Multiple Forms and Fragments of Cytosolic Glucocorticoid Receptors from Human Leukemic Cells and Normal Lymphocytes

Merry R. Sherman, Yee-Wan Stevens, and Fe B. Tuazon

Endocrine Biochemistry Laboratory, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

Therapy with glucocorticoids is generally more effective in acute lymphoblastic leukemia than in other types of human leukemia. Previous studies, however, have not revealed any consistent relationship between clinical responsiveness and the cellular or cytosolic concentration of glucocorticoid-binding sites. The objectives of this study were to determine whether there are intrinsic structural differences among the glucocorticoid receptors in various types of leukemic cells and normal lymphocytes and to investigate the role of endogenous peptidases in receptor degradation.

Cytosols were prepared from fresh or rapidly frozen leukocytes from 6 healthy adults and 35 high-risk leukemia patients (median white blood cell count, 150,000 cells/μl; median age, 13 years). Receptors were labeled with [3H]triamcinolone acetonide and quantitated by charcoal-dextran treatment or Sephadex LH-20 chromatography. Mean and median cytosolic receptor concentrations in 12 acute lymphoblastic leukemia specimens lacking the standard B-cell or T-cell markers ("null cells") were ~4-fold higher than in 23 other leukemic cell specimens. No other consistent differences in receptor content were observed.

Agarose filtration and ultracentrifugation in hypotonic buffers containing 20 mM Na2MoO4 revealed complexes of similar size and shape in all clinical specimens tested and two established leukemic cell lines. They had Stokes radii (R₀) of 8.1 ± 0.5 (S.D.) nm (n = 50), sedimentation coefficients of 9.5 ± 0.3 (n = 40), molecular weights of ~330,000, and axial ratios (a/b) of ~12. In hypotonic, molybdate-free buffer, these oligomeric complexes were dissociated into subunits with R₀ of 5.9 ± 0.3 nm (n = 12) and a/b of 11 to 12, as observed previously for other receptors. Fragmentation of the oligomer and the subunit was evident in some cytosols.

High activities of peptidases of various specificities were detected in leukemic cell cytosols, as in other cytosols, by fluorometric assays with derivatives of 7-amino-4-methylcoumarin. Receptor cleavage by these and other endogenous enzymes may account for previous observations of "abnormal" receptors in cytosols from some leukemic specimens. We conclude that intrinsic structural defects in the receptors are unlikely explanations for the unresponsiveness of some types of leukemia to steroid therapy.

INTRODUCTION

Differences in the responsiveness of various types of leukemia and lymphoma to treatment with glucocorticoids became evident soon after the introduction of cortisone therapy (reviewed in Refs. 2 and 54). Given the essential role of receptors in steroid hormone action, the plausible explanations for unresponsiveness include a reduction in the number of receptors and defects in receptor structure (2, 9, 11, 16-18, 21, 29, 30, 54, 55). To date, however, the evidence presented in support of either of these hypotheses has not been definitive.

The steroid receptors detected in hypotonic tissue extracts (cytosols) are large oligomeric proteins composed of subunits bearing at least 3 types of functional domains. These are responsible, respectively, for the binding of steroid, the binding of nuclear components, and the binding of the subunits to each other (6, 37, 44, 48, 49, 52, 55). It is a remarkable fact that the oligomeric forms of the receptors for several classes of steroids in a variety of species and tissues are nearly identical (36, 46, 47, 49, 52). We may infer that this highly conserved structure serves an essential function in steroid hormone action. Evaluation of the physicochemical parameters of this receptor form has been complicated by the ease with which the oligomer is dissociated into subunits in vitro and the vulnerability of these structures to cleavage by endogenous proteases. For these reasons, early attempts to characterize either the oligomeric or the monomeric form of the receptors from various tissues produced a wide range of results and led to considerable confusion about the intact structure. Many of the technical problems have been resolved by the discovery that the oligomer is stabilized not only by protease inhibitors (45, 48, 50, 51) but also by sodium molybdate and related salts (12, 19, 28, 32, 36, 46, 47, 51, 52, 55).

In pursuit of the molecular basis of steroid resistance, we began this detailed physicochemical study of the glucocorticoid receptors in cytosols from various hematopoietic cancers and normal lymphocytes several years ago. The samples analyzed have included 2 established cell lines derived from human leukemias and purified leukocytes from 35 patients with CML, CLL, diffuse poorly differentiated lymphoma, and several subtypes of ALL and ANLL. Most of the patients had extremely high WBC;...
the median value was 150,000 cells/μl. The data for receptor content and structure in these specimens have been analyzed in the context of results obtained on cells from the same patients by other laboratories at this Center. They have also been compared with our previous results for receptors from several healthy and malignant human and rodent tissues.

The following specific questions were addressed in the present study. Are there intrinsic or apparent structural differences among the glucocorticoid receptors extracted from normal lymphocytes and various hematopoietic cancers? If so, are such differences correlated with clinical findings or with morphological, cytogenetic, cytochemical, enzymatic, or immunological characteristics of the cells? In attempting to answer these questions, we have evaluated and compared the Stokes radii and sedimentation coefficients of the molybdate-stabilized glucocorticoid-receptor complexes in the various cytosols and calculated the molecular weights and axial ratios from these parameters. Similarly, we have characterized the salt-dissociated forms and proteolytic fragments of the receptors and have investigated the effects on receptor structure of rapidly freezing the cells in the absence of preservatives. Finally, in view of the observed degradation of the receptors in certain cytosols, we have begun to quantitate, characterize, and look for possible differences among the peptidases that are active in these cytosols at physiological pH.

MATERIALS AND METHODS

Materials. Most of the materials and methods of analysis of steroid receptors and cytosolic peptidases have been described in detail in a recent report (47). In addition to the materials used in that study, we obtained Ficoll-Metrizoate (Lymphoprep) from Accurate Chemical (Hicksville, NY). HBSS (15) and RPMI 1640 Tissue Culture Medium (33) were prepared by the Media Laboratory of this Center.

Buffers. The following buffers were prepared with Tris and had a pH of 7.4 at 24° and 7.9 at 4°: TE buffer; TEG buffer; TTE buffer; TTEG buffer; and TTES buffer. Solutions of 20 mM Na2MoO4 are designated as +Mo. Control solutions (−Mo) had KCI concentrations of 0 to 50 mM, as indicated. Buffers containing 0.4 M KCI were referred to as hypertonic, and those with KCl concentrations of 0 to 50 mM were referred to as hypotonic, regardless of the concentrations of other components.

Cell Lines. Frozen cells from 2 established cell lines derived from human nonlymphocytic leukemias were generously provided by Dr. David W. Golde, University of California Medical School, Los Angeles. Line KG-1 was established by Koeffler and Golde (24) from a patient with acute myeloid leukemia. Line HL-60 was established by Collins et al. (8) from a patient with acute promyeocytic leukemia.

Clinical Specimens (Purification, Characterization, and Storage of Cells). Most of the specimens consisted of 12 to 33 ml of heparinized peripheral blood from untreated pediatric patients with leukemia or leukemia-lymphoma syndrome, 13 to 240 ml of leukapheresis preparations from adolescent or adult leukemia patients, or 200 to 500 ml of blood from healthy adult donors. In order to obtain a sufficient quantity of cells to permit multiple physicochemical analyses of the receptors, we requested specimens primarily from high-risk patients (having a WBC exceeding 50,000 cells/μl). The median and mean WBC for the 35 malignant specimens used in this study were 150,000 and 240,000 cells/μl, respectively. A few specimens, which arrived late in the day, were diluted with RPMI 1640 and stored overnight at room temperature (−24°) prior to purification of the leukocytes. Most specimens were diluted promptly with 1 to 9 volumes of HBSS and centrifuged over Ficoll-Metrizoate at 24° for 30 min at −150 × g, as described previously (5, 56). In some leukemic cell preparations, the interface layer from the initial centrifugation contained visible RBC contamination. This was reduced, but not eliminated, by resuspension of the cells in HBSS and recentrifugation over Ficoll-Metrizoate. Cells from the interface layer were washed twice with 20 ml of HBSS, resuspended in 50 ml of RPMI 1640, and incubated for 30 min at 37° to promote dissociation of endogenous steroids (10).

