Physicochemical Differences between Glucocorticoid-binding Components from the Corticoid-sensitive and -resistant Strains of Mouse Lymphoma P1798

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ABSTRACT

The physicochemical properties of nuclear and cytosolic glucocorticoid-binding components from corticoid-sensitive (CS) and corticoid-resistant (CR) mouse lymphoma P1798 cells have been compared. Nuclei or cytosol fractions were prepared from lymphocytes that had been labeled at 37 or 4°C, respectively, with 30 nM [3H]triamcinolone acetonide ([3H]TA). [3H]TA was extracted with 0.6 M KCI, 10 mM spermidine, or 4.5 mM MgCl2 from CS nuclei and with 0.6 M KCI or 10 mM spermidine from CR nuclei. As reported previously, nuclear-associated [3H]TA in CR cells was resistant to extraction with mM concentrations of MgCl2. Loss of bound steroid during extraction with 0.6 M KCI was minimized by including the chymotrypsin inhibitor, carbobenzoxy-L-phenylalanine, in the extraction buffer. The inhibitor was not required during extraction with spermidine or MgCl2. Nuclear and cytosolic extracts were examined by analytical agarose gel filtration and glycerol density gradient centrifugation under high salt (0.6 M KCI) conditions. The glucocorticoid-binding component in KCl, spermidine, and MgCl2 extracts from CS nuclei was considerably larger and more asymmetrical [Stokes radius, 57 to 59 Å; sedimentation coefficient, 3.64 to 3.70S; molecular weight, 90,000 daltons; frictional ratio, 1.8; axial ratio (prolate ellipsoid), 15] than the [3H]TA-nacromolecule complex in KCl and spermidine extracts from CR nuclei [Stokes radius, 29 Å; sedimentation coefficient, 3.23 to 3.30S; molecular weight, 40,000 daltons; frictional ratio, 1.25; axial ratio (prolate ellipsoid), 5]. Control experiments showed that the smaller size of the glucocorticoid-binding component in CR nuclei was probably not due to cleavage of a larger, CS-like complex during the extraction procedure. The larger size of the CS [3H]TA complex did not appear to result from aggregation of a smaller species. No difference in physicochemical parameters of the binding component was observed if cells were labeled with [3H]dexamethasone instead of [3H]TA. However, [3H]dexamethasone complexes were less stable than those formed with [3H]TA as indicated by considerable dissociation of [3H]dexamethasone during gel filtration and gradient centrifugation. This may be due to the 3- to 5-fold lower relative binding affinity of [3H]dexamethasone. Analysis of [3H]TA-labeled cytosol by gel filtration and gradient centrifugation revealed the presence of a single binding component with physicochemical properties similar to those of nuclear [3H]TA complexes from the same strain of tumor. These results suggest that previously described differences in extractability of nuclear-associated [3H]TA between the CS and CR strains of mouse lymphoma P1798 and the lack of response of CR P1798 to glucocorticoid administration may be due, at least in part, to the presence of an altered glucocorticoid-binding component in the resistant tumor cells.

INTRODUCTION

Since the discovery of the beneficial effects of corticosteroids in the treatment of human leukemia, the glucocorticoids have been used widely in the management of a variety of leukemias and lymphomas, originally as single agents and since the 1960's as integral parts of combined chemotherapy schedules (8, 10, 12). Several hypotheses have been advanced to explain the lympholytic action of the glucocorticoids (for a recent review, see Ref. 35), but no direct link has been found between the multiple biochemical changes caused by corticosteroids and the lethal effect of these hormones on susceptible lymphocyte populations. Equal in importance to understanding the mechanism of glucocorticoid action in CS lymphocytes is the problem of elucidating the basis for resistance of lymphocytes that do not respond to steroid therapy.

Evidence from many laboratories suggests that hormone-receptor complex interaction with nuclear chromatin is a key requirement for expression of steroid effects in a variety of target tissues (42). Although all CS lymphocytes examined to date contain glucocorticoid-binding proteins or receptors that undergo temperature-dependent binding to the nucleus after they have complexed with an active steroid (21), occurrence of nuclear glucocorticoid binding does not always guarantee sensitivity. Thus, several groups have described CR lymphocytes that, under whole cell conditions, display "normal," increased, or only moderately reduced levels of nuclear binding (4, 15, 20, 36, 43). However, such binding may display anomalous characteristics as suggested by the pioneering work of Yamamoto et al. (43). These authors showed that cytoplasmic [3H]dexamethasone-receptor complexes from "nuclear transfer-increased" CR S.49 lymphoma mutants bound more tightly to DNA-cellulose than did hormone-receptor complexes from wild-type CS cells. In contrast, hormone-receptor complexes from "nuclear transfer-deficient" CR mutants bound less tightly to DNA-cellulose.

Further evidence for altered nuclear-glucocorticoid interactions in receptor-positive CR malignant lymphocytes was recently obtained by Bourgeois et al. (4) with S.49 lymphoma

1 The abbreviations used are: CS, corticoid-sensitive; CR, corticoid-resistant; TA, triamcinolone acetonide; CBZ-L-Phe, carbobenzoxy-L-phenylalanine; BSA, bovine serum albumin; DTT, dithiothreitol; TES, tri(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; TES-Mg, 10 mM tri(hydroxymethyl)methyl-2-aminoethanesulfonic acid-4.5 mM MgCl2; TES-EDTA, 10 mM tri(hydroxymethyl)methyl-2-aminoethanesulfonic acid-4 mM EDTA; TES-EDTA-DTT, 10 mM tri(hydroxymethyl)methyl-2-aminoethanesulfonic acid-4 mM EDTA-20 mM dithiothreitol.

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and W7 thymoma cells, by McPartland et al. (20), and by ourselves with P1798 mouse tumor lymphocytes (36). Bourgeois et al. (4) showed that receptor-positive CR mutants of the S.49 and W7 lines could be distinguished from wild-type CS cells on the basis of differences in salt extractability of nuclear-associated steroid. In some CR clones, extractability was increased, whereas in others, it was decreased. In the P1798 variants studied by McPartland et al. (20), low concentrations of NaCl extracted more [3H]dexamethasone from CR nuclei than from CS nuclei. In the P1798 variants examined in our laboratory, nuclear glucocorticoid binding appeared to be tighter in CR than in CS lymphocytes. We found (36) that low concentrations of monovalent (KCl), divalent (Mg2+ or Ca2+), or polyvalent (spерmidine) cations released less [3H]TA from CR than from CS P1798 nuclei.

