Glucocorticoid-resistant Human Acute Lymphoblastic Leukemic Cell Line with Functional Receptor

Robert Zawidykowski, Jeffrey M. Harmon, and E. Brad Thompson

ABSTRACT

A receptor-containing, steroid-resistant clone of CEM cells, CEM-C1, was isolated without selective pressure from the wild-type population. The biological and physicochemical properties of glucocorticoid receptors in CEM-C1 cells were compared to those from a clone (CEM-C7) sensitive to glucocorticoid-mediated lysis. In a whole-cell binding assay, CEM-C1 cells exhibited high affinity for \( ^{3}H \)Dexamethasone \( (K_{d}, 22 \text{ nm}) \), nuclear translocation of steroid:receptor complex \( (n, 43\%) \) and were found to contain, on the average, 12,000 receptor sites/cell \( (R_{0}) \). These steroid-binding parameters were similar to those displayed by wild-type CEM-C7 cells: \( K_{d}, 19 \text{ nm}; nt, 47\%; \) and \( R_{0}, \sim 14,000 \) sites/cell. The ion-exchange and gel permeation profiles were indistinguishable from those of identically treated CEM-C7 cytols. Thus, diethylaminoethyl cellulose chromatography of CEM-C1 cytosol showed that \( ^{3}H \)triamcinolone acetonide:receptor complex was eluted at 50 \text{ mw} \) phosphate and 220 \text{ mw} phosphate under "activating" and "nonactivating" conditions, respectively. Receptor complex of activated CEM-C1 cytosol bound to DNA-cellulose and was eluted at 100 \text{ mw} salt. Filtration of unactivated CEM-C1 cytosol over Sephadryl S-300 generated a single peak of radioactivity for receptor complex with a calculated Stokes' radius of 55 to 59 A. Dexamethasone induced glutamine synthetase in CEM-C1. The dose dependence (50\% effective dose, \( \sim 20 \text{ nm} \)) and maximal fold increase \( (1.9, 1 \mu \text{m dexamethasone}) \) were comparable to those observed in CEM-C7. Since CEM-C1 cells contain apparently normal, functional cytosolic receptor, the results suggest that resistance to glucocorticoid in these cells involves a defect(s) at another locus.

INTRODUCTION

Certain immature lymphoid cells are sensitive to the lytic effects of glucocorticoids (3). This response not only forms part of the basis for clinical use of corticosteroids in the treatment of lymphoproliferative disorders but also provides an attractive system in which to study the mechanism of steroid hormone action through the isolation and characterization of unresponsive variants. In principle, such an analysis would be expected to yield mutants with defects in each step of the lytic pathway. Almost without exception, however, resistant variants selected from sensitive clones have proven to contain receptor abnormalities (6, 48, 64). A few mouse clones with possibly normal receptors have been described (64). Almost without exception, however, resistant variants selected in vivo yield mutants with defects in each step of the lytic pathway. In principle, such an analysis would be expected to yield mutants with defects in each step of the lytic pathway. Almost without exception, however, resistant variants selected from sensitive clones have proven to contain receptor abnormalities (6, 48, 64). A few mouse clones with possibly normal receptors have been described (64).

We have made use of the cultured T-cell ALL\(^{5}\) line, CEM, as a model for study of human leukemia (20, 70, 71). We have observed that most of various unselected clones are killed by glucocorticoids (46).\(^{6}\) One sensitive clone, CEM-C7, has been used to show that these cells possess glucocorticoid receptors the occupancy of which correlates with direct cytotoxicity of potent glucocorticoids (46), that cell kill does not begin until at least 24 hr after addition of steroid and is coincident with irreversible G\(_{1}\) arrest (19), that resistance develops by spontaneous independent events (22), and that resistant clones have residual but aberrant receptors (21, 57). No resistant subclones with normal receptors have yet been found after steroid selection, with or without prior mutagenesis. However, such cells would be, in theory, of great value in studying the biochemistry and genetics of steroid action and resistance in general and in human leukemia in particular. Consequently, we studied CEM-C1, a unique clone of dexamethasone-resistant CEM cells found among the original set of unselected clones derived from CEM (46).

We show here that CEM-C1 cells are karyologically, and by membrane markers, almost certainly derived from the same original leukemic cell source as are CEM-C7. The steroid-resistant CEM-C1 cells contain glucocorticoid receptors physically identical to those of wild-type cells by several tests (tests chosen for the most part because in one or another murine system they have revealed receptor abnormalities in resistant cells). Furthermore, the receptors of CEM-C1 cells appear to function, in that they mediate the steroidial induction of GS, which we have shown to correlate with the occupancy of normal receptors by active steroids (23). Therefore, the steroid resistance of CEM-C1 cells appears to be due to a "nonreceptor" lesion. Because they are so similar to their sensitive clone, CEM-C7, CEM-C1 cells may prove to be useful in the biochemical and genetic analysis of steroid-induced cell lysis.

