Covalent Labeling of Rat Thymocyte and Human Lymphoid Glucocorticoid Receptor

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ABSTRACT

Lymphoid cells contain specific receptors for glucocorticoids. We have used [3H]dexamethasone-21-mesylate to label covalently glucocorticoid receptors in rat thymic lymphocytes and in neoplastic cells obtained from patients with acute lymphoblastic leukemia and malignant lymphoma. The covalently labeled glucocorticoid receptors were identified by polyacrylamide gel electrophoresis (in the presence of 0.1% sodium dodecyl sulfate). In cytosolic fractions prepared from rat thymic lymphocytes, [3H]-dexamethasone-21-mesylate labels a protein (M, \( \approx 95,000 \)) which was identified as the glucocorticoid receptor by the following criteria: (a) labeling of this moiety is inhibited by treatment with a 100-fold molar excess of glucocorticoids, such as dexamethasone and triamcinolone acetonide; and (b) the covalently labeled M, \( \approx 95,000 \) protein is activated (by heating at 20° for 30 min) to a form that binds to DNA-cellulose. When intact thymocytes are treated with [3H]-dexamethasone-21-mesylate, an M, \( \approx 95,000 \) moiety is also labeled covalently. Approximately 35% of the glucocorticoid receptors can be labeled covalently when intact thymocytes are treated with 100 nm [3H]dexamethasone-21-mesylate for 30 min at 4°.

Neoplastic cells from acute lymphoblastic leukemia and malignant lymphoma were treated with [3H]dexamethasone-21-mesylate. In all samples, an M, \( \approx 95,000 \) moiety was labeled covalently; labeling was inhibited by excess glucocorticoid. Smaller moieties were also identified by competition experiments; these may represent proteolytic fragments of the M, \( \approx 95,000 \) receptor. Thus, in rat and human lymphoid cells, [3H]dexamethasone-21-mesylate can be used to label covalently the glucocorticoid receptor.

INTRODUCTION

Glucocorticoids are used widely in the treatment of lymphoid cancers, such as acute lymphoblastic leukemia and lymphocytic lymphoma. The therapeutic effects of glucocorticoids may involve specific glucocorticoid receptors found in lymphoid cells (5, 10, 16). However, not all lymphoid cancers are responsive to glucocorticoids (3, 8). Defects of glucocorticoid receptors in different malignant cell types may be responsible for the variation in sensitivity of leukemia and lymphoma to glucocorticoid therapy. Such defects have been described in murine and human lymphoma and leukemia cell lines (14, 16, 22) and have been suggested recently in human leukemia (1, 11).

Until recently, glucocorticoid receptors in lymphoid cells have only been detectable by use of radiolabeled steroids (such as dexamethasone), which form noncovalent steroid:receptor complexes that dissociate readily. In order to study receptor differences in leukemic cells, it would be beneficial to use a receptor label which remains stable under analytical conditions. Affinity labeling has been used to label covalently glucocorticoid receptors in rat liver (7), rat HTC3 cells (20, 21), and mouse lymphoma (13). In the present study, we have used [3H]DM to label covalently the glucocorticoid receptor in rat thymocytes. These techniques were then applied to cells from patients with lymphoma and acute lymphoblastic leukemia.

MATERIALS AND METHODS

Chemicals. [3H]TA (37 Ci/mmol) and [3H]DM (46 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. TA dexamethasone, sodium molybdate, and 3-[tris(hydroxymethyl)methyl]aminopropane sulfonic acid were purchased from Sigma Chemical Co., St. Louis, Mo. Ready-Solv MP scintillation fluid was purchased from Beckman Instruments, Inc., Berkeley, Calif. Hydroxylapatite was purchased from LKB Instruments, Inc., Rockville, Md. Analytically pure DM was obtained as a gift from Dr. S. Simons.

