values from a representative experiment.

been used so extensively to characterize the glucocorticoid receptor.

Figure 3 shows results of a competitive binding analysis in which the tritiated probe was varied. As expected, if tritiated triamcinolone acetonide was the probe, both corticosterone and progesterone but neither estradiol nor testosterone were able to compete. This is consistent with binding to a glucocorticoid receptor (10). On the other hand, if either tritiated cortexolone or progesterone was used, the specificity was not so clear. Total binding was often high and a large fraction of label was associated with nondisplaceable sites. It was difficult to demonstrate clearly and reproducibly displaceable binding. Furthermore, when displaceable binding was seen, the steroid specificity was broader than one would expect if the binding were exclusively to a glucocorticoid receptor.

High performance liquid chromatography was used to assay the size of the steroid receptor complexes. In these trials a fairly rapid flow rate and only one column was used in order to increase the likelihood of detecting labile steroid receptor complexes. Incubates with tritiated agonists produce clear peaks eluting close to the void volume (Fig. 4). Tritiated progestins, however, did not act similarly. No reproducible peaks were seen. In other experiments (not shown) the conditions (concentration of steroid or buffer) were changed but the results were the same.

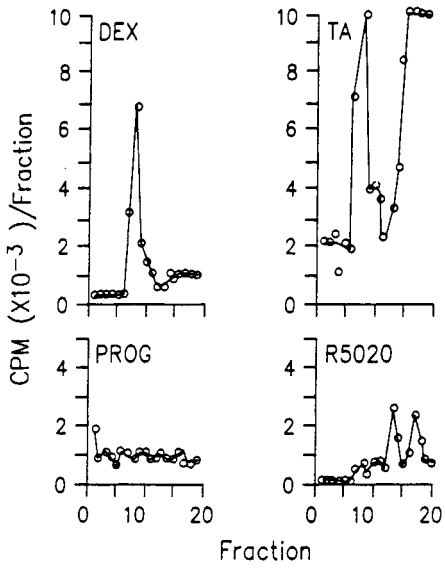

Figure 4. HPLC of AtT-20 cytosol treated with tritiated agonist or antagonist. AtT-20 cytosol in Buffer III was incubated for 2 hr at 0°C with 20 n*M* of the indicated tritiated steroid. Samples (0.5 ml) were injected onto the HPLC system and eluted at 1 ml/min into 1-ml fractions. Aliquots were removed for determination of radioactivity.

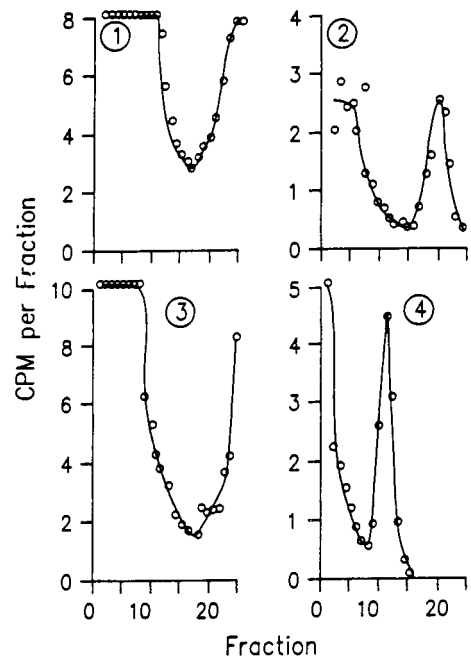

Figure 5. Vertical tube rotor ultracentrifugation of AtT-20 cell cytosol labeled with tritiated steroid. AtT-20 cytosol was prepared in Buffer III and incubated for 2 hr at 0°C with 20 n*M* tritiated progesterone or dexamethasone. Samples were then layered atop sucrose gradients which were either 5 to 20% sucrose (Panels 1 and 2) or 10 to 40% sucrose (Panels 3 and 4). After 80 min at 75,000 rpm, the bottoms of the tubes were punctured and fractions collected. The amount of radioactivity in each fraction is indicated. The top of the tube is Fraction 1. Panels 1 and 3 are of the progesterone-treated sample and Panels 2 and 4 are of the dexamethasone-treated sample. The samples were treated with charcoal for 1 min before application to the gradient.

Vertical tube rotor ultracentrifugation was used to assay the shape of the receptor. Panel 2 of Figure 5 shows that tritiated dexamethasone forms complexes that sediment in the 8 to 10S range. Analysis of a similar incubate using tritiated progesterone (Panel 1) did not reveal such a peak. Instead, radioactivity was found at the bottom of the tube. Not shown is that inclusion of an excess of nonradioactive dexamethasone in this tritiated progesterone incubate did not change the results; no displaceable progesterone binding was seen and radioactivity accumulated in the bottom of the tube. In an attempt to determine whether a very rapidly sedimenting complex was present but obscured, another experiment was conducted in which the gradient was increased from 5 to 20% to 10 to 40% sucrose. Panel 4 shows that under these conditions, as expected, the tritiated dexamethasone complex did not migrate as far. However, the results in Panel 3 show that progesterone's behavior was unchanged.