MATERIALS AND METHODS

Steroids and Chemicals. \([1,2,\text{H}]\)DEX (20 Ci/mmol) and \([1,2,4,\text{H}]\)TA (29 Ci/mmol) were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Nonradioactive steroids were purchased from Sigma Chemical Co., St. Louis, Mo. Other chemicals, unless indicated otherwise, were of reagent grade and were obtained through either Sigma or Fisher Scientific Co., Fair Lawn, N. J.

Cells and Cell Culture Methods. CCRF-CEM (12) is a human ALL line of T-cell lineage (31, 39); CEM-C1 and CEM-C7 are DEX\(^{-}\) and DEX\(^{+}\) phenotypes; TA, triamcinolone acetonide (11), 21-dihydroxy-9-fluoro-16\(\alpha\),17\(\alpha\)-isopropylidenedioxy-1.4-pregna- 4,14-diene-3,20-dione); HBSS, Hank's balanced salt solution; KPD, 5 mw potassium phosphate:0.5 mw dithiothreitol, pH 7.2 to 7.4.

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3865

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column was eluted at a rate of 6 to 8 ml/hr; 1-ml fractions were collected equilibrated with 10 mM KPD buffer modified to contain 0.3 M KCl, 1 mM EDTA, 50 mM Na2MoO4, and 10% (v/v) glycerol (elution buffer). The phacryl S-300 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). Cytosols (0.4 ml) were applied to a 1- x 49-cm column (38-ml bed volume) of receptor complex was achieved by heat-salt treatment (20°, 0.2 M KCl). Free steroid was removed either by passage of labeled cytosols over KPD-washed Sephadex G-25M columns (PD-10; Pharmacia Fine Chemicals AB, Uppsala, Sweden) or by adsorption onto dextran-coated charcoal. Cytosols were loaded, columns were eluted, and fractions were collected in cpm between parallel incubations with and without a 200-fold excess unlabeled TA) typically exceeded 80% of total bound radioactivity; 

Preparation, Labeling, and Activation of Cytosols. Preparative procedures were performed at 0–4°. Cells in exponential growth (1 to 2 liters, 4 to 8 x 10^8/ml) were harvested, washed once in 15 ml ice-cold HBSS, and homogenized (1.5 volumes) in KPD buffer with a TenBroeck ground glass homogenizer (2 series of 10 strokes, separated by a 30-sec interval). "Cytosols" were isolated by centrifugation of the homogenate for 1 hr at 50,000 rpm (type 65 rotor) in a Beckman Model L-65 refrigerated ultracentrifuge and collection of the supernatant fraction. Cytosols were labeled with [3H]TA at 5 x 10^{-6} M for 2 hr. At this concentration, specific glucocorticoid-bound (determined as the difference in cpm between parallel incubations with and without a 200-fold excess unlabeled TA) typically exceeded 80% of total bound radioactivity; cytosols contained 0.2 to 1 pmol receptor per mg protein. "Activation" of receptor complex was achieved by heat-salt treatment (20°, 0.2 M KCl). Free steroid was removed either by passage of labeled cytosols over KPD-washed Sephadex G-25M columns (PD-10; Pharmacia Fine Chemicals AB, Uppsala, Sweden) or by adsorption onto dextran-coated charcoal.

**Assay of Plasma Membrane Fragility.** Cells were pulse-labeled (20 μCi/ml suspension, 2.5 hr, 37°) with Na2[3H]CrO4 (379.3 μCi/ml; New England Nuclear), harvested, then washed and resuspended in HBSS (μ, 0.158, calculated) at 2 x 10^8/ml. Aliquots of 0.5 ml were diluted 1:20 with NaCl solutions of varying hypotonicity (fortified with MgCl2 to a final concentration of 1.5 mM, for maintenance of nuclear integrity), and were allowed to stand at room temperature for 10 min. Portions were removed for determination of cell viability by dye exclusion and 51Cr release. Radioactivity was counted in both the supernatant and pellet fractions in a Packard Prias PGD Auto-Gamma counter (about 2% efficiency). Counts were expressed relative to maximum label released and were corrected for spontaneous loss of radiochromium.