These differences could be due to presence of a modified steroid-binding component, to changes at the nuclear level, or to a combination of both factors. Elucidation of the nuclear contribution requires use of a cell-free nuclear-binding system in which all the properties of "whole-cell" nuclear-glucocorticoid interactions are preserved. Such a system is currently unavailable. On the other hand, physicochemical studies on glucocorticoid receptors may provide information concerning the role played by these binding components in giving rise to altered forms of nuclear-glucocorticoid interaction. In this regard, Yamamoto et al. (43) demonstrated that cytoplasmic [3H]dexamethasone-receptor complexes from some CR S.49 lymphoma mutants differed in their physicochemical properties from wild-type, CS receptor complexes. However, attempts to characterize nuclear glucocorticoid-binding components have been hampered by cleavage of the complexes (6) or by considerable loss of bound steroid (2, 13, 24, 40) when nuclei were extracted with the usual concentrations of NaCl or KCl (0.3 to 0.6 M). We have described recently a reliable method for obtaining high yields of macromolecule-associated [3H]TA from lymphoid nuclei based on the use of the chymotrypsin inhibitor, CBZ-L-Phe, (7) to prevent loss of bound hormone during extraction of nuclei with 0.6 M KCl (36). In this paper, we have compared the physicochemical properties of glucocorticoid-binding components isolated from CS and CR lymphoma P1798 nuclei using this procedure. Our results indicate that CR P1798 lymphocytes contain a smaller and less asymmetrical nuclear and cytoplasmic [3H]TA-binding component than do CS P1798 cells. The altered properties of the CR [3H]TA complex may contribute, at least in part, to the differences in extractability of nuclear-associated [3H]TA between CS and CR P1798 lymphocytes.

MATERIALS AND METHODS

Chemicals and Radioisotopes. Agarose gels (Bio-Gel A-0.5m, 100 to 200 mesh and Bio-Gel A-1.5m, 100 to 200 mesh) were purchased from Bio-Rad Laboratories, Rockville Centre, N. Y. Sephadex G-25 (coarse), Blue Dextran 2000, and nonradioactive standard proteins (except myoglobin) used for calibrating the agarose columns were from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. In some experiments, we used cadmium-free horse spleen ferritin from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The following compounds were obtained from the Sigma Chemical Co., St. Louis, Mo.: nonradioactive BSA; CBZ-L-Phe; DTT; whale muscle myoglobin; nonradioactive TA; TES; and Triton X-100. RNase-free sucrose was from Research Plus, Denville, N. J., while spermidine trihydrochloride was obtained from Calbiochem, La Jolla, Calif. Leupeptin was generously provided by the United States-Japan Cooperative Cancer Research Program through the courtesy of Dr. W. A. Troll and Dr. M. Levitz (New York University, New York, N. Y.). Nonradioactive dexamethasone was obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. DMEM was purchased from Grand Island Biological Co., Grand Island, N. Y. [3H]TA (21 to 37.4 Ci/mmol) and [3H]dexamethasone (21 to 41 Ci/mmol) were obtained from Amersham/Searle Corp., Arlington Heights, Ill., or from New England Nuclear, Boston, Mass. Purity of radioactive steroids was routinely checked by thin-layer chromatography as described previously (36) and was always >98%. [14C]Methylated ovalbumin (11.1 μCi/mg), [14C]methylated BSA (13.2 μCi/mg), and [14C]-valine (246 mCi/mmol) were supplied by New England Nuclear. All radioisotopes were stored at the same concentration at which they were received and at the temperature recommended by the manufacturer. ACS counting fluid was from the Amersham/Searle Corp. All other chemicals were products of J. T. Baker Chemical Co., Phillipsburg, N. J., except for methylcellulose (15 centipoises) which was from Fisher Scientific Co., Pittsburgh, Pa.

Tumors. The CS and CR strains of lymphoma P1798, an estrogen-induced thymoma in mice (17), were maintained in female BALB/c mice (Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.) as described previously (36).

Buffers. Buffers used were TES-Mg and TES-EDTA. The pH of all buffers was 7.5 at 4°C. On the day of experiment, DTT was added to a final concentration of 5 mM (column elution buffer) or 20 mM (all other buffers). CBZ-L-Phe (10 mM) was dissolved by vigorous shaking after the addition of 12 mM NaOH. The pH of buffers containing leupeptin was readjusted to 7.5 with an appropriate volume of 1 N NaOH.

Nuclear Binding at 37°C. The method for labeling P1798 tumor lymphocytes with [3H]TA and subsequent isolation of nuclei from the radioactive cells has been described in detail elsewhere (36). All steps were performed at 4°C unless indicated otherwise. Briefly, cells were incubated for 60 min at 37°C with 30 nM [3H]TA in DMEM, washed twice with ice-cold medium, and homogenized in 2 mL sucrose-0.1% Triton X-100 (final concentrations) in TES-Mg. Purified nuclei were recovered in >70% yield by layering the homogenate on a cushion of 2.1 M sucrose in TES-Mg (no Triton X-100) and by centrifuging 10 min at 25,000 × g. We have shown (36) that under these conditions, the amount of [3H]TA associated with nuclei from cells incubated with 30 nM [3H]TA plus 100-fold excess nonradioactive TA (nonspecific binding) was only 2% of the amount of [3H]TA in nuclei of cells exposed to 30 nM [3H]TA alone. Hence, it was not necessary to analyze nuclear extracts from cells incubated with tritiated steroid plus unlabeled hormone. In some experiments, 60 nM [3H]dexamethasone was used instead of [3H]TA.

Extraction of Nuclei with KCl. The surface of the nuclear pellet and inside walls of the tube were rinsed with TES-EDTA. After addition of 0.6 M KCl-10 mM CBZ-L-Phe in 5% glycerol (w/v), TES-EDTA, containing BSA (2 mg/ml) and 20 mM DTT (1.3 ml for each portion of nuclei from 5 to 6 × 10^7 cells), the
nuclei were extracted by shaking at top speed for 15 min on a Burrell wrist-action shaker (Arthur H. Thomas and Co., Philadelphia, Pa.). The supernatant obtained by centrifuging the nuclei at 27,000 × g for 15 min was recentrifuged for 30 min at 100,000 × g and used for further studies. KCl extracts from CS and CR nuclei contained 80 to 90% of total nuclear-associated [3H]TA (KCl-extractable [3H]TA plus radioactivity released from the 27,000 × g pellet by overnight incubation at room temperature with absolute ethanol).