Taken together, these results suggest that if progestins do bind to the glucocorticoid receptor they are quite unstable and that even these relatively rapid techniques cannot be used to characterize the complexes. Hence, another tact had to be used to capture these antagonist-receptor complexes.

The approach involved covalently attaching steroid to the binding species. Specifically, irradiation with uv wavelengths was used (20). Progesterone, R-5020, triamcinolone acetonide, and dexamethasone all have a structure that allows for covalent attachment. The goal of the work is to prepare and partially purify covalently labeled steroid-receptor complexes, determine whether they involve the glucocorticoid receptor, and then determine some of the physical properties of these complexes.

Ultraviolet radiation was conducted using an approach similar to that outlined by Ilenchuk and Walters (20). After uv radiation the product was partially purified. The first step involved DEAE cellulose chromatography. The peak of radioactivity eluting from the column in each case was collected and precipitated with ammonium sulfate. The recoveries of these two steps is reported in Table 1; over two thirds of the initially bound charcoal-resistant dexamethasone was lost and only 10% of the bound progesterone was recovered, for example. The redissolved precipitates were subject to HPLC. The peak of high molecular weight progesterone-labeled or dexamethasone-labeled material was isolated and then characterized.

The first characterization step was designed to determine whether the covalently labeled material involved the glucocorticoid receptor. To accomplish this, the monoclonal antibody BuGR-2 was used (21). This antibody is directed against the glucocorticoid receptor. The approach was similar to that which Harrison's

Table I. Recovery of Covalently Labeled Receptor during Purification[a]

Step	Steroid			
	Triamcinolone acetonide	Dexamethasone	Progesterone	R-5020
DEAE	46	41	13	13
$(NH_4)_2SO_4$	27	33	9	11
HPLC	9	8	0.7	2

[a] AtT-20 cytosol was labeled covalently with the indicated tritiated steroid. After treatment with charcoal to remove free steroid, the samples were partially purified by the sequential application of DEAE cellulose chromatography, ammonium sulfate precipitation, and HPLC. The recovery at each step is compared with the amount originally resistant to charcoal. The values reflect the average of two separate experiments.

group used when they demonstrated that the antibody interacted with the glucocorticoid receptor (21). The results are seen in Figure 6. Samples of partially purified receptor covalently labeled with either tritiated dexamethasone or progesterone steroid migrate as single peaks with a 3 to 4S value on a sucrose gradient (left-hand panels). Preincubation with BuGR-2 antibody causes some of the radioactivity in each case to sediment in an intermediate position (middle panels). Exposure of antibody receptor incubates with goat anti-mouse IgG antibody causes most of the covalently labeled material to sediment at the bottom of the tube (right-hand panels). These results suggest that the covalently labeled material in each case contains the glucocorticoid receptor.

Some of the properties of these partially purified receptor complexes labeled with either agonist or antagonist are shown in Figure 7. DEAE chromatography of the progesterone or dexamethasone-labeled material

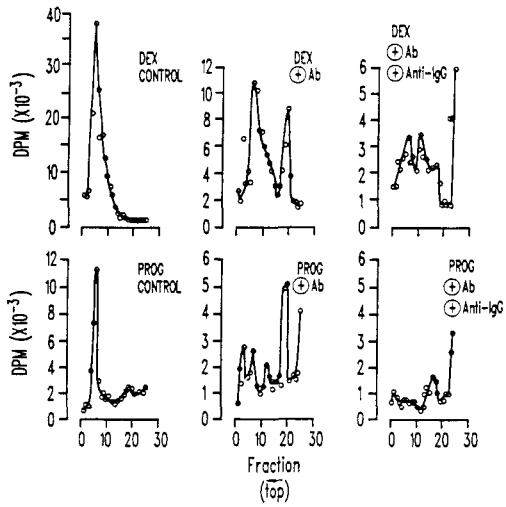

Figure 6. Effect of BuGr-2 antibody on covalently labeled receptor. AtT-20 cytosol was labeled overnight with 40 n*M* tritiated progesterone or dexamethasone and then treated with uv radiation for 20 min. After partial purification, aliquots of each were treated with BuGr-2 antibody for 2 hr at 0°C. Portions of these samples were removed and incubated for another 2 hr at 0°C with goat anti-mouse IgG antibody. Samples of each were placed atop a linear sucrose gradient and centrifuged for 16 hr. Eleven drop fractions were collected and the radioactivity in each fraction assayed. The top of the tube begins with Fraction 1.