**Surface Membrane Properties.** Binding of monoclonal antibodies was measured by indirect immunofluorescence. Cells (100 μl, 10^7/ml) were suspended in HBSS containing 0.1% albumin:0.1% NaN3 and incubated with antibody (αHuLyt-1,-2,-3 and αHula; New England Nuclear) at a final dilution of 1:15 for 40 min at 0°. Cells were washed twice, and the wet pellet was equilibrated for an additional 40 min with 10 μl saturated solution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, F(ab')2 fragment (provided by Dr. Richard Asofsky, National Institute of Allergy and Infectious Diseases). Stained cells were washed and suspended in Aquasol LSC (Perkin-Elmer Life Sciences, Inc., Norwalk, Conn.) and assayed for the presence of homologous erythrocytes (Clinical Center Blood Bank, NIH) (51).

**Zawydiowski et al.**

Clones independently isolated from the parent line (46). Cells were grown as stationary-suspension cultures at 37° in a humidified atmosphere of 95% air:5% CO2 in Roswell Park Memorial Institute Medium 1640 (NIH Media Unit or Biofluids, Inc., Rockville, Md.) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Biofluids, Inc., or North American Biologicals, Miami, Fla.) or with 10% (v/v) of a 50:50 mixture of fetal and newborn calf sera (Biocell Laboratories, Carson, Calif.). Cells were maintained in logarithmic growth by manual dilution to densities between 10^6 and 10^7 cells/ml, as determined by a Model Z8 Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). Size distributions were made by a Coulter Channelizer calibrated with polystyrene microspheres and coupled to an X-Y recorder. Cell viability was measured by the exclusion of trypan blue (49) and by the release of 51Cr (24) essentially as previously described.

**Cytogenetics.** Chromosomal analyses of metaphase spreads from Colcemid-arrested cells were performed by conventional Giemsa staining, G-banding (60), and Giemsa-11 (76) banding techniques.

**Whole-Cell Steroid-Binding Assay.** Specific binding of [3H]DEX was determined in cells harvested in mid-logarithmic growth (57). Results were plotted according to the method of Scatchard (54), and kinetic parameters (Kd, Rmax) were calculated following linear regression of the data by the method of least squares. Nuclear translocation of receptor complex was measured by a minor modification (58) of the procedure of Munck and Wira (43).

Glutamine Synthetase Activity. Glutamine synthetase (EC 6.3.1.2) was assayed spectrophotometrically (59) by measuring glutamyltransferase activity essentially according to the method of Thordikne and Reif-Lehrer (72). Enzyme specific activity was expressed as nmol γ-glutamylhydroxamate formed per min per mg protein and was computed from the "effective" molar extinction coefficient (calculated each experiment) of γ-glutamyl acid-γ-monoxyhydroxamate (Sigma) measured under the conditions of the assay. Protein was determined by the method of Lowry et al. (35) with bovine serum albumin (Sigma) as the standard.

**Steroid Metabolism.** Cultures, seeded at a cell density of 10^6 cells/ml, were treated with 1 μM DEX containing tracer [3H]DEX (5 μCi; 2.5 x 10^{-8} M final concentration) and incubated for 48 hr. Cells were collected by centrifugation, washed once with HBSS, resuspended in distilled water (2 ml), and sonically disrupted (Braun-sonic Model 1510; B. Braun Instruments, San Francisco, Calif.) with two 10-sec bursts at 15 watts. The sonically disrupted cells were extracted 3 times with 5 ml ethyl acetate. The organic layers were dried over anhydrous Na2SO4 and were evaporated to dryness with argon (<5 ppm oxygen). The extracts were chromatographed in chloroform:ethanol (4:1) on Silica Gel GF plates (250 μm thick; Analtech, Inc., Newark, Del.) previously washed (air dried) in developing solvent. The plates were scraped at 5-mm intervals, and the silica was suspended in Aquasol LSC and counted. Supematant media and cell washes were pooled and treated in an identical manner. An equivalent amount of labeled and unlabeled DEX added to cell-free incubation medium served as a control for serum-mediated steroid transformation and/or artefacts of extraction and drying.

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**Erythrocyte rosette formation was assayed according to the method of Weiner et al. (75) with neuraminidase (Sigma)-treated sheep RBC (Veterinary Resources Branch, NIH). Detection of soybean agglutinin receptor was performed by quantitation of the fraction of cells agglutinated by soybean lectin (P-L Biochemicals, Inc., Milwaukee, Wis.) in the presence of homologous erythrocytes (Clinical Center Blood Bank, NIH) (51).
RESULTS