By supplementing the extraction buffer with 5% glycerol and BSA (2 mg/ml), we were able to increase the percentage of macromolecule-associated [3H]TA in CS and CR 0.6 m KCl nuclear extracts to about 90% from the previously reported value of 70 to 80% (36). Furthermore, in the presence of glycerol and BSA, [3H]TA complexes were stable for at least 20 to 22 hr at 4°C, whereas in the absence of glycerol and BSA, there was considerable loss of bound steroid. Glycerol and BSA were ineffective in the absence of CBZ-L-Phe.

Extraction of Nuclei with MgCl2 and Spermidine. Nuclei from 5 to 6 × 107 cells were extracted for 15 min with 2 ml of TES-Mg-20 mM DTT containing BSA (2 mg/ml) or with 2 ml of TES-EDTA-DTT plus BSA (2 mg/ml) at one-quarter speed on the wrist-action shaker. No protease inhibitor was included in either extraction buffer. The extracts were centrifuged at 27,000 × g for 15 min, and the supernatant was recentrifuged for 30 min at 100,000 × g before being analyzed by gel filtration or glycerol gradient centrifugation. Ninety % or more of radioactivity in MgCl2 and spermidine extracts was in the form of a macromolecule complex, compared to only 50 to 70% when extraction was carried out in the absence of BSA (36).

Intracellular Distribution of Bound [3H]TA at 37°C. Cell suspensions were incubated with [3H]TA and divided into 2 portions. Nuclei were isolated from one portion as described above. For convenience, the nuclei were extracted directly with absolute ethanol at room temperature rather than with 0.6 m KCl, and an aliquot of the ethanol extract was counted. This appears to be a legitimate procedure since 0.6 m KCl extracts 90% of nuclear-associated [3H]TA (36), and 90% of the radioactivity in the extract is present as a macromolecular complex when the improved methodology described in this paper is used. At most, levels of nuclear binding may be overestimated by 20%. The remaining cells were homogenized in TES-EDTA-DTT containing BSA (2 mg/ml) and centrifuged for 10 min at 27,000 × g to obtain the cytosolic fraction. We have shown (36) that TES-EDTA only minimally extracted [3H]TA from nuclei of cells incubated with labeled steroid at 37°C. Hence, there was little or no contamination of cytoplasmic extracts with nuclear glucocorticoid-binding components under these conditions. Bound [3H]TA in the 27,000 × g supernatant was determined by gel filtration on columns of Sephadex G-25 (22). No increase in the fraction of bound steroid was achieved by including 10 mM CBZ-L-Phe in the homogenizing buffer. Nuclear-associated [3H]TA was expressed as a percentage of total intracellular (nuclear plus cytoplasmic) bound [3H]TA.

Cytosol Binding at 4°C. Cells (8 to 9 × 106/ml) were incubated for 2 hr at 4°C in DMEM supplemented with methylcellulose (1.2 mg/ml) containing 30 nM [3H]TA alone or 30 nM [3H]TA plus 3 μM nonradioactive TA to estimate the level of nonspecific binding. A constant level of binding was achieved within this period of time. The cells were kept in suspension by continuous rotation of the flasks at 2 rpm on a Rugged Rotator (Kraft Apparatus, Mineola, N. Y.). At the end of incubation, the lymphocytes were washed 5 times with 3 ml of DMEM for each portion of 5 to 6 × 107 cells. The washed cells were homogenized in 2 ml of TES-EDTA-DTT-BSA (2 mg/ml) per 5 to 6 × 107 cells with 10 strokes of a motor-driven, glass-Teflon, Potter-Elvehjem homogenizer. The homogenizer was immersed in an ice bath, and 10-sec intervals were allowed between strokes to ensure adequate temperature control. In some experiments, the homogenizing buffer contained 10 mM CBZ-L-Phe or 20 mM leupeptin also.

The homogenates were centrifuged for 15 min at 27,000 × g, and the resulting supernatants were spun at 100,000 × g for 30 min to obtain the cytosol fraction. Levels of bound [3H]TA in cytosol from cells incubated with 30 nM [3H]TA plus 3 μM nonradioactive TA were 1% or less of the levels of macromolecule-associated [3H]TA in cytosol from lymphocytes exposed to [3H]TA alone. Therefore, cytosol from cells incubated with radioactive plus nonradioactive TA was not analyzed further.

Agarose Gel Filtration. Three columns of agarose A-0.5m (1.5 × 80 cm) and 3 of agarose A-1.5m (1.5 or 1.6 × 66 to 70 cm) were poured in glass-distilled H2O and equilibrated with 0.6 m KCl in 10% glycerol (w/v)-TES-EDTA-0.02% NaN3 (elution buffer). The columns were eluted at flow rates of 8 to 12 ml/hr. Fraction volumes ranged from 1.75 to 2.25 ml except for the experiments in Chart 1. Void volume (V0) was determined with Blue Dextran 2000. Total liquid volume (Vt) was taken to be the elution volume of glucose or [14C]valine (32). The agarose A-0.5m columns were calibrated with RNase A (Stokes radius, 16.4 Å), myoglobin (Stokes radius, 20.2 Å), chymotrypsinogen A (Stokes radius, 20.9 Å), ovalbumin...
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were calibrated with chymotrypsinogen A, ovalbumin, BSA, aldolase, and ferritin (Stokes radius, 61.0 Å). The Stokes radius of myoglobin was that used by Sherman et al. (30). All other values were those listed by the manufacturer of the calibration kits. Distribution coefficients of standard proteins and glucocorticoid-binding components were estimated according to Sherman (29). Calibration curves [log Stokes radius versus distribution coefficient (14)] of the agarose A-0.5m columns were linear over the entire range of standards. The calibration curves of agarose A-1.5m columns were linear between ferritin and ovalbumin. Sample volume was 3 ml for columns with a 1.5-cm diameter and 4 ml for the column with a 1.6-cm diameter. Columns were calibrated in the presence of 5 mM DTT inasmuch as the elution buffer was routinely supplemented with 5 mM DTT when tumor samples were analyzed.