Buffers and Solutions. TEG buffer consisted of 25 mm 3-[tris(hydroxymethyl)methyl]aminopropane sulfonic acid, 1 mm EDTA, and 10% glycerol, pH 8.2. SDS sample buffer consisted of 2% SDS, 20% glycerol, 2% dithiothreitol, and 0.6 M Tris (pH 8.85).

Preparation of Cells. Five male Sprague-Dawley adrenalec- tomized rats were sacrificed by decapitation, and thymus glands were removed. The glands were minced in 10 ml Dulbecco's PBS (pH 7.5), and the cells were separated with one stroke of a Corning glass tissue homogenizer. The cell suspension was filtered through fine nylon mesh and centrifuged at 400 × g for 10 min. The cell pellets were washed twice and resuspended in PBS. At least 95% of the cells were viable by trypan blue exclusion.

Cells were obtained from patients at the University of Minnesota and received at ambient temperature within 24 hr at the NIH in Bethesda, Md. Peripheral blood was diluted at least 1:5 in Roswell Park Memorial Institute Tissue Culture Medium 1640 with penicillin (50 μg/ml), gentamicin (50 μg/ml), glucose (300 μg/ml), 25 mm 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid, and heparin (100 units/ml) prior to shipment. The mononuclear white cells were obtained by Ficoll-Hypaque differential centrifugation by the method of Boyum (4). Cells were 95% viable by trypan blue exclusion. Single-cell suspensions from neoplastic lymphoid tissue were prepared at the University of Minnesota. If the single-cell suspension contained at least 50% malignant cells, the cells were shipped to Bethesda, Md., in Roswell Park Memorial Institute Tissue Culture Medium 1640. The preparations were centrifuged at 400 × g for 10 min and resuspended in PBS. Lymph node preparations were at least 80% viable by trypan blue exclusion.

The abbreviations used are: HTC, hepatoma tissue culture; DM, dexamethasone-21-mesylate; TA, triamcinolone acetonide; TEG, 3-[tris(hydroxymethyl)methyl]aminopropane sulfonic acid; EDTA, glycerol; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

[1] This work was supported in part by the NIH Grant CA 26273, American Cancer Society Grant CH-167, and the Coleman Leukemia Research Fund.

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Received April 15, 1983; accepted August 5, 1983.
Preparation of Cytosolic Fractions. Cell suspensions were centrifuged at 400 \( \times \) g for 10 min; cell pellets were frozen at -70° and then thawed at 4° \( \times \) in 2.5 volumes of TEG buffer containing 50 mM NaCl. When completely thawed, the pellet was suspended (vortexed) and centrifuged at 100,000 \( \times \) g for 30 min, and the supernatant (cytosolic) fraction was collected.

Labeling of Intact Lymphoid Cells with \([^{3}H]DM\). After initial preparation of cells, the cell pellets were suspended in 10 volumes of PBS. Additions were made (as indicated in the text), and the cell suspensions were incubated on ice. The cell suspensions were centrifuged at 400 \( \times \) g for 5 min; supernatant media were aspirated, and the cell pellets were frozen at -70°. The frozen cell pellets were thawed at 4° \( \times \) in 2.5 volumes of TEG buffer containing 20 mM sodium molybdate and then centrifuged in an Eppendorf microcentrifuge for 5 min. Supernatant fractions (140 \( \mu \)l) were treated immediately with SDS sample buffer (60 \( \mu \)l).

PAGE. Polycrylamide gels (9%) in the presence of 0.1% SDS were prepared as described by Laemmli (9). Each sample in SDS sample buffer was heated at 100° for 1 min before applying an aliquot (40 \( \mu \)l) to the gel. A Bio-Rad slab gel apparatus was used, and electrophoresis was carried out at 100 V/slab. Gels were fixed and stained in 50% methanol, 7.5% acetic acid, and 0.2% Coomassie Brilliant Blue R250 and destained in 10% methanol and 7.5% acetic acid. Gel lanes were cut (1 mm) and dissolved in 0.6 ml of 30% hydrogen peroxide at 40°. Radioactivity was determined with Ready-Solv MP and a Tracor Mark III spectrometer (counting efficiency, 40 to 50%).