Whole-Cell Steroid-Binding Activity. CEM-C1 cells, which were isolated in the absence of steroid selective pressure, grow in suspension culture in 1 μM DEX without a reduction in cell density (46). Our initial cell-free binding studies revealed specific and saturable binding of [3H]DEX and indicated that resistance to the lytic effect of glucocorticoid was not due to the lack of or decreased affinity for cytosolic receptor. In order to determine whether resistance was the result of a defect at the level of hormone uptake and/or transport, we measured steroid binding by whole cells. The data, summarized in Table 1, show that DEX1 CEM-C1 cells accumulated steroid in both the cytosolic and nuclear compartments to an extent slightly less than and with an apparent affinity comparable to sensitive CEM-C7 cells. In 2 parallel experiments, total cellular receptor sites were indeed found to be slightly lower in CEM-C1 (≥80% of sites in CEM-C7). This observation is consistent with the reduced level of cytoplasmic binding of glucocorticoid found in cell-free extracts of CEM-C1 (46). Both clones exhibited a 2-fold range in DEX-binding sites (cf. 9,000 to 23,000 and 8,000 to 19,000 sites/cell for DEX* and DEX* cells, respectively) when determined over a period of about 1 year. The large experimental deviation is possibly due to receptor regulation by variable levels of endogenous hormone (8, 10, 40, 55, 61, 67) and/or other components (27) present in different batches of serum.

Physicochemical Properties of Receptor. Although CEM-C1 receptor was able to bind DEX and undergo "activation" as evidenced by nuclear accumulation, the possibility that it was nonetheless aberrant, generating a complex incapable of association with specific nuclear acceptor sites, was subsequently considered. Presumably, such a receptor would possess altered biochemical and biophysical properties. We therefore analyzed receptor by ion-exchange and gel permeation chromatography. Receptor abnormalities in murine and human systems have been detected by each of these techniques (37, 57, 66, 77).

DEAE-cellulose chromatography of glucocorticoid-receptor complexes of various cell types resolves the complexes into 2 forms: a low-salt eluting form (Peak I) which binds to DNA, nuclei, and chromatin and is thought to represent the "activated" form of the hormone-receptor complex, and a high-salt eluting form (Peak II) which is devoid of these activities and probably represents the "unactivated" form of the complex (42, 53). Chart 1 depicts typical elution profiles of [3H]TA:receptor complexes formed under activating and nonactivating conditions. Peaks I and II of CEM-C1 characteristically were eluted at the same salt concentrations (0.05 and 0.22 M potassium phosphate, respectively) as were the corresponding peaks for the cytoplasmic receptor complexes of CEM-C7. In the experiment shown, CEM-C1 cytosol, however, exhibited less residual Peak II activity (<10% compared to >30% for CEM-C7) and total recoverable material (cf. 40 to 60% versus >80%). Because these experiments were not performed in parallel, it was unclear whether such differences were significant and indicated a subtle structural defect in receptor of the DEX* cells or simply reflected biological or experimental variation. To distinguish between these possibilities, we monitored the time course of receptor activation by analyzing sequential binding of labeled cytosols to slurries of DNA-cellulose, DEAE-cellulose, and hydroxylapatite. DNA binding thus detects activated receptor, DEAE-cellulose, residual unactivated holoreceptor, and hydroxylapatite binds membrerceptor (42). CEM-C1 and CEM-C7 showed no discernible difference in either the rate of formation or quantity of complex bound to the gels from 5 to 30 min postactivation (data not shown). The increase in receptor complex bound to DNA was accompanied by a corresponding reduction in the species bound to DEAE-cellulose.
both clones generated a small but detectable increase in receptor associated with hydroxylapatite within the initial 5 min; this fraction remained constant thereafter. In addition, mixed cytosols produced upon activation a pattern of binding which was unchanged. Thus, by these criteria, CEM-C1 does not appear to have an abundance or deficiency of cytoplasmic regulatory factors governing receptor activation and/or stability.

Since CEM-C1 receptor might possess a reduced potential for binding to nuclear components, we examined the relative affinity of steroid:receptor complex for DNA in vitro. Recently, it has been demonstrated (37) that glucocorticoid:receptor complexes of cells from some leukemic patients yield abnormal DEAE-cellulose chromatograms, characterized either by a multiplicity of peaks or by a single-peak profile. Cytosols having the latter pattern lacked Peak II activity but showed a low-salt-eluting fraction in the vicinity of Peak I which failed to bind DNA. Chart 2 shows that the heat-salt-activated [3H]TA:receptor complex of CEM-C1 not only bound to DNA-cellulose but also was eluted from the matrix at the same ionic strength (0.1 M phosphate) as did CEM-C7 receptor complex.