Glycerol Gradient Centrifugation. Aliquots (0.4 ml) of nuclear extracts or cytosol were layered on duplicate linear, 8 to 35% (w/v) glycerol density gradients made up in TES-EDTA containing 0.6 M KCl and 20 mM DTT. To one gradient was added 1 μl of 14C-ovalbumin [3.53S (30)] and to the other was added 1 μl of 14C-BSA [4.4S (30)] as internal markers. When necessary, samples were adjusted to 5% glycerol-0.6 M KCl by adding appropriate volumes of 100% glycerol and 3 M KCl in TES-EDTA. Gradients were centrifuged for 2 hr at 48,000 rpm in a SW 50.1 rotor in a Beckman L5-75 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) after which fractions of 3 drops each were collected by gravity. Sedimentation coefficients were estimated according to the method of Tott and Sherman (38) with correction for the width of the initial sample layer. Values obtained for duplicate samples within the same run, one extrapolated from the 14C-ovalbumin standard and the other from the 14C-BSA standard, differed by 3% or less.

For recycling gradient peaks onto agarose columns, 2 out of 6 gradients were run with internal markers. Sedimentation coefficients were calculated from these gradients. The other 4 gradients, which contained no internal marker, were collected in 4 × 20-drop fractions (CS P1798) or in 4 × 21-drop fractions (CR P1798) designated 1 to 4 from bottom to top. Fraction 3, which corresponded to the peak area, was applied to the column. In order to obtain sufficient volume, fractions from 4 gradients were pooled.

Apparent molecular weights and frictional ratios (f/fo) were calculated from the Stokes radius and sedimentation coefficient as described by Siegel and Monty (33). We assumed a partial specific volume of 0.74 ml/g (34) and a ‘‘compromise’’ value of 0.2 g of solvent per g of protein for the solvation factor (37). Axial ratios were obtained from the values listed by Schachman (26).

RESULTS

Agarose Gel Filtration. Chart 1 shows the elution profiles of 0.6 M KCl-10 mM CBZ-L-Phe extracts from [3H]TA-labeled CS and CR P1798 tumor lymphocyte nuclei on calibrated columns of agarose A-1.5m. The major peak of bound [3H]TA in the extract from CS nuclei (Chart 1a) eluted shortly after the ferritin marker. In contrast, the glucocorticoid-binding component in the CR nuclear extract (Chart 1b) was markedly retarded and eluted between BSA and ovalbumin. However, in other experiments, the [3H]TA-binding component from CR nuclei eluted from the agarose A-1.5m columns coincident with or slightly after the ovalbumin standard (Chart 2, Peak II). The reason for this variability probably is due to the fact that the CR [3H]TA complex eluted at the end of the linear portion of the calibration

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curve for the agarose A-1.5m gel. As described later, more reproducible determinations of the molecular size of CR binding components were obtained using agarose A-0.5m. Radioactivity in the void volume probably corresponds to a small amount of aggregated material. Free [3H]TA eluted with a distribution coefficient of 1.1 to 1.2 and represented about 10% of input radioactivity. The reduced amount of macromolecule-associated [3H]TA in the CR extract reflects the lower level of nuclear glucocorticoid binding by CR P1798 lymphocytes (36). These results suggest that the 0.6 m KCl-extractable glucocorticoid-binding component in CR nuclei is substantially smaller than the glucocorticoid-binding component in CS nuclei. Alternatively, CR nuclei might have contained a larger [3H]TA-macromolecule complex that was degraded during the extraction procedure. Experiments were designed to distinguish between these possibilities.

Nuclei were isolated from separate portions of [3H]TA-labeled CS and CR cells and extracted with 0.6 m KCl-10 mM CBZ-L-Phe, and the ratio of bound radioactivity in the CS extract to bound radioactivity in the CR extract was determined. In the same experiment, intact [3H]TA-labeled CS and CR lymphocytes were mixed in equal proportions. Nuclei were isolated from this mixture of cells and extracted with 0.6 m KCl in the presence of CBZ-L-Phe. Chart 2 shows that 2 major peaks of bound [3H]TA (I and II) were detected when the mixed extract was passed through a column of agarose A-1.5m. Peak I eluted in the position expected for bound [3H]TA in a KCI extract from CS nuclei while Peak II eluted in the position expected for a CR nuclear extract. The amount of radioactivity in Peak I was 1.8 times the amount of radioactivity in Peak II. Similarly, the amount of bound [3H]TA extracted with 0.6 m KCl-10 mM CBZ-L-Phe from the CS nuclei that were processed separately (90,014 dpm) was 1.74 times higher than the amount of bound [3H]TA obtained from the separately extracted CR nuclei (51,855 dpm). These results suggest that CR nuclei did not contain activity that in the presence of 10 mM CBZ-L-Phe was capable of degrading the large binding component characteristic of CS nuclei. Therefore, it is unlikely that the smaller binding component in the 0.6 m KCl-10 mM CBZ-L-Phe extracts from CR nuclei arose by cleavage of a larger species with properties similar to those of the CS [3H]TA complex. Further evidence that the CR [3H]TA complex was probably not a degradation product was obtained by performing a mixing experiment similar to the one described above, except that female BALB/c mouse thymocytes were used as a source of nuclear glucocorticoid receptor from a normal corticoid-sensitive tissue. Gel filtration of the KCI extract from thymus nuclei alone gave a single symmetrical peak with a Stokes radius of 59 Å plus a small amount of aggregated material in the void volume. The [3H]TA-binding component from CR P1798 nuclei had a Stokes radius of 30.5 Å. The mixed extract yielded an elution profile similar to the one shown in Chart 2. The amount of radioactivity in each of the 2 major peaks was exactly that expected from the levels of bound [3H]TA present in individual thymus and CR P1798 nuclear extracts that were run in parallel. Hence, CR P1798 lymphocyte nuclei did not contain an activity that in the presence of 10 mM CBZ-L-Phe was capable of cleaving the normal, 59 Å mouse thymus glucocorticoid receptor.