The size and yield of the cells varied among the different types of leukemia. The mean ± S.D. of the mass of cells recovered from the peripheral blood of ALL patients was 240 ± 110 mg (n = 16)/10° cells (e.g., a 10-ml sample with a WBC of 100,000 cells/μl). The mass of cells recovered from the same number of ANLL cells, 440 ± 170 mg (n = 7), was generally higher than from the ALL specimens (p < 0.01), as expected from the size of the cells.

Our initial studies of normal specimens confirmed previous reports that cells fractionated as described above include a significant percentage of monocytes (27). Therefore, in more recent preparations of donor cells, the lymphocytes were further purified by allowing the monocytes to adsorb to plastic flasks (Corning: 150 sq cm) for 1 hr at 37° (35).

For practical reasons, many preparations of purified leukocytes were stored at −85° for periods of 1 day to more than 1 year prior to homogenization. Following the final wash with RPMI 1640, the cells were centrifuged for 10 to 15 min at 200 × g into weighed Nunc tubes (Vanguard International, Neptune, NJ) and placed in a Revco freezer. As shown in "Results and Discussion," this procedure caused significant alterations in receptor structure and is not recommended for future studies.

Bone marrow specimens from most patients were classified by morphological and cytochemical criteria according to the French-American-British system (1, 3). Peripheral blood or bone marrow specimens were characterized with respect to immunological markers by the Lymphocyte Surface Markers Laboratory (28); the activities of Tδ2 were determined by the Department of Clinical Chemistry (31), and cytogenetic analyses were performed by the Laboratory of Cancer Genetics and Cytogenetics (7) at this Center.

Cytosol Preparation, Labeling of Receptors, and Removal of Free Steroid. Frozen cells were thawed at 0-4° and were generally suspended in 3 to 5 volumes of TTES buffer containing no added salt or 20 mM KCl (−Mo cytosols) or 20 mM Na2MoO4 (+Mo cytosols). They were homogenized on ice in a Potter-Elvehjem glass-Teflon homogenizer. As fresh cells are more resistant than frozen cells to rupture in isotonic buffer, the fresh cells were usually homogenized in TTE buffer (±Mo) and the homogenate was promptly adjusted to isotonicity by the addition of one-third volume of 1 M sucrose in the same buffer. Cell breakage under these conditions was confirmed by light microscopy. For studies involving comparisons of fresh and frozen cells from the same specimen, the fresh-cell protocol was used for both preparations. Cytosols were prepared by centrifugation at 0° for 25 min at 246,000 × g or for 37 min at 166,000 × g (the equivalent of 1 hr at 100,000 × g).

The steroid-binding specificity of glucocorticoid receptors in normal human lymphocytes and leukemic cells has been established in several previous studies (11, 22, 29, 56) and was not reinvestigated here. [3H]TA was used to label the receptors in all of our experiments, because it has been shown to have high affinity for the receptors and a slow rate of dissociation. All incubations of cytosols with steroids and all analyses of the labeled complexes were performed at 0-4°. Aliquots of cytosol were incubated with various concentrations of [3H]TA in the absence or presence of a 200-fold excess of unlabeled TA, to assay the total and nonspecific binding, respectively. Samples were incubated for 90 min, unless otherwise indicated. For routine physicochemical analyses, the receptors were labeled with a high concentration of [3H]TA (−40 to 100 nm). Free steroid was removed either by agitation for 20 min with a charcoal-dextran suspension (final concentrations, −2% Norit A; −0.2% Dextran T-40) and centrifugation for 5 min at 8700 × g ("charcoal treatment") (36) or by chromatography of labeled cytosol (up to 2.4 ml) on a column of Sephadex LH-20.(0.7 x 23 cm) (45, 51). Labeled cytosols were sometimes frozen prior to the removal of free steroid and physicochemical analysis. The effects of freezing the cytosol on the quantity and structure...
of the steroid-receptor complexes were investigated.

For the evaluation of the dissociation constant (Kd) and the concentration of specific binding sites (B0), the total concentration of [3H]TA was varied from 2 to 75 nM. The total binding observed at each steroid concentration was corrected for the nonspecific binding evaluated at the same concentration of free [3H]-steroid. This provides a considerably lower (and more appropriate) estimate of nonspecific binding than that measured at the same total concentration of [3H]-steroid, under conditions in which a significant proportion of the total [3H]-steroid is bound. Values of Kd and B0 were determined by unweighted linear regression analysis of data for the ratio of specifically bound steroid (B) to free steroid (F) as a function of B. Most cytosols were labeled with a single, nearly saturating concentration of [3H]TA, and B0 was estimated using the equation (41)

$$B_0 = \frac{B}{F} (K_d + F)$$

and a value of 10 nM for Kd (10). The reported values of Kd for the binding of dexamethasone to leukemic cell receptors vary considerably (10, 54). With a free steroid concentration of 60 nM, however, the resultant estimates of B0 based on values of 5 or 20 nM for the Kd of [3H]TA would be 93 or 114%, respectively, of those based on a Kd of 10 nM.

Fluorometric Peptide Assays. We used the following substrates, all synthesized from L-α-amino acids and AMC: leucyl-AMC, glutaryl-glycyl-arginyl-AMC, succinyl-alanyl-alanyl-prolyl-phenylalanyl-AMC, succinyl-leucyl-tyrosyl-lysyl-AMC, and β-butyloxycarbonyl-valyl-leucyl-glycyl-arginyl-AMC (23, 34). Stoch solutions of the substrates (10 mM in dimethyl sulfoxide) were stored at 4°C prior to dilution in assay buffer to concentrations of 10 μM.

Assays were performed at room temperature (~24°C) in 2.5 ml of TEG buffer, unless otherwise indicated. The reaction product, AMC, was detected in a Perkin-Elmer spectrofluorometer at excitation and emission wavelengths of 375 and 438 nm, respectively, as described previously (47, 50). Reaction velocities, in RFU/hr, were determined by linear regression analysis of at least 3 readings, made at intervals of 20 min to 1 hr, during the linear portion of the reaction. This included a period of at least 3 hr after the addition of leukocyte cytosols or charcoal-treated cytosols to all of the substrates tested. Under these conditions, significant rates of cleavage of all of these substrates except the arginine derivative were detected using 20 μM substrate and 20 μl or less of cytosol. Following the filtration of cytosols on agarose columns (see Chart 9), the rates of cleavage of the phenylalanine derivative were increased ~20-fold by the presence of 20 μM CaCl2 in the assay buffer, and the kinetics of cleavage was nonlinear. Therefore, a linear portion of the assay, from 3 to 5 hr after the addition of enzyme, was used to evaluate the relative activities in the column fractions (47).

Physicochemical Analyses (Gel Filtration, Ultracentrifugation, and Ion-Exchange Chromatography). Three or 4 internal standards were routinely included with the samples for gel filtration or density gradient centrifugation. These proteins were detected either spectrophotometrically (myoglobin, hemoglobin, catalase, ferritin) or radiometrically, after reductive methylation with [14C]formaldehyde (40). The tissue sources and primary references for the parameters of the standards have been published (13, 32, 50).

Gel filtration was performed on columns of Agarose A-1.5m (100 to 200 mesh; 1.27 x 90 to 100 cm) at flow rates of 15 to 18 ml/hr in TTEG buffer containing various salts. The void volume (V0) and the total liquid volume (Vt) were determined as described previously (47). These parameters and the elution volume (Ve) were used to calculate the distribution coefficient:

$$K_d = \frac{V_t - V_e}{V_e - V_0}$$

The Stokes radii (Rd) of the standards used to calibrate the columns were: myoglobin, 2.01 nm; carbonic anhydrase, 2.28 nm; serum albumin, 3.59 nm; β-amyrase, 4.81 nm; ferritin, 6.15 nm; and thyroglobulin, 8.61 nm. The Stokes radii of the receptors and peptidases were computed from the linear dependence of log Rd on Kd for the standards in each column (43). Estimates of the width of the peaks were obtained by calculating the values of Rd corresponding to the left and right sides of the peaks at two-thirds of the maximum height (see Chart 2). Comparisons of the extent of receptor cleavage in various preparations were based on the percentage of the labeled complexes characterized by Rs of <4 nm (see Charts 3, 4, and 7).