In the study cited above, leukemic samples exhibiting the abnormal single-peak DEAE-cellulose profiles were also found by glycerol gradient analysis to have a lower sedimentation coefficient than did cytosols showing normal chromatograms. Furthermore, examples of abnormalities in molecular size and shape (determined by density gradient centrifugation and gel permeation chromatography) in receptors having altered nuclear and DNA-binding properties have been reported for corticosteroid-resistant variants of the S49 (64) and P1798 (66) mouse lymphoma cells. Whereas CEM-C1 receptor appeared unremarkable in both its DEAE- and DNA-binding characteristics, it is nevertheless possible that the receptor could be deformed or truncated and yet retain these and other wild-type properties. Such a molecule might fail to properly "present" or could altogether lack a domain governing lytic activity. To probe for this possibility, we sized receptor complex by gel filtration. Sephacryl S-300 chromatography of unactivated, molybdate-stabilized, CEM-C1 cytosol generated a peak of specific, competable material, being eluted between the calibration proteins, ferritin and catalase (Chart 3). The gel filtration profile was virtually superimposable upon that obtained with similarly treated CEM-C7 cytosol. Assuming these proteins to be globular, we calculated Stokes' radii of 53 to 59 Å and 55 to 59 Å (n = 3) for the receptor complexes of the DEXr and DEXy clones, respectively.

GS induction. Although the receptor of DEXy CEM-C1 seemed normal by the biochemical criteria applied, we sought to determine whether, in fact, it was functional. GS is induced by glucocorticoids in a variety of cells: GH3 rat pituitary cells (68, 69), mouse L-cells (2, 69), HTC cells (32), Chinese hamster lung cells (73), embryonic chick neural retina (41), rat glial C6 cells (50), and mouse primary astrocytes (29). We recently reported that GS is also inducible in CEM-C7 cells and that this is a glucocorticoid-specific, receptor-mediated effect which occurs independently of DEX-mediated growth inhibition (23). We therefore chose to examine CEM-C1 for the expression of this steroid response.

The dose-response curve for DEX (Chart 4) clearly indicates an elevation in GS activity with half-maximal and maximal activity occurring at about 2 x 10^-8 M and 10^-7 M steroid, respectively. These concentrations were similar to those required to elicit comparable enzyme activity in CEM-C7 and essentially paralleled the concentrations necessary for half- and full occupancy of wild-type receptor (46). Moreover, the fold induction of GS produced by 1 µM DEX was found to be identical in CEM-C1 and CEM-C7 [cf. 1.9 ± 0.6 (12 determinations) and 1.9 ± 0.6 (9 determinations)]. As expected, the steroid specificity for GS induction, and hence for receptor, was the same in CEM-C1 and CEM-C7 (data not shown).

Steroid Metabolism. The striking similarity in receptor properties of clones CEM-C1 and CEM-C7, together with the apparent rarity of such a variant (6, 48, 64), prompted us to consider
alternative explanations for the resistance of CEM-C1 to DEX. Reports in the literature of more active than usual metabolism of cortisol by lymphocytes of murine lymphoma (11) and leukemic patients (11, 13, 28) raised the speculation that CEM-C1 could have an inherently normal lytic response to hormone but that resistance is due instead to accelerated steroid biotransformation.

In CEM-C7 cells, inhibition of growth (46), loss of viability, and the concomitant arrest of cells in G1 (19) become apparent after 24 hr of DEX addition; GS induction reaches maximum levels at 12 hr (23). CEM-C7 cells can be rescued from the lethal effects of DEX by steroid washout after 1 day (19, 45) but are irreversibly blocked in G1 and committed to death by 48 hr. Other wild-type CEM clones, on the other hand, require a longer duration of DEX treatment (e.g., up to 4 days) before full growth inhibition is achieved (71). It is possible then, that a depletion of intracellular steroid to suboptimal concentrations by metabolic inactivation and/or elimination within the appropriate time frame might result in the abrogation of lytic activity in sensitive cells without affecting enzyme induction. Our data, however, do not support such an interpretation for CEM-C1. We observed no appreciable difference between CEM-C1 and CEM-C7 in the capacity to transform DEX to more polar (extracellular fraction) or nonpolar (cellular fraction) compounds (Chart 5). The latter observation appears to rule out conversion of DEX to a derivative possessing high receptor affinity and antagonist activity. Furthermore, extracellular medium from DEX-treated CEM-C1 cells, when added to cultures of CEM-C7 in amounts calculated to yield DEX concentrations between 5 and 100 nM (assuming unmetabolized steroid), produced the same degree of growth inhibition and cell lysis as did identical concentrations of ethanolic DEX (data not shown). It seems unlikely then that such steroid modification as does occur is, in itself, a significant factor in contributing to glucocorticoid resistance in CEM-C1.