Next, we explored the possibility that extraction of CR nuclei with 0.6 m KCl might release degradative activity that was resistant to the inhibitory action of CBZ-L-Phe. For this purpose, [3H]TA-labeled CR nuclei were extracted with 0.6 m KCl in the presence of 20 or 40 mM leupeptin. Leupeptin is a protease inhibitor of microbial origin (39) that is extremely effective in preventing cleavage of a variety of steroid-binding proteins from normal and malignant target tissues (31, 32). The Stokes radius of [3H]TA-binding components extracted in the presence of leupeptin (30.5 Å) was similar to the Stokes radius of complexes isolated with 0.6 m KCl-10 mM CBZ-L-Phe (31 Å).

Attardi and Ohno (1) found that the size of [3H]dihydrotestosterone receptors in brain homogenates from normal female mice had a Stokes radius of 53 Å in concentrated cytosol preparations compared to about 30 Å in more dilute homogenates. Therefore, we were concerned that the larger size of the [3H]TA-binding component isolated from CS nuclei might be an artifact due to aggregation of a smaller species. However, this does not seem to be the case. In 2 closely agreeing experiments, the Stokes radius of [3H]TA complexes extracted from CS nuclei with the usual volume of buffer (1.3 ml) averaged 57 Å, practically identical to the Stokes radius (55 Å) of complexes extracted in parallel from the same number of nuclei with twice the usual volume of buffer.

Comparison of the KCl-, Spermidine-, and MgCl2-extractable Nuclear [3H]TA-binding Components. We have reported (36) that 5 mM spermidine released substantial amounts of macromolecule-associated [3H]TA from CS and CR P1798 nuclei whereas mM concentrations of MgCl2 and low levels of spermidine were effective only in releasing [3H]TA from CS but not from CR nuclei. Therefore, it was of interest to compare the physicochemical properties of the spermidine- and MgCl2-extractable glucocorticoid-binding complexes with those of complexes extracted with 0.6 m KCl. In these and subsequent experiments, extracts from CR nuclei were analyzed on columns of agarose A-0.5m instead of on columns of agarose A-1.5m so that bound [3H]TA would elute closer to the middle portion of the calibration curve. Chart 3a shows that the elution profiles of the spermidine and MgCl2 extracts from CS nuclei were similar to the profile of the 0.6 m KCl CS nuclear extract in Chart 1a except for the absence of aggregated material in the void volume. This similarity was verified in each experiment by gel filtration of the 0.6 m KCl extract from a parallel sample of nuclei. Glycerol gradient centrifugation revealed that the sedimentation coefficient of the spermidine- and MgCl2-extractable species (3.6 to 3.7S) was identical to the sedimentation coefficient of the KCl-extractable [3H]TA-binding component (Chart 3b). Chart 4a illustrates the similarity in size of the [3H]TA-macromolecule complexes isolated from CS nuclei with 0.6 m KCl and 10 mM spermidine. The corresponding glycerol density gradient profiles are presented in Chart 4b. A sedimentation coefficient of approximately 3.3S was obtained for the bound material in both CR extracts. Although no protease inhibitor was present during extraction of CS and CR nuclei with spermidine or during extraction of CS nuclei with MgCl2, there was no evidence for degradation of [3H]TA-binding components on the columns or gradients.

Table 1 summarizes the Stokes radii and sedimentation coefficients of the binding components obtained in several experiments of the kind described above. This table shows also that, in agreement with our previous results (36), 4.5 mM MgCl2 released 50 to 60% of the [3H]TA in CS nuclei but only 16 to 17% from CR nuclei. MgCl2 extracts from CR nuclei were
thermore, only a single, symmetrical peak of bound steroid was detected. These results indicate that neither CS nor CR nuclear glucocorticoid-binding components were degraded appreciably during the extended period of ultracentrifugation.

Based on the data in Table 1, and assuming a partial specific volume of 0.74 ml/g (34) and a “compromise” value of 0.2 g of solvent per g of protein (37), the \(^{3}H\)TA-binding component from CS P1798 nuclei had an apparent molecular weight of approximately 90,000 daltons, a frictional ratio \((f/f_0)\) of 1.8, and an axial ratio (prolate ellipsoid) of about 15. In contrast, the apparent molecular weight of the \(^{3}H\)TA-binding component from CR nuclei was about 40,000 daltons with a frictional ratio of 1.25 and an axial ratio (prolate ellipsoid) of approximately 5.

**Gel Filtration and Density Gradient Centrifugation of Cytosolic \(^{3}H\)TA-binding Components.** In view of the striking differences in physicochemical properties between nuclear \(^{3}H\)TA-binding components from CS and CR P1798 lymphocytes, it was of interest to determine whether similar differences existed between cytosolic \(^{3}H\)TA complexes from the 2 strains of tumor. Chart 5 shows the elution profiles, on agarose gels, of cytosol from CS and CR cells that were homogenized in the

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**Chart 3.** Comparison of KCl-, spermidine-, and MgCl₂-extractable \(^{3}H\)TA-binding components from CS P1798 nuclei. a, gel filtration profiles of spermidine and MgCl₂ extracts on columns of agarose A-1.5m. The profiles of parallel runs on 2 separate columns have been superimposed. Sample volume was 3 ml for the spermidine extract and 4 ml for the MgCl₂ extract.

Even though 10 mM spermidine and 0.6 M KCl extracted similar percentages of nuclear-associated \(^{3}H\)TA, radioactivity in the spermidine sample was considerably lower than in KCI samples (cf. to Chart 1 a) because of greater dilution during extraction of the nuclei (2 ml of spermidine-containing buffer were used as opposed to 1.3 ml of 0.6 M KCl buffer). The spermidine extract was further diluted by addition of 0.2 volume of 3 M KCl to adjust the salt concentration to 0.6 M KCl before analysis by gel filtration or gradient centrifugation. The same applies to the MgCl₂ extract. Furthermore, MgCl₂ extracted less \(^{3}H\)TA than did spermidine or KCl. Arrows, position of protein standards used to calibrate the columns. Abbreviations as in Chart 1. b, superimposed glycerol density gradient profiles of KCI, spermidine, and MgCl₂ extracts. Arrow, position of the \(^{14}C\)-ovalbumin internal marker.