Density gradient centrifugation was performed at 0° in a Beckman L2-65B ultracentrifuge. Samples of up to 0.5 ml were layered onto 3.8-ml linear gradients of 16 to 41% (w/v) glycerol in various buffers and centrifuged for 17 to 22 hr at 246,000 × g to 360,000 × g in a Beckman SW56 or SW60Ti rotor, as indicated in “Results and Discussion.” Samples of 1 ml were layered onto 12-ml gradients of 16 to 41% (w/v) glycerol and centrifuged for 42 hr at 148,000 × g in a Beckman SW40 rotor. The sedimentation coefficients (sd) of the standards were: myoglobin, 2.05; carbonic anhydrase, 3.2S; hemoglobin, 4.4S; serum albumin, 4.4S; aldolase, 7.9S; β-amyrase, 9.4S; and catalase, 11.3S. Values of sd were for the receptors were computed from the linear correlation of sd with fraction number for the internal standards.

The molecular weight of each receptor form was calculated from 4224 (sd, Rg, Rd), where sd were in Svedberg units, Rg was in nm, and the factor was based on a value of 0.732 cu cm/g for the partial specific volume, as reported for serum albumin (43, 50). The frictional ratio due to shape, (sf/fo), was calculated from 13.93 (Rs/Mril3), in which a hydration of 0.2 g/g of protein was assumed (43). The axial ratio (a/b) of a hydrodynamically equivalent prolate ellipsoid was determined from (sf/fo) and a published table (42). Ion-exchange chromatography was performed on columns of DEAE-cellulose (Whatman DE52; 1.5 cm diameter x 6 cm) in TTEG +Mo buffer, as described previously (47, 51). Adsorbed proteins were eluted with a linear gradient prepared from 80 ml each of 0.10 M and 0.6 M KC1 in the buffer.

Other Methods. Protein concentrations were determined using fluorescamine (4), with bovine serum albumin as the standard. In cytosols prepared from frozen cells, the protein concentrations were consistently higher (13 to 37%) than in the corresponding preparations from fresh cells. In some experiments (as indicated in “Results and Discussion”), the cytosolic protein concentration was supplemented by the addition of ovalbumin, in order to minimize proteolysis of the receptors. Radioactivity in aqueous samples of up to 0.8 ml was measured in 5 ml of Aquasol, and the data were analyzed as described previously (47). The results reported for most parameters indicate the number of determinations (n) on which the mean and the standard deviation were based. Calculations of statistical significance were based on the two-tailed Student’s t test. Coefficients of determination of linear regressions (r2) and the 95% confidence intervals of the slopes and intercepts were described as described by Sneden and Cochran (53). Our computer programs are available upon request.

RESULTS AND DISCUSSION

Overview. We have examined the relationship of cytosolic receptor content to leukemic cell subtype, the structure of the glucocorticoid-receptor complexes, and the properties of several cytosolic peptidases. The results have been evaluated in relation to the conditions under which the specimens were stored, processed, and analyzed. Very high concentrations of receptor were found in cytosols from one immunological subtype of leukemic cells (Table 1). The major forms and fragments of the receptors from various types of leukemic cells and normal lymphocytes have been characterized by physicochemical techniques. The receptor forms analyzed are: the large complex, which is stabilized by protease inhibitors and by molybdate in buffers of low and moderate ionic strength (Charts 1 to 4; Table 2); a subunit...
of this complex, which is detected in molybdate-free, hypertonic buffers (Charts 7 and 8; Table 3); the smallest fragment which retains the bound steroid, called the mero-receptor (Charts 5 and 6); and fragments of intermediate size (Charts 7 and 8). Because the fragments appear to be the products of endogenous peptidases, several of these enzymes have been characterized (Chart 9). In the course of these studies, several technical issues were resolved. Two methods for the removal of free steroid from labeled cytosols were compared; the effects of freezing the labeled cytosols on the amount of bound steroid subsequently detected were analyzed; the effects of the presence of molybdate in the homogenate and/or the gel filtration buffer were investigated (Charts 2 to 5); and effects on receptor structure of rapidly freezing the cells in the absence of preservatives were examined (Charts 6 and 7).

Comparison of Methods Used to Remove Free Steroid from Labeled Cytosols. While adsorption to dextran-coated charcoal permits the rapid removal of free and loosely bound steroid from labeled cytosols, a chromatographic technique is sometimes advantageous. Chromatography on small columns of Sephadex or Sephadex LH-20 is preferable for cytosols or other receptor preparations with very low protein concentrations, from which there may be a significant loss of receptors by adsorption to charcoal (44). In addition, the receptors can be rapidly equilibrated with a new buffer during chromatography. We have reported previously that better resolution of bound from free steroid is obtained with the hydrophobic derivative, Sephadex LH-20, than with unmodified Sephadex (51). Moreover, under the conditions used in this study, the elution of bound steroid from small columns of Sephadex LH-20 is nearly as rapid as the recovery of the supernatant following charcoal treatment and centrifugation.

In a preliminary experiment, we compared the Scatchard plots (41) of data obtained by charcoal treatment and by chromatography on Sephadex LH-20 for [3H]TA binding to a leukemic cell cytosol with a high protein concentration (14 mg/ml). A slightly lower estimate of $K_d$ was obtained by the chromatographic technique, and the difference was just significant at the level of 0.05. The results for $K_d$ and the 95% confidence intervals were 16.1 (14.5 to 18.2) nM using Sephadex LH-20 and 21.1 (18.3 to 24.8) nM using charcoal. We also noted that the amount of nonspecific binding determined by charcoal adsorption was higher than that determined chromatographically. At the highest concentration of [3H]TA used in this experiment (~70 nM), the nonspecific binding corresponded to 8% of the total binding detected by charcoal treatment but was <1% of that detected with Sephadex LH-20. On the other hand, the values of the binding capacity ($B_0$) obtained by these techniques were not significantly different at the level of 0.05. From these results and analogous data for another ALL specimen, we inferred that similar amounts of binding would be detected by both methods if the cytosols were incubated with a nearly saturating concentration of [3H]TA. The method used to remove the free steroid did, however, affect the form of the complex detected by centrifugation in hypotonic --Mo buffer (see Table 3).

Preliminary experiments also indicated that the amount of specific binding was not decreased by freezing the labeled +Mo cytosols before removing the free steroid. This was tested using aliquots of 4 leukemic cell cytosols which were analyzed immediately after incubation with [3H]TA and after storage at -85°C for up to 1 year. The binding detected after freezing was 113 ± 7% ($n = 4$) of that detected before freezing.

Relationship of Receptor Concentration to Diagnosis and Other Characteristics of Leukemic Cells. We have correlated our results for cytosolic receptor content with clinical data and results obtained by other laboratories at this Center. Diagnoses were based on clinical and hematological findings, morphological and cytochemical criteria (1, 3), and cytogenetic data, particularly the presence or absence of the Philadelphia chromosome (7). In addition, we considered the following parameters: WBC (31 of 35 of our leukemic specimens had >50,000 cells/μl); age of the patient (24 of 27 of our acute leukemic specimens were from children); TdT activity (31); and immunological classification (26). Among the 22 specimens of ALL which were studied, 8 were classified as T cells, 12 were classified as "null," and one was reported to be "abnormal inconclusive" by the Lymphocyte Surface Markers Laboratory. One ALL specimen was not evaluated immunologically.

Although the major focus of our research was the structure rather than the quantity of receptors, we noticed that all cytosols that had very high concentrations of receptors (>900 fmol/mg protein) were from ALL cells classified as "null." Such cells lack the standard B-cell and T-cell markers; i.e., they lack surface immunoglobulins and are unable to form rosettes with sheep erythrocytes (26). Despite the absence of these surface markers, however, most "null-cell" leukemias appear to be precursors committed to the B-cell lineage at the gene level (25). Our results, which are summarized in Table 1, are consistent with those of

### Table 1

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Cytosolic receptor concentrations in subsets of leukemic cells and in normal lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosols were incubated with several concentrations of [3H]TA or with a single, nearly saturating concentration. Binding was evaluated by charcoal-dextran treatment or by Sephadex LH-20 chromatography, and the data were analyzed to determine the concentration of binding sites, as described in &quot;Materials and Methods.&quot; The resultant values were divided by cytosolic protein concentration. The data presented are based on the mean value for each specimen, regardless of whether the cells or cytosols were frozen or whether the cytosols contained molybdate.</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Specimen</strong></td>
<td><strong>No. of specimens</strong></td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>&quot;Null-cell&quot; ALL</td>
<td>12</td>
</tr>
<tr>
<td>T-cell ALL</td>
<td>8</td>
</tr>
<tr>
<td>Other leukemic cells</td>
<td>15</td>
</tr>
<tr>
<td>Normal lymphocytes</td>
<td>6</td>
</tr>
</tbody>
</table>

*Indicates a total of 2 specimens of unclassified ALL, 5 of ANLL, 2 of CLL, 5 of CML, and 1 of diffuse poorly differentiated lymphoma.