RESULTS

Affinity Labeling of Rat Thymocyte Glucocorticoid Receptors in Cytosolic Preparations. DM has been used previously to label covalently glucocorticoid receptors in HTC cell cytosol (20, 21) and in rat liver cytosol (7), presumably through reaction with thiol anions of cysteine residues present in the steroid binding sites of the glucocorticoid receptor (18). Although \([^{3}H]DM\) reacts covalently with many proteins in crude cytosol, covalent binding between \([^{3}H]DM\) and the glucocorticoid receptor can be inhibited selectively by unlabeled glucocorticoid, such as TA or dexamethasone (19). \([^{3}H]DM\) binding to other cytosolic proteins (e.g., albumin) is not inhibited competitively by excess glucocorticoid (20).

Previous work with rat liver has shown that small-molecular-weight compounds present in crude cytosol prevent \([^{3}H]DM\) binding to the glucocorticoid receptor (7). In order to determine whether DM labels glucocorticoid receptor in rat thymocytes, crude cytosol was incubated with 25 nM \([^{3}H]TA\) and increasing concentrations of DM (Chart 1). DM (200 nM) inhibited effectively \([^{3}H]TA\) binding to the receptor. If thymocyte preparations were contaminated with RBCs (known to contain high levels of glutathione), such competition was not observed. In such preparations, gel filtration could be used to remove low-molecular-weight moieties that interfered with receptor labeling (data not shown).

Cytosolic fractions were treated with 200 nM \([^{3}H]DM\), alone or in combination with a 100-fold molar excess of TA, for 2 hr at 4° and analyzed by SDS-PAGE (Chart 2). Several \( ^{3} H \)-labeled proteins were detected in sliced gels, but labeling of only one major protein band (\( M_s 95,000 \)) was inhibited by a 100-fold molar excess of TA. Smaller proteins, also inhibited by excess unlabeled TA, were not detected when other techniques were used to label rat thymocytes (see below). The saturaability of the \( M_s 95,000 \) moiety provides evidence that this protein is the specific glucocorticoid receptor in rat thymocyte cytosol.

The \([^{3}H]DM\)-labeled glucocorticoid receptor in both rat liver and HTC cells has been identified by its ability to undergo temperature-dependent "activation" to forms that bind to DNA-cellulose (12, 19). Rat thymocyte glucocorticoid receptor was identified similarly by warming \([^{3}H]DM\)-labeled cytosol (20° for 30 min) and then cooling to 4°, followed by chromatography on DNA-cellulose. The \( M_s 95,000 \) moiety was adsorbed to DNA-cellulose and could be eluted by buffer containing 0.5 M NaCl (Chart 3). Similar chromatography with unheated cytosol showed virtually no adsorption of the \( M_s 95,000 \) moiety (data not shown).

Affinity Labeling of Glucocorticoid Receptor in Intact Thymocytes. Human lymphoid glucocorticoid receptors appear to be unstable in cell extracts (6) and therefore should be labeled in intact cells. We used rat thymocytes to study conditions for
**Rat and Human Lymphoid Glucocorticoid Receptors**

The effects of concentration of [3H]DM and of time of treatment on receptor binding were assessed (Chart 5). Receptor labeling was detectable at 10 min and was maximal after 30 min; 100 nm [3H]DM was sufficient to label the receptor maximally. The labeling efficiency of [3H]DM was determined by labeling rat thymocyte suspensions for 1 hr at 4° with 100 nm [3H]DM or 50 nm [3H]TA ± a 100-fold excess of TA. Specific labeling determined directly from [3H]TA-treated cytosol was 14,800 ± 300 dpm. Specific labeling from SDS-PAGE-analyzed [3H]DM-treated cytosol was 5,300 ± 500 cpm. Therefore, [3H]DM labeling of the glucocorticoid receptor was 35% of that found for [3H]TA.