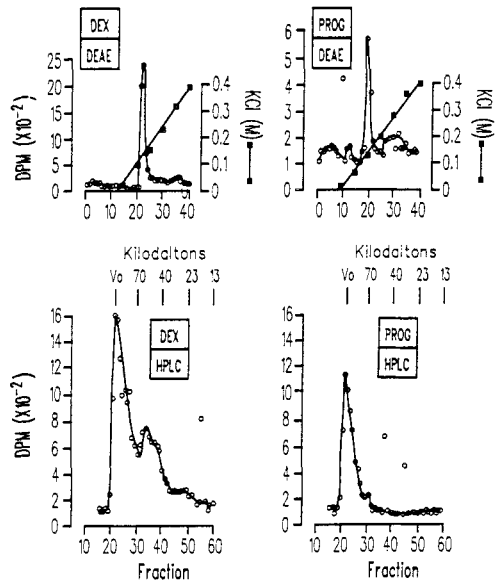

Figure 7. DEAE cellulose chromatography and HPLC of covalently labeled receptor. AtT-20 cytosol labeled overnight with 40 n*M* tritiated dexamethasone or progesterone and treated for 20 min with uv radiation. Samples were purified. Aliquots were placed atop DEAE cellulose columns or HPLC columns. The amount of radioactivity in each eluted fraction is recorded.

shows similar results. On HPLC both elute as high molecular weight complexes. (There was a shoulder of lower molecular weight matter in the dexamethasone preparation that may reflect partially proteolyzed receptor.)

Discussion

The properties of antagonist glucocorticoid receptor complexes have been deduced using synthetic steroids with high affinities for the receptor. Whether these compounds give accurate predictions of how potential "natural" antagonists like progesterone or cortexolone work is uncertain. For example, the mesylate derivative of cortisol can act as either an agonist or antagonist (5). RU 486, the most studied antiglucocorticoid, is an antiprogestin as well as an antiglucocorticoid (6). Currently, the data for RU 486 suggest that antagonists bind to the agonist site of the glucocorticoid receptor and stabilize the nontransformed form. On the other

hand, in the early studies of cortexolone binding, Turnell and associates (22) reported that this compound readily formed a 4S glucocorticoid receptor species even at 0°C. Schmidt and Davidson (23) reported that cortexolone-glucocorticoid receptor complexes do not bind to DNA as readily as do those of RU 486. Hence, there are reasons to question whether synthetic and potential natural antiglucocorticoids form similar structures with glucocorticoid receptors.

Although we could clearly demonstrate that progesterone and cortexolone interfere with agonist binding to the glucocorticoid receptor, we were not able to characterize the binding of the tritiated species directly to the receptor using the standard techniques of steroid incubation followed by HPLC and vertical tube rotor ultracentrifugation. We have recently used these techniques to demonstrate directly the binding of the labile agonist corticosterone to similar receptor preparations (1). We hypothesize that the receptor, labeled with an antiglucocorticoid, is even more labile. It must be noted that others have reported some positive results, although overall there are very few such reports in the literature. Lefebvre et al. (24) have reported HPLC studies of the glucocorticoid receptor labeled with tritiated progesterone and cortexolone. We cannot explain these different results except to note that their studies involved analysis of cytosol preparations derived from intact cells that had been incubated with tritiated steroid as opposed to our studies in which cell cytosol was exposed to steroid *in vitro*. It is possible that intact cells metabolize some of the labeled steroid.

Several groups have used uv radiation to attach steroids to the glucocorticoid receptor (24–26). The molecular details of this step are not known completely but probably involve a covalent linkage through the C-3 position of the steroid. Using this approach we have been able to attach a glucocorticoid or a progestin to a cytosolic species. That at least some of this binding is to a glucocorticoid receptor is demonstrated by the BuGR-2 antibody study. The partially purified covalently labeled species that bind tritiated progesterone interacts with the antibody in the same way as the species that bind tritiated dexamethasone. Thus, most likely, the species binding progesterone is a glucocorticoid receptor. The gross properties of these receptors are similar regardless of whether they are labeled with an agonist or an antagonist. In this respect our work confirms that of others.

What remains to be investigated is whether the two classes of steroids usually interact with the very same region of the receptor. We (8–11) and others (27–30) have presented evidence that the glucocorticoid receptor contains two close, but distinct, sites that bind either agonists or antagonists but not both. Binding to one site inhibits binding at the other site through allosteric mechanisms. We have presented preliminary evidence that the proteolytic digestion patterns of receptors covalently labeled with agonists or antagonists are different (31), suggesting that the two types of steroids bind to different regions.

Other groups have evidence which may be complementary. Carlstedt-Duke et al. (32) note that with uv activation, triamcinolone acetonide binds to two cysteine sites that are over 130 amino acids apart on the glucocorticoid receptor. On the other hand, dexamethasone mesylate binds to only one site and it is at least 34 amino acids away from either of the two other sites that bind the agonist. They interpret this data as indicating that the receptor, when bound with an agonist, folds back along itself so that two widely distant sites along the receptor are brought close together. An alternate explanation for the different regions of interaction is that there are two separate sites on the receptor that can bind steroid. Quite recently, Srivastava and Thompson (33) reported that the synthetic agonist steroid cortivazol recognizes two steroid binding sites on the glucocorticoid receptor.

All of these data are with the glucocorticoid receptor. Others, however, have evidence which may be interpreted similarly involving other systems. Moudgil et al. (34) and Jensen et al. (35), for example, have evidence that can be interpreted as showing separate sites for agonists and antagonists along progesterone and estrogen receptors, respectively.

The work in this article demonstrates that potential natural antiglucocorticoids do interact with the glucocorticoid receptor as predicted by the current models of steroid hormone action. Whether the receptor contains two close, but separate, sites for the binding of agonists and antagonists still awaits direct proof.

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