ND, not determined. The mean WBC for normal adult specimens under these conditions was 6500 ± 300 cells/μl ($n = 100$).
Yarbro et al. (57), who detected higher whole-cell binding levels in "null-cell" ALL than in T-cell ALL, using specimens from patients with lower WBC. In the latter study (57), the median WBC for the "null-cell" specimens was <18,000 cells/μl, compared with a median value of 93,000 cells/μl in our specimens. Other groups have also reported higher receptor levels in "null-cell" ALL specimens than in T-cell ALL (reviewed in Refs. 21 and 54). We conclude that cytosolic receptor levels are generally higher in "null-cell" than in T-cell ALL specimens, regardless of the WBC.

Our results for cytosolic receptor concentration, in units of fmol/mg protein, are not directly comparable to results for glucocorticoid binding to whole cells, expressed as sites/cell. Some investigators have made such correlations by assuming a certain value for the mass of cytosolic protein/cell. In our opinion, however, such an assumption is not appropriate for comparative studies of different types of leukemic cells, since they may vary greatly in size and morphology, and hence in the mass of cytosolic protein/cell. Furthermore, the amount of protein recovered in the cytosol may vary with the methods of cell storage and cytosol preparation. In view of previous reports of discrepancies between whole-cell binding and cytosolic binding (measured in the absence of molybdate) (22), more extensive comparisons of receptor levels in "null-cell" ALL and other types of leukemia should be done by both techniques.

Sequential Fractionations of the Molybdate-stabilized Receptor from ALL Cells. We reported previously that the presence of 20 mM Na₂MoO₄ stabilizes the oligomeric form of the glucocorticoid receptors in rat liver and kidney cytosols during fractionation in hypotonic buffers by several techniques in sequence (46, 47, 51). Similar analyses of the receptor in charcoal-treated cytosol from frozen ALL cells are shown in Chart 1. The initial fractionation on an agarose column in hypotonic +Mo buffer revealed a single, fairly symmetrical peak of bound steroid (Kₒ of 0.13) and a small peak of free steroid (Kₒ of 1.1), corresponding to ~8% of the total steroid eluted from the column. The recovery of [³H]TA from the agarose columns under these conditions was excellent, with a mean value of 93 ± 10% (n = 50). The large size of the steroid-receptor complex was indicated by its coelution with ¹⁴C-methylated thyroglobulin, which has a Stokes radius of 8.61 nm (13). More rigorous evaluation of receptor size was obtained by linear regression analysis of data for log Rₛ versus Kₒ for all of the internal standards. In replicate analyses of leukocyte receptors on these columns with 3 or 4 of the standards listed in "Materials and Methods," the coefficient of determination (r²) of these regressions was 0.997 ± 0.005 (n = 49). Hence, the values of Rₛ summarized in Table 2 were determined with considerable precision.

From the peak of bound steroid shown in Chart 1a, fractions corresponding to Rₛ of 8 to 9 nm were combined for further analysis. As shown in Chart 1b, glycerol gradient centrifugation of this material also revealed a single peak of bound steroid. This complex comigrated with an internal standard, ¹⁴C-methylated β-amyase, which has a sedimentation coefficient of 9.4S (32). Under these conditions, ~40% of the recovered [³H]TA remained at the top of the gradient. This radioactivity presumably represents steroid that dissociated from the receptor either during the time elapsed between the charcoal treatment and the time when the sample was layered onto the gradients (~24 hr) or during the early hours of centrifugation. In replicate analyses of leukocyte receptors on gradients prepared in this buffer, the recovery of [³H]TA was 87 ± 12% (n = 40). The linear correlation of sₑₑₑₑ with fraction number for 3 or 4 internal standards was also excellent; r² was 0.997 ± 0.003 (n = 36) in gradients prepared in this buffer (TTE +Mo).

The partially purified receptor from the agarose column shown in Chart 1a was also analyzed by chromatography on DEAE-cellulose (Chart 1c). The fraction of the recovered radioactivity that was eluted in the wash (~40%) was similar to that detected at the top of the gradient in Chart 1b. The absence of protein-bound radioactivity in this peak was demonstrated by chroma-
tography on Sephadex LH-20 (not shown). The receptor-bound radioactivity was eluted by a KCl gradient from the DEAE-cellulose column as a single, slightly asymmetrical peak, with some trailing of radioactivity on the high-salt side. The KCl concentration corresponding to the maximum in the elution profile (155 mM) was close to that determined for the receptor in unfractionated cytosol from fresh ALL cells (143 mM, not shown) and was within the range previously found for the receptors in rat liver and kidney cytosols (47).

In summary, the data in Chart 1 and studies of other specimens by either size-dependent or charge-dependent techniques revealed the presence of a single large form of the glucocorticoid receptor in most ALL cell cytosols that were analyzed in buffers of 20 mM Na2MoO4 with moderate concentrations of salt (0 to 0.2 mM KCl). From the values of Rs (8.4 nm) and s20,w (9.4S) determined in this experiment, a molecular weight of 330,000 and an axial ratio of 13 (for a prolate ellipsoid) were calculated, as described in "Materials and Methods." Our accumulated data from hydrodynamic analyses of molybdate-stabilized receptors in leukocyte cytosols are summarized in Table 2.

The Stokes radii of the oligomeric receptor forms in cytosols from fresh or frozen leukemic cell specimens varied from 7.2 to 8.5 nm. These values are within the range of results reported previously for Rs of the glucocorticoid receptors from 2 healthy tissues of the rat, the kidney [Rs of 7.1 ± 0.3 nm (n = 21)] and the liver [Rs of 8.2 ± 0.4 nm (n = 12)] (47). The data in Table 2 revealed no consistent difference in size or shape between the molybdate-stabilized receptors in cytosols prepared from fresh versus frozen ALL or CLL cells. In general, the parameters of the cytosolic receptors from lymphoid leukemias resemble those of the receptors in rat liver cytosol. In comparison with the receptors from fresh or frozen lymphoid leukemic cells, which had axial ratios of 12 to 13, the receptors from frozen myeloid leukemic cells appeared to be somewhat less asymmetrical, having axial ratios of 9 to 10. These receptors resemble those in rat kidney cytosol. Only a few myeloid leukemic samples have been analyzed to date, since most of our specimens were from children, in whom myeloid leukemias are rare. Nevertheless, these preliminary data suggest that homogenates and/or cytosols prepared from such specimens may be rich in receptor-cleaving enzymes, as found in previous studies of the rat kidney (47, 52).

Effects of Molybdate on the Degradation of Receptors prior to and during Fractionation. In the experiments described above, 20 mM Na2MoO4 was present in all of the buffers. It was of interest to determine at which stage(s) in the procedure the presence of molybdate was actually required, i.e., during homogenization of the cells, the preparation and labeling of cytosol, and/or the physicochemical analyses. Previous studies of the glucocorticoid receptor in cytosols from frozen rat liver revealed the stabilizing effect of molybdate on the large receptor form during agarose filtration, but little effect of molybdate during the extraction and labeling of the receptor (28). The results of analogous experiments on the receptor from frozen ALL cells were qualitatively similar. In this case, however, there was less extensive degradation of the receptor in the absence of molybdate than that observed for the rat liver receptor.