Plasma Membrane Fragility. Another potential explanation for the resistance of CEM-C1 invokes the elaboration of lysis-resistant cell membranes rather than a defect(s) in the biochemistry of steroid action. Based initially on observations with nuclei of corticosteroid-sensitive and -resistant murine P1798 lymphosarcoma cells, a hypothesis was proposed which attributed the ability of the latter to better withstand the destructive effects of glucocorticoids, as measured by a "nuclear fragility" assay (17), to the presence of "hardier" membranes (44). These findings were subsequently extended to the plasma membranes of normal rat thymocytes, and the greater structural integrity of cells more resistant to osmotic rupture was suggested as reflecting a generalized membrane phenomenon in cells insensitive to glucocorticoid-mediated lysis (78). We therefore considered the possibility that DEX+CEM-C1 might simply be of stronger biophysical constitution than is its sister clone, DEX+CEM-C7. Using an assay analogous to that of Young et al. (78), we were unable, however, to demonstrate differential susceptibility of untreated CEM-C1 and CEM-C7 cells to hypotonic shock. Both the dye uptake and 51Cr release methods yielded curves with similar midpoint inflections corresponding to salt concentrations (μ, 0.07 to 0.08) resulting in 50% stained cells and 50% release of radiochromium (Chart 6). We conclude that CEM-C1 does not possess an intrinsically more lysis-resistant cell plasma membrane. Nevertheless, these observations do not preclude structurally dissimilar surface membranes in the sensitive and resistant cells which could be correlated with glucocorticoid cytotoxicity (4, 5).

Cell Lineage. Lymphocyte sensitivity to the lytic effects of DEX. Cells were exposed (18 hr) to various concentrations of DEX, washed in medium without glutamine, and then resuspended in buffer (25 mM citrate, pH 6.4) and frozen over dry ice. Cell pellets were thawed and disrupted by sonication, and the particle-free cytosol was assayed for enzyme activity. Data are normalized for differences in basal and maximal (10 to 17 and 19 to 30 nmol/min/mg, respectively) GS activity, the latter set at 100. Bars, range of replicate determinations from 2 separate experiments.

Chart 4. Dose dependence of GS induction by DEX. Cells were exposed (18 hr) to various concentrations of DEX, washed in medium without glutamine, and then resuspended in buffer (25 mM citrate, pH 6.4) and frozen over dry ice. Cell pellets were thawed and disrupted by sonication, and the particle-free cytosol was assayed for enzyme activity. Data are normalized for differences in basal and maximal (10 to 17 and 19 to 30 nmol/min/mg, respectively) GS activity, the latter set at 100. Bars, range of replicate determinations from 2 separate experiments.

Chart 5. Thin-layer chromatograms of radioactivity extracted from [3H]DEX-treated cells. Cell cultures were incubated with [3H]DEX for 48 hr; the cells were collected, washed, and disrupted by sonication. The sonically disrupted cells and supernatant incubation media were extracted with organic solvent (at >90% and 1.6 M) and collected, washed, and disrupted by sonication. The sonically disrupted cells and supernatant incubation media were extracted with organic solvent (at >90% and 1.6 M) and collected, washed, and disrupted by sonication.

Chart 6. Plasma membrane fragility as a function of ionic strength of medium. Cells were suspended in salt solutions of varying hypotonicity, allowed to stand at room temperature, and then assayed for cell viability by trypan blue exclusion and 51Cr release.
Table 2

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<th>Growth and size</th>
<th>Surface membrane markers (% of cells)</th>
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<td>Karyotype</td>
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<td>CEM-C1 (DEX&lt;sup&gt;*&lt;/sup&gt;)</td>
<td>25 ± 3&lt;sup&gt;a&lt;/sup&gt; (6)</td>
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<tr>
<td>CEM-C7 (DEX&lt;sup&gt;*&lt;/sup&gt;)</td>
<td>24 ± 3 (6)</td>
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<sup>a</sup> Mean ± S.D.

<sup>b</sup> Numbers in parentheses, number of determinations.

**Fig. 1.** Giemsa-stained chromosome 9 of CEM-C1 (a) and CEM-C7 (b). The abnormal homologue is the right-hand member of each chromosome pair.

Corticosteroids varies with the state of cell maturation and/or differentiation and morphological or immunological classification (9, 25, 56). Since CEM-C1 insensitivity could be ascribed to these factors and not to an abnormality of steroid response, we undertook to establish the origin of the resistant phenotype.