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not subjected to further analysis. Before the data in Table 1 could be used to calculate molecular weights, frictional ratios, and axial ratios of CS and CR nuclear glucocorticoid-binding components, it was necessary to verify that the \(^{3}H\)TA complex obtained by density gradient centrifugation corresponded in size to the complex eluted from the agarose columns. To this end, the peak of bound \(^{3}H\)TA from gradients of KCl extracts was subjected to gel filtration. The Stokes radius of the recycled material was compared to that of the binding component in KCl extracts that were chromatographed on agarose columns without prior ultracentrifugation. In 2 closely agreeing experiments, the Stokes radius of CS nuclear complexes averaged 58 Å before centrifugation and 56 Å after isolation from the gradients. For CR nuclear extracts, we obtained average values of 28 Å both before and after gradient analysis. Further
absence of protease inhibitor. A single 57 Å binding component was detected in CS cytosol (57.5 ± 0.6 Å; n = 4), and a single 30 Å [3H]TA complex was present in CR cytosol (29.7 ± 0.2 Å; n = 3). Identical results were obtained when cytosol was prepared in the presence of 10 mM CBZ-L-Phe or 20 mM leupeptin (not shown). As shown in Chart 6, the [3H]TA macromolecular complex in CS cytosol sedimented at 3.64 to 3.67S (n = 2) compared to 3.26 to 3.33S (n = 2) for macromolecule-associated [3H]TA in CR cytosol. Recycling experiments, identical in design to those described in the previous section, established that no significant cleavage or degradation of the binding components took place during density gradient centrifugation. Thus, CS and CR P1798 lymphocytes differ also with respect to the physicochemical properties of their cytosolic [3H]TA-binding components. Furthermore, within each strain of tumor, the nuclear and cytosolic [3H]TA binders appear to be of comparable molecular weight and shape. In spite of these differences in physicochemical properties, the intracellular distribution of the [3H]TA-binding component in cells incubated with [3H]TA at 37° was similar in both strains of tumor. Thus, 86 ± 3% (n = 3) of bound radioactivity in CS cells and 79 ± 3% (n = 3) of bound radioactivity in CR cells were located in the nucleus. These values were not significantly different at the 0.05 level.

Comparison of [3H]TA and [3H]Dexamethasone Binding.

Dexamethasone, like TA, is a synthetic, fluorinated glucocorticoid that has been widely used for steroid-binding studies in normal and malignant lymphocytes (21). Dexamethasone differs from TA in that it has a 16α-methyl- rather than a 16α-hydroxyl substituent. Also, dexamethasone lacks the 16,17-acetone function present in TA. Therefore, it was of interest to compare the physicochemical properties of nuclear [3H]TA and [3H]dexamethasone-binding components. For this purpose, a suspension of CS (or CR) P1798 lymphocytes was divided into 2 portions. One portion was incubated for 1 hr at 37° with [3H]TA while the other was incubated with [3H]dexamethasone. Nuclei from the [3H]TA- and [3H]dexamethasone-labeled cells were extracted with 10 mM spermidine, and the extracts were analyzed by agarose gel filtration and glycerol.

Table 1

<table>
<thead>
<tr>
<th>Stokes radius (Å)</th>
<th>Sedimentation coefficient</th>
<th>% of nuclear-associated [3H]TA-extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>57.4 ± 0.7^a (10)^b</td>
<td>3.70 ± 0.02 (8) 86 ± 1 (10)</td>
</tr>
<tr>
<td>CR</td>
<td>29.1 ± 0.3^c</td>
<td>3.30 ± 0.003 (5) 88 ± 1 (5)</td>
</tr>
<tr>
<td>Spermidine extract</td>
<td>58.8 ± 0.6 (6)</td>
<td>3.64 ± 0.01 (4) 81 ± 1 (6)</td>
</tr>
<tr>
<td>CR</td>
<td>28.9 ± 0.2^d</td>
<td>3.23 ± 0.05 (3) 82 ± 1 (3)</td>
</tr>
<tr>
<td>MgCl2 extract</td>
<td>59.2 ± 1.2</td>
<td>3.66 ± 0.02 (3) 54 ± 4 (3)</td>
</tr>
</tbody>
</table>

^a Mean ± S.E.
^b Numbers in parentheses, number of experiments.
^c p < 0.001 versus CS.
^d ND, not done.
^e p < 0.005 versus CS.

Gradient centrifugation. Chart 7 depicts the results obtained with nuclei from cells labeled with [3H]dexamethasone. Profiles of extracts from parallel samples of [3H]TA-labeled nuclei (not shown) were identical to the ones already presented in Charts 3 and 4 for CS and CR nuclei, respectively. No major differences were detected between the [3H]TA- and [3H]dexamethasone-binding components as judged by gel filtration or glycerol gradient centrifugation. However, there appeared to be
Chart 7. Agarose gel filtration and glycerol density gradient centrifugation of nuclear-associated \[^{3}H\]dexamethasone-binding components from CS and CR P1798 lymphocytes. Nuclei were isolated from cells that had been incubated for 1 hr at 37° with 60 nM \[^{3}H\]dexamethasone. The nuclei were extracted with 10 mM spermidine as described in "Materials and Methods." One portion of the extract was subjected to gel filtration. Another portion was analyzed by glycerol density gradient centrifugation. a, profile of the extract from CS nuclei on a column of agarose A-1.5m. b, profile of the extract from CR nuclei on a column of agarose A-0.5m. Sample volume was 3 ml for both columns. Arrows, position of standard proteins. Abbreviations as in Charts 1 and 4. Glycerol density gradient profiles of spermidine extracts from CS (c) and CR (d) P1798 nuclei. Arrow, position of the \[^{14}C\]ovalbumin internal marker.

considerably more dissociation of \[^{3}H\]dexamethasone than of \[^{3}H\]TA during column elution and the 21-hr centrifugation. Work by Schaumburg (27) and by Bell and Munck (3) with rat thymocytes and data of MacDonald and Cidlowski (19) obtained with spleen cell suspensions suggest that this may be due to lower affinity of \[^{3}H\]dexamethasone for its binding sites. Results of the competition experiments depicted in Chart 8 are consistent with this possibility. Thus, 4- to 5-fold higher concentrations of nonradioactive dexamethasone than of unla
teled TA were required to cause 50% suppression of nuclear binding of \[^{3}H\]TA in CS P1798 cells. Conversely, the concentration of nonradioactive TA that reduced nuclear binding of \[^{3}H\]dexamethasone by 50% was 4 to 5 times lower than the necessary concentration of unlabeled dexamethasone. Similar results were obtained in a second experiment with CS cells and in 2 experiments of identical design with cells from the CR tumor. Chart 9 shows that nonradioactive dexamethasone was a competitive inhibitor of \[^{3}H\]TA nuclear binding by CS cells and that unlabeled TA competitively suppressed nuclear uptake of \[^{3}H\]dexamethasone. Identical results were obtained with CR P1798 lymphocytes (not shown). Each of these competition experiments was performed twice with each strain of tumor.