As shown in Chart 2a, the bound steroid in leukemic cell cytosol was eluted as a broad peak from the control columns, which contained 50 mM KCl, regardless of whether the homogenate contained 30 mM KCl or 20 mM Na2MoO4. Only a small increase in the apparent proportion of the larger components within the mixture was observed when molybdate was present prior to gel filtration. The width of each distribution was calculated as described in "Materials and Methods." The range of values of Rs was 5.6 to 9.5 nm for both samples. Despite the use of frozen cells for this experiment, only 8% of the labeled complexes had Rs of <4 nm (compare Charts 5 and 6). The heterogeneity of receptor forms seen in Chart 2a may be due to limited proteolysis and/or the dissociation of the oligomeric complex into subunits. The occurrence of both processes is suggested by the results of gel filtration in hypertonc- Mo buffer (see Charts 7 to 9 and

Table 2
Parameters of the major molybdate-stabilized receptor form in cytosols prepared from fresh or frozen leukocytes.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>No. of specimens</th>
<th>Cells frozen</th>
<th>Rs (nm)</th>
<th>s20,w (S)</th>
<th>Mw x 10^-6</th>
<th>a/b</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>11</td>
<td>-</td>
<td>8.0 ± 0.5 (10)</td>
<td>9.5 ± 0.3 (8)</td>
<td>320 ± 12</td>
<td>12</td>
</tr>
<tr>
<td>CML</td>
<td>2</td>
<td>+</td>
<td>8.5 ± 0.1 (4)</td>
<td>9.6 ± 0.2 (13)</td>
<td>340 ± 12</td>
<td>12</td>
</tr>
<tr>
<td>ANLL</td>
<td>1</td>
<td>+</td>
<td>8.4 ± 0.3 (6)</td>
<td>9.4 ± 0.2 (2)</td>
<td>330 ± 13</td>
<td>13</td>
</tr>
<tr>
<td>CML</td>
<td>3</td>
<td>+</td>
<td>8.3 ± 0.2 (9)</td>
<td>9.3 ± 0.1 (3)</td>
<td>330 ± 13</td>
<td>13</td>
</tr>
<tr>
<td>ANLL</td>
<td>2</td>
<td>+</td>
<td>7.4 ± 0.4 (2)</td>
<td>9.8 ± 0.2 (2)</td>
<td>320 ± 10</td>
<td>9</td>
</tr>
<tr>
<td>CML</td>
<td>2</td>
<td>+</td>
<td>8.0 ± 0.6 (2)</td>
<td>9.6 ± 0.2 (5)</td>
<td>290 ± 9</td>
<td>9</td>
</tr>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL-60</td>
<td>+</td>
<td>8.5 ± 0.1 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KG-1</td>
<td>+</td>
<td>7.9 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined data: leukemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>+</td>
<td>8.2 ± 0.5 (17)</td>
<td>9.5 ± 0.3 (11)</td>
<td>330 ± 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal lymphocytes</td>
<td>6</td>
<td>+</td>
<td>7.9 ± 0.4 (7)</td>
<td>9.2 ± 0.2 (6)</td>
<td>310 ± 12</td>
<td></td>
</tr>
<tr>
<td>Combined data: leukemic and normal cells</td>
<td>+</td>
<td>8.2 ± 0.5 (17)</td>
<td>9.5 ± 0.3 (12)</td>
<td>330 ± 12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Some of these data have been published previously in conference proceedings (36, 46, 52).
* b fresh; +, frozen at -85°C for up to 1 year.
* d Mean ± S.D.
Control Column

Chart 2. a, degradation and/or dissociation of the receptor into subunits during gel filtration in the absence of molybdate; b, stabilization of a single, large complex in its presence. Frozen cells from a 16-year-old patient with ALL were used for the preparation of cytosol in TTES buffer containing 30 mM KCl (O, D) or 20 mM Na₂MoO₄ (•, •). Following incubation with 38 nM [³H]TA, 2.4-ml aliquots were chromatographed on Sephadex LH-20 in TTE buffer containing the same salt as the cytosol, and fractions containing the peak of bound steroid were combined. Aliquots (1 ml) of each preparation were filtered on agarose columns in TTEG buffer containing 50 mM KCl (a) or 20 mM Na₂MoO₄ (b). The internal standards were ferritin (F), myoglobin (M), [³C]-methylated thyroglobulin (T) and either serum albumin (S) or carbonic anhydrase (CA). The indicated Rs values were calculated at two-thirds of the receptor peak height.

The bound steroid was eluted as much narrower, more symmetrical peaks from columns containing 20 mM Na₂MoO₄ (Chart 2b). The peaks were characterized by Rs of ~8.4 nm, and the range of Rs was only 7.3 to 9.6 nm. Virtually identical patterns were obtained whether the cells were homogenized in −Mo or +Mo buffer. These results do not exclude the possibility that limited proteolysis of the receptors had occurred and that the apparent integrity of the large complex under these conditions was due to noncovalent interactions. Regardless of the mechanism of stabilization by molybdate, the inclusion of Na₂MoO₄ at a concentration of 20 mM in the gel filtration buffer clearly increases the recovery and facilitates the characterization of the large receptor form.

Stabilization by Molybdate of Receptors from Normal Lymphocytes, CLL Cells, and Leukemic Cell Lines. The agarose filtration patterns obtained after continuous exposure to either −Mo or +Mo buffers are shown for receptors extracted from fresh CLL cells and frozen normal lymphocytes in Charts 3 and 4, respectively. In discussing these and other gel filtration patterns, we have considered labeled complexes to be receptor fragments if their apparent Rs was <4 nm. This criterion was based on our determination that the intact steroid-binding subunit of the receptor has Rs of ~6 nm (see Table 3). As a consequence of these results, we propose that the largest complex observed in the absence of molybdate is a subunit of a large receptor that has been partially degraded and that the large complex observed in the presence of molybdate is an intact steroid-binding subunit of the receptor.
of diffusion, the elution profile of a 6-nm protein includes detectable concentrations of protein in fractions corresponding to Rs of 4 to 8 nm, under our chromatographic conditions (see Chart 7). Therefore, the proportion of steroid bound to complexes with Rs of <4 nm provides a conservative estimate of the proportion of receptor fragments.

The elution pattern of the molybdate-stabilized CLL cell receptor (Chart 3) was indistinguishable from those shown in Charts 1a and 2b for ALL cell receptors. There was no evidence of degradation or subunit dissociation of the large complex, which had Rs of 8.5 nm. The pattern obtained in -Mo buffer included a somewhat higher proportion (~20%) of complexes with Rs of <4 nm than the analogous patterns for ALL cell receptors (Chart 2a). The fragments detected in CLL cell cytosol, however, were not so small as the mero-receptor (Rs of 2.3 nm; see Charts 5 and 6 and the discussion below).

The elution profiles of the receptor in cytosols from frozen normal lymphocytes (Chart 4) differed in a few respects from the preceding results. The proportion of labeled complexes with Rs of <4 nm in the control buffer (~25%) was slightly higher than in +Mo buffer (~17%), but there was clear evidence of receptor cleavage under both conditions. The Rs value of the largest complex detected in +Mo buffer (7.3 nm) was smaller than those calculated from the data in Charts 1a, 2b, and 3. It was also the smallest of those obtained in 7 analyses of cytosols from frozen normal specimens, in which Rs varied from 7.3 to 8.5 nm. The proportion of labeled complexes with Rs of <4 nm also varied considerably among these preparations, from 6 to 24%. These results indicate that significant and variable cleavage of the receptors can be observed in cytosols from normal cells, as well as leukemic cells (see below), despite the continuous presence of molybdate. These normal cell preparations, however, were purified only by centrifugation over Ficoll-Metrizoate. Thus, contamination of the lymphocytes to different degrees by cells richer in proteases may account for the variable extent of receptor cleavage observed (18).

Studies of receptor metabolism, e.g., the evaluation of rates of receptor synthesis and degradation, are facilitated by the use of established cell lines (38). Therefore, it was of interest to analyze the structures of the glucocorticoid receptors in human leukemic cell lines that had been maintained in culture for several years. Cytosols were prepared in TTES +Mo buffer from frozen cells from 2 lines of nonlymphocytic leukemia, KG-1 (24) and HL-60 (8). We found the concentrations of glucocorticoid-binding sites in these cytosols to be <100 fmol/mg protein. These values are within the range of the receptor concentrations in cytosols from most normal lymphocyte preparations but are lower than those in cytosols from most leukemic cell specimens (see Table 1). Despite their low concentration, the molybdate-stabilized receptors from the cultured cells were indistinguishable in structure from those obtained from the clinical specimens. The Rs values of the major receptor forms in KG-1 and HL-60 cells were 7.9 and 8.5 nm, respectively (Table 2). In comparison with the patterns shown for the receptor from frozen normal lymphocytes (Chart 4), there was little fragmentation of these receptors. At most, 10% of the bound steroid had Rs of <4 nm in cytosol from either cell line.