CCRF-CEM was initially obtained as an uncloned ALL cell line. It exhibited a bimodal chromosome distribution (38) and expressed T-cell-specific membrane characteristics (31, 39). CEM-C1 and CEM-C7 were independently isolated from the parent line (46); the DEX<sup>*</sup> variants described to date, however, have all been derived from CEM-C7 (22) and were determined to have receptor abnormalities (22, 57). It seemed a distinct possibility, then, that CEM-C1 might not be a clonal relative of CEM-C7 which had developed resistance through random spontaneous mutation (22, 62) but had instead evolved from a second leukemic population that was innately insensitive to glucocorticoid-mediated cytolysis. However, several lines of evidence suggest that CEM-C1 and CEM-C7 share a common ancestry. (a) The morphological and growth properties of both clones were found to be indistinguishable. Upon microscopic examination, each clone showed intracellular features (not shown) typical of the lymphoblasts from which they were derived (12). Furthermore, the cells were found to be comparable in size and were observed to grow in stationary-suspension culture at identical rates (Table 2). (b) Cytogenetic analysis showed no difference in cell karyotype (Table 2), CEM-C1 having the same modal chromosome number and chromosome abnormality as does CEM-C7. The unique structural rearrangement of chromosome 9 (Fig. 1), involving both a pericentric inversion and partial deletion of the short arm,<sup>a</sup> provides strong evidence that these cells arose from the same transformed clone. This abnormality has been identified in all clones of CEM that we have studied thus far including DEX<sup>*</sup> variants isolated from a DEX<sup>*</sup> clone other than CEM-C7. (c) both clones expressed similar biochemical markers on their surface membranes. When screened against a panel of 4 monoclonal antibodies, CEM-C1 and CEM-C7 yielded a qualitatively similar spectrum of reactivity (Chart 7). Virtually all the cells of each clone bound αHuLyt-2; a subpopulation of both clones, some-

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<sup>a</sup> D. E. Moore, R. Zawydiwski, and E. B. Thompson, manuscript in preparation.
what variable in number (Table 2), exhibited lower binding of αHulLy-t and αHulLy-t. These immunoglobulins detect different antigens found largely on normal human T-cells and T-cell leukemias (18, 30, 36). A minor but comparable fraction of CEM-C1 and CEM-C7 cells reacted with αHula, which recognizes a determinant found predominantly on the non-T-cell population, i.e., B-cells, monocytes, and null cells (18). CEM-C1 failed to form erythrocyte rosettes, and only a small number of CEM-C7 cells did so (Table 2). CEM-C1 and CEM-C7 alike were strongly aggregated by soybean agglutinin, a response characteristic of the helper subset of T-lymphocytes (51). Collectively, these observations reveal no dramatic differences in the membrane or in cellular or biological properties of either clone, thus lending support to our interpretation of a common lineage for these cells.

The membrane phenotype, in particular, the inability to form erythrocyte rosettes, suggests that CEM-C1 is not a more differentiated lymphocyte and argues against DEX resistance by reason of cell maturation.

**DISCUSSION**

The phenotype of DEX clone CEM-C1 is atypical of steroid-selected, mutagenized, or drug-induced lymphoid variants which are resistant to glucocorticoid-induced cytolysis. Resistant cells of established murine lymphoma lines S49 and WEHI-7 (W7) either lack glucocorticoid-binding activity altogether (r-) (48, 63) or exhibit aberrant nuclear translocation of receptor (nt-; nt) (7, 16, 26, 48, 77). Cells in the latter category possess receptors with altered affinity for DNA or altered molecular size or shape (16, 64, 77); they fail to demonstrate positive complementation of defects in somatic cell hybrids (15, 47). Resistant cells of the P1798 transplantable mouse lymphoma contain an abnormally small receptor which displays greater than parental nuclear- and DNA-binding properties (66).

In the human ALL cell line CEM, spontaneously resistant clones derived from DEX clone CEM-C7 are receptor positive but exhibit a wide range in content (22). Preliminary characterization of some clones reveals diminished or nonexistent nuclear transfer (21) which, in at least one example (57), can be attributed to the inability to form stable activated steroid-receptor complex (act*). Hence, while the DEX derivatives of murine and human lymphoid cells show considerable phenotypic variability, physicochemical and genetic evidence alike indicates that they all contain defects in receptor. Resistant clone CEM-C1, on the other hand, has near wild-type levels of receptor, comparable affinity for DEX, and efficiency of nuclear transfer. Biochemically, the receptor of CEM-C1 is indistinguishable from that of sensitive clone CEM-C7 as assessed by ion-exchange and gel filtration chromatography. Furthermore, CEM-C1 receptor can mediate another glucocorticoid-specific effect, GS induction. None of the receptor-containing DEX variants of CEM examined to date has demonstrated this response. Thus, CEM-C1 receptor not only appears normal but is also functional, at least by this test.