As shown in Table 2, determination of apparent dissociation constants for nuclear binding in whole cells at 37° by the method of Scatchard (25) revealed also that \[^{3}H\]TA was bound more tightly than was \[^{3}H\]dexamethasone. The 3-fold difference in dissociation constants observed between the 2 steroids agrees reasonably well with the results of the competition experiments in Chart 8. The number of binding sites is higher than was reported previously because calculations are based on total ethanol-extractable nuclear counts at infinite steroid concentration rather than on the fraction of bound steroid in KCl extracts of nuclei from cells exposed only to a subsaturating concentration of \[^{3}H\]TA (36). However, the difference in binding between CS and CR cells is still highly significant. The somewhat lower levels of binding observed with \[^{3}H\]dexamethasone may be due to partial dissociation of \[^{3}H\]dexamethasone during cell fractionation and nuclear isolation. In view of the complexity of nuclear binding by whole cells, no conclusion can be drawn from this type of analysis (which was performed...
for comparative purposes only) concerning saturability, possible subclasses, and affinity of nuclear acceptor sites.

**DISCUSSION**

This study shows clearly that 0.6 M KCl extracts of purified CR P1798 nuclei from cells labeled with [3H]TA at 37° contain a smaller and less asymmetrical glucocorticoid-binding component (40,000 daltons; axial ratio, −5) than do 0.6 M KCl extracts from purified CS P1798 nuclei (90,000 daltons; axial ratio, −15). Several lines of evidence suggest that the smaller CR nuclear [3H]TA-binding component was not an artifact that arose during the extraction procedure. (a) The 0.6 M KCl extraction buffer was supplemented with glycerol, BSA, and the protease inhibitor CBZ-L-Phe (7) to maximally stabilize binding activity. (b) No increase in size of the CR [3H]TA complex was achieved by extracting nuclei in the presence of concentrations of leupeptin known to block cleavage of glucocorticoid and other steroid receptors (31, 32). (c) Extraction of nuclei with low-ionic-strength buffers containing 10 mM spermidine (CS and CR nuclei) or 4.5 mM MgCl2 (CS nuclei), conditions unlikely to release chroatin-associated proteases (9), yielded [3H]TA macromolecule complexes of which the Stokes radii and sedimentation coefficient of the complexes formed with [3H]dexamethasone, another widely used synthetic glucocorticoid. The higher relative binding affinity of [3H]TA compared to that of [3H]dexamethasone makes [3H]TA the ligand of choice for studies on glucocorticoid binding in lymphoid tissues when dissociation of the steroid is an undesirable feature.

In an earlier study, Kaiser et al. (15) reported that 0.15 M KCl extracts of the 27,000 x g (crude chromatin) pellet from CR P1798 tumor cells that had been labeled with [3H]TA at 37° contained a more slowly sedimenting [3H]TA complex (3.7S) than the [3H]TA-binding component in similar extracts from CS cells (4.1S). This difference was not apparent when intact, [3H]TA-labeled cells, rather than the 27,000 x g pellet, were extracted directly with 0.15 M KCl. Furthermore, Kaiser et al. (15) observed no difference in the presence of 0.15 M KCl between the sedimentation coefficients of cytoplasmic [3H]TA-binding components from CS and CR P1798 tumor cells that were used for comparative purposes only.)

We reported previously that low concentrations of spermide were much less effective in releasing [3H]TA from CR than from CS P1798 nuclei (36). One mM spermidine extracted only 20% of [3H]TA from CR nuclei compared to about 60% from CS nuclei. This difference was abolished when the spermidine concentration was increased to 5 mM. Under these conditions, 60 to 70% of [3H]TA was extracted from both CS and CR nuclei. Furthermore, close to 70% of [3H]TA in 5 mM spermidine extracts was bound to macromolecules even though no protease inhibitor was included in the extraction buffer. These results suggested that extraction with spermidine might be a convenient method for isolating undegraded, lymphoid, nuclear glucocorticoid-binding components under relatively mild conditions in the absence of an antiprotease. As shown in this paper, extraction of either CS or CR P1798 nuclei with 10 mM spermidine-BSA (2 mg/ml) in the absence of CBZ-L-Phe or leupeptin released 80% of nuclear-associated [3H]TA of which 90% or more was recovered as a macromolecular complex. The physicochemical properties of [3H]TA complexes extracted with spermidine were identical to those of [3H]TA-binding components extracted with 0.6 M KCl-10 mM CBZ-L-Phe. Using the spermidine extraction procedure, we established that the [3H]TA-binding component was indistinguishable from those of the complexes formed with [3H]dexamethasone, another widely used synthetic glucocorticoid. The higher relative binding affinity of [3H]TA compared to that of [3H]dexamethasone makes [3H]TA the ligand of choice for studies on glucocorticoid binding in lymphoid tissues when dissociation of the steroid is an undesirable feature.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Comparison of nuclear binding of [3H]TA and [3H]dexamethasone by Scatchard analysis (25)</th>
</tr>
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<tbody>
<tr>
<td>Cells were incubated 1 hr at 37° with concentrations of [3H]TA or [3H]dexamethasone ranging from 1 to 300 nM. Nuclei were isolated from the cells and extracted with absolute ethanol, and an aliquot of the ethanol extract was counted. Values were corrected for nonspecific binding as described in &quot;Materials and Methods.&quot; Three experiments were performed with each isotope and with each strain of tumor.</td>
<td></td>
</tr>
<tr>
<td>CS P1798</td>
<td>[3H]TA</td>
</tr>
<tr>
<td>[3H]TA</td>
<td>12.943 ± 562*</td>
</tr>
<tr>
<td>[3H]Dexamethasone</td>
<td>11.285 ± 580*</td>
</tr>
<tr>
<td>CR P1798</td>
<td>[3H]TA</td>
</tr>
<tr>
<td>[3H]TA</td>
<td>6.135 ± 427*</td>
</tr>
<tr>
<td>[3H]Dexamethasone</td>
<td>4.817 ± 632*</td>
</tr>
</tbody>
</table>