Mero-receptor Formation in Cytosols from Myeloid Leukemias; Effects of Rapidly Freezing the Cells. In previous studies of receptors for 3 classes of steroids in cytosols from other types of human specimens and several rodent tissues, we have observed wide variations in the extent of receptor degradation (45, 48, 50–52). Correlated studies of receptor structure and the activities of several endopeptidases in cytosols prepared from fresh and frozen rat liver and kidney were reported recently (47). The results were consistent with a relationship between the extent of receptor degradation in these cytosols and the activity of "lysine-specific" endopeptidase(s), detected fluorometrically with 1-butyloxy carbonyl-valyl-leucyl-lysyl-AMC (47). A partially purified "lysine-specific" endopeptidase from rat kidney cytosol was subsequently shown to be capable of converting the molybdate-stabilized receptor in human CLL cell cytosol into the mero-receptor, the smallest fragment that retains noncovalently bound steroid (52).

The ability of proteases in cytosols from frozen myeloblastic cells to convert the glucocorticoid receptor to fragments of various sizes during processing at 0–4°C is illustrated in Charts 5 and 6. The effects of 2 procedural variations on the extent of receptor cleavage are also documented. The cells used for the experiment shown in Chart 5 were classified as myeloblastic CML on the basis of the clinical history, the presence of the Philadelphia chromosome, the percentage of blast cells in the

M. R. Sherman et al.

Chart 4. Inhibition by molybdate of the dissociation and fragmentation of the receptor in normal lymphocyte cytosol. Frozen lymphocytes from a healthy donor were used for the preparation of cytosols in TTES buffer containing 30 mM KCl (C) or 20 mM Na2MoO4 (Q). The cytosols were labeled with 47 nm [3H]TA and chromatographed on Sephadex LH-20 columns, as in Chart 2. An aliquot (1.1 ml) of each preparation was filtered on an agarose column in TTES buffer containing 50 mM KCl (C) or 20 mM Na2MoO4 (Q), with femtin (F), myoglobin (M), *-methylated thyroglobulin (T) and serum albumin (S) as internal standards. The Rs values of the major and minor receptor forms detected in +Mo buffer were 7.3 and 2.4 nm, respectively. The Rs of the fragment detected in -Mo buffer was 2.4 nm, and that of the mixture of larger forms was 4.2 to 8.2 nm, measured at two-thirds of the peak height. For approximately 17% (Q) and 25% (C) of the labeled complexes, Rs was <4 nm (- - - -).
Filtration patterns in Chart 5 reflects processes that occurred during the preparation of the cytosols.

The following inferences can be drawn from these results: (a) endogenous enzymes in some myeloid leukemic specimens are capable of cleaving the receptor into the mero-receptor; (b) much of the observed cleavage occurred within the first hr after homogenization of the cells; (c) this process was retarded, but not prevented by the presence of molybdate in the homogenate; (d) the largest receptor form observed under these conditions ($R_s$ of 7.0 to 7.1 nm) was smaller than those detected in cytosols of 7.0 to 7.1 nm) was detected in cytosol prepared from fresh cells, the $R_s$ values were 7.0 nm (O) and 7.1 nm (•) for the larger complexes and 2.3 nm for the mero-receptor in both preparations.

The data in Chart 7 and the results of corollary experiments indicate that the use of cells frozen in this way can result in cleavage of the receptor into fragments that are intermediate in size between the mero-receptor ($R_s$ of 2.3 nm) and the intact

of fragmentation. In contrast, the pattern obtained using frozen cells from the same specimen revealed a conspicuous peak of the mero-receptor and a decrease in $R_s$ of the larger complex, compared with that obtained from fresh cells. This decrease, from 8.3 to 7.3 nm, was readily detected with reference to the 2 internal standards which migrate close to the receptor in these columns: ferritin [$R_s$ of 6.15 nm (50)]; and carbonic anhydrase (CA) as internal standards. The Stokes radii of the major receptor forms are indicated. The mero-receptor ($R_s$ of 2.3 nm) was detected in cytosol from frozen cells. Free steroid was eluted with a $K_o$ = 1.1.

**Characterization of Salt-dissociated Receptor Forms; Effect of Freezing the Cells.** In contrast with the results shown in Table 2, agarose filtration in the presence of 0.4 M KCl revealed extensive degradation of the receptors from frozen ALL cells (Chart 7). While a single, symmetrical peak of bound steroid ($R_s$ of 6.1 nm) was detected in cytosol prepared from fresh cells, the cytosol from frozen cells contained a mixture of receptor forms, of which the $R_s$ of ~25% was <4 nm. Chromatography of cytosol from the same frozen cells in TTEG +Mo buffer provided no evidence of receptor cleavage (not shown). The bound steroid was characterized by $R_s$ 8.3 nm, and the shape of the peak was indistinguishable from that obtained using fresh cells from the same specimen (see Chart 9e).

**Table 2**

<table>
<thead>
<tr>
<th>Cytosol</th>
<th>Distribution Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Cells</td>
<td>[3H]TA and ovalbumin to final concentrations of 55 nM and 16 mg/ml, respectively, the cytosols were incubated for 90 min. Aliquots of the charcoal-treated cytosols (1.4 ml) were filtered on agarose columns in TTEG +Mo buffer, with ferritin (F), myoglobin (M), [14C]methylated thyroglobulin (T), and carbonic anhydrase (CA) as internal standards. The Stokes radii of the major receptor forms are indicated. The mero-receptor ($R_s$ of 2.3 nm) was detected in cytosol from frozen cells. Free steroid was eluted with a $K_o$ = 1.1.</td>
</tr>
</tbody>
</table>
monomer ($R_s$ of $\sim$6 nm) (see Table 3). These intermediate fragments, which are revealed by chromatography in hypertonic buffer, may not be formed or may escape detection during most analyses in hypotonic buffer. Receptor fragments of similar size have been observed in cytosols from rat thymocytes (20) and from fresh and frozen rat liver and kidney (28, 47, 51) and have been produced by chymotryptic digestion of larger receptor forms (6, 55). It should be noted that the effects of freezing the cells on the form of the receptors from some specimens of ALL and CLL cells were less dramatic than those illustrated in Charts 6 and 7.

Analyses of Receptor Form before and after Freezing Labeled Cytosols. We have done only a few studies of the effects on receptor structure of freezing the labeled +Mo cytosols, as opposed to freezing the intact cells. For example, no difference was found between the elution patterns obtained by gel filtration in hypotonic +Mo buffer of aliquots of cytosol from a specimen of fresh CLL cells, before or after storage for 3 months at $-85^\circ$. Cytosol was also prepared in +Mo buffer from frozen cells from the same CLL patient and labeled with $[^3H]$TA, and aliquots were analyzed by filtration in hypotonic +Mo buffer on the day of preparation and after 3 days at $-85^\circ$. No difference was detected in the apparent $R_s$ (6.0 nm) or in the shape of the elution profile. Similarly, portions of labeled cytosol from a fresh ALL specimen were analyzed by filtration in hypertonic --Mo buffer before and after storage for 6 months at $-85^\circ$. The resultant patterns were nearly identical with respect to $R_s$ of the labeled complex (6.1 to 6.2 nm), the width of the peak (5.2--7.3 nm), and the percentage of receptor fragments (7 to 10%). In more systematic studies of the glucocorticoid receptor in rat liver cytosol, the large receptor form in +Mo cytosol appeared to be stable during several months of storage at $-85^\circ$ (47).

Instability of Salt-dissociated Receptor Forms during Sequential Fractionations. The data in Chart 8 were obtained with --Mo cytosol prepared from the same frozen leukopheresis specimen that was used in Chart 1. The cytosol was incubated with steroid at 4° for 3 hr, rather than the usual 90 min, in order to increase the quantity of receptor fragments available for further characterization. Following charcoal treatment, the cytosol was filtered on an agarose column in TTEG buffer containing 0.4 M KCl. Two receptor forms, which were partially resolved by the initial fractionation (Chart 8a), were further analyzed by rechromatography under the same conditions (Charts 8b, b and c) or by centrifugation through glycerol gradients in hypertonic --Mo buffer (Chart 8e). No Aliquots of the unfractionated, charcoal-treated cytosol were also subjected to centrifugation under the same conditions (Chart 8d).