The presence of a functional receptor in CEM-C1 raised the possibility that resistance may not, in fact, be due to a lesion(s) in the mechanism of glucocorticoid hormone action. We consequently considered and subsequently ruled out as unlikely several alternative explanations, among them steroid metabolism, "hardier" plasma membranes, cell ploidy, growth rate, and cell lineage. Collectively, these observations suggest that resistance in clone CEM-C1 is not associated with a receptor defect, but results from a postreceptor lesion in a steroid-sensitive cell line. CEM-C1 possesses properties of the murine "deathless" variant (63) and may prove to be the first bona fide example of this variant in human lymphoid cells, the murine equivalents of which have been shown only limited study (64).

Evidence for the presence of functional glucocorticoid receptor in some lymphoid cells inherently insensitive to lysis has recently been presented. The human lymphoblastoid cell line, IM-9, while not growth inhibited by DEX, responds with a receptor-mediated stimulation of 5'-nucleotidase (52). SAK8, a murine T-cell line derived from AKR leukemic cells, shown previously to contain apparently normal steroid receptor (34), responds with cell aggregation and an increase in mRNA and leukemia virus proteins (14). Although likewise not susceptible to the lethal activity of glucocorticoids, SAK8 has receptor capable of complementing the lytic defect in somatic cell hybrids formed with a receptorless variant of the W7 cell line. In addition, treatment with 5-azacytidine generates DEX sensitivity. It has therefore been suggested that glucocorticoid resistance in this line involves inactivation of non-receptor genes through methylation of DNA at a "lysis" locus (14). Methylation and/or demethylation may, of course, cause much more general effects. Nevertheless, the probability that glucocorticoid resistance in human clone CEM-C1 can be reversed by 5-azacytidine has also been explored. Such treatment, however, failed to restore DEX sensitivity. Thus, there is a clear difference in phenotype between the 2 receptor-positive but lysis-resistant lines, the CEM-C1 (human) and the SAK8 (mouse). This may reflect different mechanisms of resistance between the 2 lines, and may also reflect a tendency of the 2 species to acquire resistance by different mechanisms, as other data suggest (19–23, 37, 70, 71). CEM-C1 is but one of over 100 spontaneously resistant and mutagenized clones isolated from CEM in this laboratory. The majority of these DEX variants contain drastically reduced (<30% wild-type) receptor levels; a few contain 50% or more and, of these, at least 2 possess greater than parental (CEM-C7) levels of receptor (22). Because only a limited number have been studied, it is not possible to estimate the frequency of this new phenotype. Presumably, many of these cells would exhibit the recently characterized "activation-labile" receptor defect. However, it is also conceivable that some of the above-mentioned clones possessing near wild-type steroid binding might have functional receptor. It remains to be determined, therefore, whether the CEM-C1 phenotype constitutes a larger proportion of the resistant population than is presently indicated. Furthermore, it would be of considerable interest to learn whether this class of resistance has a restricted distribution (is confined to the CEM line) or is widely manifest in human leukemias.

The CEM-C1 phenotype of glucocorticoid resistance has important implications for the hormonal management of lymphoproliferative disorders. First and foremost, it indicates that resistance need not necessarily be associated with diminished content of and/or structural abnormality in receptor. Thus, biochemical assays for altered receptor properties might not be reliable indicators of cytolytic sensitivity and therapeutic response. Also, it questions the utility of GS induction as a prognosticator of cytolytic sensitivity. We have suggested previously that GS

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*J. C. Gasson and S. Bourgeois, personal communication.*
induction may provide a useful marker for receptors capable of mediating cell death in leukemic cells (59). Clearly, the data show that cell death and GS induction in CEM-C1 cells are noncoordinately regulated. Nonetheless, the infrequency with which we have isolated cells expressing the CEM-C1 phenotype suggests that this phenotype may be the exception rather than the rule. Only direct analysis of samples from leukemic patients will provide the answer as to the usefulness of GS induction as a marker for therapeutic response.

Although our data suggest that CEM-C1 contains a "postreceptor" lesion, they do not exclude entirely the possibility that resistance is due to a subtle receptor defect. Confirmation awaits the demonstration of either structural identity with wild-type receptor or functional capacity to mediate cytostasis. Studies toward resolving this question are currently in progress. Should CEM-C1 prove to possess a nonreceptor defect, it would be invaluable in the elucidation of the molecular basis of resistance and in the identification of genetically distinct components of glucocorticoid hormone action in somatic cell hybrids between variants of similar phenotypes.

While these cells do not meet the genetic ideal of being derived directly from a sensitive clone by single-step selection, they nevertheless appear to be of considerable potential value in the study of steroid action. They are closely related to their sensitive clone, have functional receptors, and yet are steroid resistant. CEM-C1 cells could ultimately prove to be a well-defined model for the chemotherapy of certain classes of leukemias refractory to glucocorticoids.

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Glucocorticoid-resistant Human Acute Lymphoblastic Leukemic Cell Line with Functional Receptor

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