* Mean ± S.E.  
* Not significant versus molecules of [3H]TA per CS nucleus.  
* p < 0.025 versus dissociation constant of [3H]TA.  
* p < 0.001 versus molecules of [3H]TA per CS nucleus.  
* p < 0.005 versus molecules of [3H]dexamethasone per CS nucleus.  
* Not significant versus molecules of [3H]TA per CR nucleus.
had been labeled at 4°. From the data of Kaiser et al. (15), it would seem also that cytoplasmic [3H]TA-binding components from CS and CR cells incubated with steroid at 4° were larger than the [3H]TA-binding components extracted from the 27,000 x g pellet of cells that had been labeled at 37° (CS, 4.7S versus 4.1S; CR, 4.6S versus 3.7S). These values were obtained also in the presence of 0.15 M KCl. Contrary to these observations, we found that the [3H]TA-binding component in cytosol from CS cells (Stokes radius, 57.5 Å; sedimentation coefficient, 3.66S) was identical in size to the nuclear-associated [3H]TA complex in CS P1798 cells. Similarly, [3H]TA complexes in cytosol from CR cells (Stokes radius, 29.7 Å; sedimentation coefficient, 3.30S) were identical in size to those in CR nuclear extracts. The different results obtained by Kaiser et al. (15) and ourselves could be due to differences in methodology or to the use of different variants of the P1798 tumor (36).

The molecular weight of the CS P1798 glucocorticoid-binding component (approximately 90,000 daltons) is comparable to that of the cytosolic [3H]dexamethasone complex from CS S.49 lymphoma cells (43). The molecular weight of the [3H]TA-binding component from CR P1798 lymphocytes (40,000 to 46,000 daltons) appears to be somewhat lower than that of the cytosolic [3H]dexamethasone complex from “nuclear transfer-increased” CR S.49 mutants (50,000 daltons). The strain of CR P1798 used in the present study cannot be characterized as a “nuclear transfer-increased” variant since the fraction of bound [3H]TA associated with the nucleus at 37° was very similar (about 80%) in both CS and CR cells. Rather, the strain of CR P1798 used here is a variant in which altered, nuclear glucocorticoid interactions are associated with an overall reduction in specific glucocorticoid-binding sites and presence of a modified steroid-binding component.

It is interesting to compare our data with the results of Carlstedt-Duke et al. (6) and of Wrangle and Gustafsson (41) on [3H]dexamethasone binding in rat liver. These authors described a 61-Å, 4S, 102,000-dalton [3H]dexamethasone-receptor complex in hepatic cytosol with a frictional ratio of 1.84. These physicochemical parameters are comparable to the ones we obtained for the cytosolic and nuclear glucocorticoid-binding component in CS P1798 tumor cells. Chymotryptic digestion of the liver [3H]dexamethasone complex led to formation of a 36 Å, 46,000-dalton fragment with a sedimentation coefficient of 3.2S and a frictional ratio of 1.38 (41). In turn, these parameters are quite similar to the values obtained for cytosolic and nuclear [3H]TA-binding components from CR P1798 tumor cells. However, it should be noted that Carlstedt-Duke et al. (6) and Wrangle and Gustafsson (41) used Sephadex G-150 and G-200 to estimate Stokes radii whereas we used agarose A-0.5m and A-1.5m. It is possible that different Stokes radii could be obtained for the same protein analyzed on different matrices.

The 36 Å fragment from rat liver had a higher affinity for DNA-cellulose than did the 61 Å complex (41). This is particularly interesting in light of our observation (36) that the 29 Å [3H]TA-macromolecule complex present in CR P1798 nuclei was less readily released with low concentrations of mono-, di-, and trivalent cations than was the 57 Å complex from CS P1798 nuclei. It is tempting to speculate that the CS P1798 [3H]TA-binding component might consist of a portion that is similar or identical to the 29 Å species characteristic of CR P1798 cells plus another portion that endows it with the capability of binding normally to nuclei, thereby initiating the events that ultimately result in cell death. This possibility is currently under investigation. Alternatively, the 57 Å complex may be a dimer that is resistant to the dissociative action of 0.6 M KCl. It is well established that chick oviduct progesterone receptor consists of 2 subunits (28). However, studies on glucocorticoid receptors from a variety of mammalian cells have failed to provide evidence for a similar structure (23).

We have found that corticoid-responsive lymphocytes from patients with chronic lymphatic leukemia contain glucocorticoid-binding components similar, if not identical, in size to those present in CS P1798 (this paper) and CS S.49 (43) mouse lymphoma cells. In addition, other glucocorticoid-responsive tissues, such as rat liver (6, 18, 41) and CS Novikoff hepatoma cells (18), contain a 90,000 to 100,000-dalton glucocorticoid binder also. We suggest that presence of a large glucocorticoid-binding component may be necessary for expression of glucocorticoid sensitivity by malignant lymphocytes and other glucocorticoid target tissues. However, other factors must be involved also, inasmuch as the [3H]dexamethasone-receptor complex from CR “deathless” S.49 mutants was physicochemically indistinguishable from the [3H]dexamethasone-receptor complex of wild-type CS cells (43). Furthermore, Novikoff hepatoma cells that were not inhibited by glucocorticoid contained a 90,000-dalton [3H]dexamethasone-receptor complex also (18). It should be pointed out that the physicochemical properties reported here and in most other studies were obtained by examination of crude nuclear and cytoplasmic preparations. However, it is encouraging to note that the physicochemical properties of purified chick oviduct progesterone receptors are very similar to those derived from the study of crude extracts (11, 16).

In summary, we have shown that lack of response of CR lymphoma P1798 to glucocorticoid administration is associated with the presence of a glucocorticoid-binding component strikingly smaller than the one characteristic of CS P1798 lymphocytes. The altered physicochemical properties of this binding component may be responsible, at least in part, for the previously described differences in nuclear-glucocorticoid interactions between CS and CR P1798 cells (36).

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Glucocorticoid Binding in Lymphocytes


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Physicochemical Differences between Glucocorticoid-binding Components from the Corticoid-sensitive and -resistant Strains of Mouse Lymphoma P1798

John Stevens and Yee-Wan Stevens


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