Several conclusions can be drawn from the results in Chart 8 and the corollary experiments. (a) The larger receptor form seen in Chart 8a ($R_s$ of 5 to 6 nm) is relatively unstable with respect to the retention of bound steroid and the size determined by refiltration on the same column. Of the total radioactivity in the pool analyzed as shown in Chart 8b, $\sim$33% appeared to be free ($K_o$ of 1.1), and $\sim$67% of the residual bound radioactivity migrated with an apparent $R_s$ of $<4$ nm. (b) The $R_s$ values calculated for both the small receptor form in Chart 8b (2.9 nm) and the single broad peak in Chart 8c (2.8 nm) were considerably smaller than those of the predominant receptor forms in Charts 7 or 8a. We infer that further proteolysis and/or more complete dissociation of the cleavage products occurred during reanalysis of the samples. (c) The $R_s$ value corresponding to the peak of bound steroid in Chart 8c (2.8 nm) is slightly larger than that of the mero-receptor (2.3 nm; Charts 5 and 6), but this broad peak may contain a mixture of the mero-receptor and larger fragments. (d) For the discrimination among the salt-dissociated receptor forms, agarose filtration is a far more sensitive technique than density gradient centrifugation. Complexes with $R_s$ values of $\sim$3 nm and $\sim$5 nm were nearly completely resolved on the column (Chart 8b), but the $s_{20\times}$ values of all were $\sim$4S (Chart 8a). (e) These results for the glucocorticoid receptor in leukemic cell cytosol resemble those obtained for the receptor in rat liver cytosol analyzed under similar conditions (47).

![Chart 7. Degradation of the receptor from frozen ALL cells revealed by gel filtration in hypertonic --Mo buffer. Purified cells from a 14-year-old patient were divided into 450-mg portions, one of which was kept at $-85^\circ$ for 6 weeks. The fresh (○) or frozen (□) cell pellets were homogenized in 3 volumes of TTE +Mo buffer, and the homogenates were adjusted to isotonicity, as in Chart 6. The cytosols had protein concentrations of 15.7 mg/ml (○) and 18.5 mg/ml (□). They were incubated with 86 nM $[^3H]$TA and treated with charcoal, and 2.4-ml aliquots were filtered on agarose columns in TTEG buffer containing 0.4 M KCl. The indicated $R_s$ values were calculated relative to the internal standards: myoglobin (M), 14C-methylated β-amylase (B), and carbonic anhydrase (CA). For approximately 7% (○) and 25% (□) of the labeled complexes, $R_s$ was $<4$ nm.](chart7.png)
In contrast, the apparent values of $s_{20,w}$ and the resultant estimates of molecular weight varied widely, depending on the experimental conditions. For both ALL cell cytosol (Patient 1) and CLL cell cytosol (Patient 6), the $s_{20,w}$ values obtained after charcoal treatment of the labeled cytosols were higher than those obtained after Sephadex LH-20 chromatography in hypertonic buffer. Furthermore, the $s_{20,w}$ value obtained for the receptor in cytosol from ANLL cells (Patient 5) was higher after Sephadex LH-20 chromatography in hypotonic +Mo buffer than after chromatography on the same type of column in hypertonic −Mo buffer. These differences could result from proteolysis of the receptor during the latter procedure and/or the incomplete dissociation of the oligomeric complex into subunits under the other conditions. Chromatography in glycerol-free buffer containing 0.4 M KCl favors dissociation of the complex, since the samples are diluted slightly and exposed to a buffer of high conductivity. In the density gradients, and to some extent in the agarose columns, the dielectric constant of the buffers and hence the effectiveness of the salt are reduced by the presence of glycerol [16 to 41% (w/v) in the gradients, 10% (v/v) in the columns] (44).

Our interpretation of the data in Table 3 is that the higher estimates of $s_{20,w}$ and $M_r$ reflect the incomplete dissociation of the oligomeric complex. Accordingly, the lower estimates of $s_{20,w}$ (4.4 to 4.8S) and of molecular weight ($M$, 100,000 to 110,000) are more likely to resemble the parameters of the monomeric subunit. The latter molecular weight values are in reasonable agreement with our results for the glucocorticoid receptor in rat liver cytosol (47), those of Holbrook et al. (19) for the rat thymocyte receptor, those of Stevens et al. (56) for the human CLL cell receptor, and those of Foster et al. (14) for the covalently labeled, denatured receptor from human leukemic cells. On the basis of their high affinity for nuclei or DNA, the monomeric forms of the receptors in these and other systems have been identified as the transformed or activated receptors (reviewed in Ref. 49).

**Relationship of Receptor Structure and Cleavage to Diagnosis.** The hydrodynamic parameters of the molybdate-stabilized and salt-dissociated receptor forms have been summarized in Tables 2 and 3, respectively. In general, the properties of the major receptor form detected in each buffer system were remarkably similar among the different types of leukemic cells. The most conspicuous difference among the gel filtration patterns was the apparent degradation of receptors in cytosols from several normal and malignant specimens. Cleavage products of 3 types were distinguished. The mero-receptor ($R_2$ of 2.3 to 2.4 nm) and slightly degraded oligomers ($R_2$ of 7.0 to 7.5 nm) were detected by filtration in hypotonic +Mo buffer (Charts 4 to 6). The latter complexes are clearly smaller than the intact oligomers that are intermediate in size between the monomer and the oligomeric complex. Accordingly, the lower estimates of $s_{20,w}$ and $M_r$ reflect the incomplete dissociation of the oligomeric complex. In order to express the extent of degradation quantitatively, we considered elution patterns in which the $R_2$ of >20% of the complexes was <4 nm and/or the radius of the molybdate-stabilized complex was <7.5 nm to reflect significant degradation. Among the fresh specimens studied, such degradation was observed in 2 of 8 cases of ALL (not shown) and 0 of 5 other leukemic cell specimens. Among the frozen specimens, degradation was observed in 0 of 7 cases of ALL, 2 of 6 specimens...
of normal lymphocytes (see Chart 4), 1 of 2 cases of CML (Chart 5), and 1 of 3 cases of ANLL (Chart 6). Although the number of samples analyzed to date is small, it is apparent that the detectable cleavage of receptors is not restricted to frozen cells or to a particular type of leukemic cell or to malignant cells, in general.

Concomitant Analyses of the Glucocorticoid Receptor and Several Peptidases in Leukemic Cell Cytosols. The observed degradation of receptors in certain cytosols stimulated our interest in the endogenous peptidases. The use of fluorescent substrates, in particular aminoacyl and peptidyl derivatives of AMC, has facilitated the detection of enzymes of various specificities in small volumes of cytosol and in fractions from the columns or gradients in which the receptor was also analyzed (47, 52). Chart 9 illustrates the results obtained by agarose filtration of [3H]TA-labeled cytosol from fresh ALL cells, under conditions in which the receptor was in either the molybdate-stabilized (oligomeric) form or the salt-dissociated (monomeric) form. Aminopeptidase activities were assayed with leucyl-AMC and the endopeptidases with oligopeptide derivatives of AMC in which the amino-terminal residues were blocked. Aminopeptidases of various specificities have been described previously in normal leukocytes and leukemic cells (27, 39). More detailed characterization of these and other enzymes from leukemic cells with respect to catalytic and physicochemical properties will be published elsewhere.4, 5 The following observations, however, are relevant to the present study.

Using the assay conditions described in "Materials and Methods," we detected significant activities of leucine aminopeptidase and at least 3 endopeptidases in cytosols from various types of leukemic cells. For example, small aliquots (2 to 50 µl) of the cytosol used for the experiments shown in Chart 9 were assayed at pH 7.4 and 25°, using 20 µM solutions of leucyl-AMC and the oligopeptide substrates in which the carboxyl-terminal residues were lysine, phenylalanine, or tyrosine. The reaction velocities, normalized to 10 µl of cytosol, were 440, 84, 140, and 590 RFU/hr, respectively. Under these conditions, velocities as low as 10 RFU/hr can be measured with reasonable precision (±1 RFU/hr).

The activities detected with the peptidyl substrates may reflect the direct cleavage of the bond between the carboxyl-terminal residue and the amino group of AMC, or a sequence of reactions, involving initial cleavage at a more distal peptide bond (34). In assays of the enzymes recovered from agarose columns, as in Chart 9, the rate of release of AMC from the phenylalanine derivative increased progressively during 5 hr, to approximately twice the initial rate. This kinetic behavior suggests that the enzyme requires activation and/or that more than one peptidase is involved in the cleavage of this substrate. Regardless of the detailed mechanisms of these reactions, the use of substrates of this type has revealed the presence of neutral peptidases of diverse specificities and sizes in leukemic cell cytosols.

When labeled ALL cell cytosol was filtrated on agarose in hypotonic +Mo buffer, the glucocorticoid receptor was eluted earlier than the predominant forms of the enzymes assayed (Chart 9, a and b). Under these conditions, the radius of the

### Table 3

<table>
<thead>
<tr>
<th>Diagnosis and patient</th>
<th>Cells frozen</th>
<th>±Mo cytosol</th>
<th>CD or LH</th>
<th>Rg (nm)</th>
<th>% fragment</th>
<th>sₑₐₓₒ(S)</th>
<th>Mₑₓ₁₀⁻⁴</th>
<th>a/b</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>+</td>
<td>±Mo</td>
<td>CD</td>
<td>5.5 ± 0.2</td>
<td>27 ± 7</td>
<td>4.8 ± 0.03</td>
<td>110</td>
<td>11</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td></td>
<td>LH–</td>
<td>5.6</td>
<td>30</td>
<td>4.4 ± 0.05</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>±Mo</td>
<td>CD</td>
<td>5.8</td>
<td>18</td>
<td>5.7 ± 0.02</td>
<td>150</td>
<td>12</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>±Mo</td>
<td>CD</td>
<td>6.2 ± 0.1</td>
<td>18</td>
<td>5.3 ± 0.04</td>
<td>150</td>
<td>12</td>
</tr>
<tr>
<td>ANLL</td>
<td>–</td>
<td>+</td>
<td>CD</td>
<td>6.1</td>
<td>11</td>
<td>5.3 ± 0.04</td>
<td>140</td>
<td>12</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>±Mo</td>
<td>LH+</td>
<td>5.7 ± 0.27</td>
<td>4.4 ± 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>±Mo</td>
<td>LH–</td>
<td>5.8 ± 0.15</td>
<td>150</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>+</td>
<td>±Mo</td>
<td>CD</td>
<td>6.1 ± 0.2</td>
<td>10 ± 1</td>
<td>4.8 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>±Mo</td>
<td>LH–</td>
<td>5.7 ± 0.15</td>
<td>150</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined data</td>
<td></td>
<td></td>
<td></td>
<td>5.9 ± 0.3</td>
<td>(12)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* CD, charcoal-dextran; LH+, Sephadex LH-20 columns in TTE buffer containing 20 mM Na₂MoO₄; LH–, Sephadex LH-20 columns in TTE buffer containing 0.4 M KCl.

† Percentage of steroid-receptor complexes with elution volumes corresponding to Rg of <4 nm; the number of determinations is the same as for Rg.

‡ Numbers in parentheses, number of determinations.

§ Mean ± S.D.

¶ Acute monocytic leukemia cells (French-American-British Class M5B) from a 1-year-old boy.

⁎ Acute monocytic leukemia cells (French-American-British Class M5B) from a 35-year-old woman.

⁎⁎ Acute monocytic leukemia cells (French-American-British Class M5B) from a 14-year-old girl.

⁎⁎⁎ Acute monocytic leukemia cells (French-American-British Class M5B) from a 35-year-old woman.

⁎⁎⁎⁎ 1 µl of cytosol used for the experiments shown in Chart 9 were assayed at pH 7.4 and 25°, using 20 µM solutions of leucyl-AMC and the oligopeptide substrates in which the carboxyl-terminal residues were lysine, phenylalanine, or tyrosine. The reaction velocities, normalized to 10 µl of cytosol, were 440, 84, 140, and 590 RFU/hr, respectively. Under these conditions, velocities as low as 10 RFU/hr can be measured with reasonable precision (±1 RFU/hr).
Human Leukemic Cell Glucocorticoid Receptors

receptor preparations obtained by gel filtration as shown in Charts 7, 8a, and 9c are contaminated with a variety of peptidases. These enzymes and/or others of similar size may contribute to the instability of the salt-dissociated receptor forms during fractionation of cytosols. This degradation was apparent in the experiments shown in Chart 8 and in analogous studies of the receptor in rat liver cytosol (47, 52).

In recent analyses of apparent receptor cleavage in human leukemic cell cytosols by a rapid minicolumn technique, Holbrook et al. (17) found that cytosols from myelocytic subtypes of ANLL cells contained a higher proportion of the mero-receptor than did cytosols from monocytic subtypes. They have also reported that a factor in CLL cells appears to stabilize the receptor from ANLL cells (18). The mechanisms underlying these observations are currently being explored in our laboratory, using direct assays of peptidase activities by the methods illustrated in Chart 9.4

Conclusions. We have demonstrated that the glucocorticoid-receptor complexes from various types of leukemic cells are nearly identical in size, shape, and subunit structure to each other and to the complexes from normal tissues including lymphocytes. It was shown, however, that numerous experimental factors can alter these structures. These results imply the need for caution in attributing clinical significance to observations, such as those of McCaffrey et al. (30), that "abnormal" receptors occur in certain human leukemic specimens, particularly when the results were based on analyses in molybdate-free buffers of receptors obtained from rapidly frozen cells. In general, we conclude that most of the apparent structural differences among the receptors in extracts of leukemic cells and other tissues are the consequence of in vitro degradation by endogenous proteases. The properties and possible physiological functions of these enzymes are intriguing subjects for future research.

ACKNOWLEDGMENTS

We are grateful to Dr. Kathleen P. Cavanaugh, Dr. Roberto E. Garcia, Regina Neal, and Joseph A. Carlin for skillful assistance with several of these experiments; to Dr. Denis R. Miller and other members of the Department of Pediatrics, Drs. Michael Andreoff and Arlene Redner of the Investigative Cytology Laboratory, Dr. Benjamin Koziner of the Lymphocyte Surface Markers Laboratory, Stephen Ellis and Claudia Little of the Hematology/Lymphoma Service, Dr. E. S. K. Chaganti of the Cancer Genetics and Cytogenetics Laboratory, Drs. Morton K. Schwartz and Martin Fleisher of the Department of Clinical Chemistry, and the Tumor Procurement Service of this Center for providing the clinical specimens and other data concerning the patients; to Dr. Susan Groshen for assistance with statistical analyses; to Drs. Allan Munck and Nikki J. Holbrook for providing their manuscripts prior to publication and for insightful reviews of this report; and to Drs. John Stevens and Alex Silberberg for many constructive discussions.

REFERENCES


10. Crabtree, G. R., Smith, K. A., and Munck, A. Glucocorticoid receptors and
11. M. R. Sherman et al.


26. Characterization of molybdate-stabilized glucocorticoid receptors in healthy
27. and malignant tissues. In: H. J. Lee and T. J. Fitzgerald (eds.). Progress in

31. and nuclear glucocorticoid-binding components in human leukemic lympho-
32. blood cells: results in 89 adult patients with lymphoid leukemias and

35. Moore, G. E., Gerner, R. E., and Franklin, H. A. Culture of normal human

37. Okret, S., Stevens, Y.-W., Carlstedt Duke, J., Wränge, Ö., Gustafsson, J.-Å.,
38. and Stevens, J. Molecular basis of glucocorticoid receptor function. J. Steroid

40. line responsive to colony-stimulating activity. Science (Wash. DC), 200: 1153–


41. Sherman, M. R., Barzilai, D., Pine, P. R., and Tuazon, F. B. Glucocorticoid


44. Sherman, M. R., Pickefester, L. A., Rollwagen, F. M., and Miller, K. L. Micro-


Multiple Forms and Fragments of Cytosolic Glucocorticoid Receptors from Human Leukemic Cells and Normal Lymphocytes

Merry R. Sherman, Yee-Wan Stevens and Fe B. Tuazon


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/44/9/3783

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.