**Affinity Labeling of the Glucocorticoid Receptor in Human Leukemic and Lymphoma Cells.** Glucocorticoid receptors from neoplastic cells were labeled covalently with [3H]DM in 5 patient samples. Patient diagnosis, specimen type, and labeling conditions are indicated in Table 1. All samples were analyzed by SDS-PAGE (Chart 6). In all cases, saturable labeling was shown for an Mr = 95,000 protein moiety. Saturable labeling was also seen in an Mr = 45,000 protein moiety in Patient A; Mr = 53,000 and Mr = 35,000 in Patient B; Mr = 75,000, Mr = 57,000, Mr = 45,000, and Mr = 31,000 in Patient D; and Mr = 75,000 in Patient E. No additional saturable protein moieties were seen in Patient C.

**DISCUSSION**

This study represents the first application of affinity labeling to the analysis of glucocorticoid receptors in human leukemia and malignant lymphoma. When malignant cells obtained from lymph node biopsy or from peripheral blood are treated with [3H]DM, labeled receptor can be analyzed under denaturing conditions. In the 5 cases studied, an Mr = 95,000 moiety showed that specific binding of [3H]DM is similar to that found for the rat

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**Table 1**

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<th>Patient</th>
<th>Diagnosis</th>
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<th>Treatment</th>
<th>Source of Sample</th>
<th>Labeling Conditions</th>
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*ALL, acute lymphoblastic leukemia; N-PDLL, nodular poorly differentiated lymphocytic lymphoma; WDLL, well-differentiated lymphocytic lymphoma.*
Chart 6. [3H]DM labeling of human lymphoma and leukemic cell glucocorticoid receptor as analyzed by SDS-PAGE. Gel lanes contain proteins from 40 μl of sample. (Abbreviations for standards are as in Chart 2.) In A, cytosol from circulating blasts of a patient with acute lymphoblastic leukemia was labeled with 200 nM [3H]DM alone (●) or in combination with 20 μM TA (○). One hundred μl of cytosol were combined with 100 μl of sample buffer. In B, a 5% cell suspension by cytocrit of circulating blasts from a patient with acute lymphoblastic leukemia was labeled with 200 nM [3H]DM alone (●) or in combination with 20 μM TA (○). The frozen pellet was resuspended in 1 ml of TEG buffer with 20 mM sodium molybdate. One hundred μl of cytosol were combined with 100 μl of sample buffer. In C, intact lymphoma cells from a lymph node of a patient with nodular poorly differentiated lymphocytic lymphoma were labeled with 100 nM [3H]DM alone (●) or in combination with 10 μM TA (○). Cytosol was prepared as in "Materials and Methods." In D, intact lymphoma cells from a lymph node of a patient with diffuse well-differentiated lymphocytic lymphoma were labeled with 100 nM [3H]DM alone (●) or in combination with 10 μM TA (○). Cytosol was prepared as in "Materials and Methods." In E, intact malignant cells from a lymph node of a patient with nodular, poorly differentiated lymphocytic lymphoma were labeled with 100 nM [3H]DM alone (●) or in combination with 10 μM TA (○). Cytosol was prepared as in "Materials and Methods."
thymocyte. Saturability with excess unlabeled steroid (such as TA) and ability to undergo activation and bind to DNA-cellulose (properties of noncovalently radiolabeled lymphoid glucocorticoid receptor) are retained by rat thymocyte receptor which has been labeled covalently with [3H]DM. These findings are in agreement with those of Simons and coworkers (7, 19) and Milgrom (12), who have demonstrated similar properties in rat liver cytosol and in rat HTC cells.

Rat thymocyte glucocorticoid receptor has $M_\text{r} \approx 95,000$ as determined by SDS-PAGE. This is consistent with previous reports of molecular weight of the glucocorticoid receptor in rat liver and in rat HTC cells (7, 19). This moiety represents the reduced denatured fundamental unit of the glucocorticoid receptor. Under nondenaturing conditions, the glucocorticoid receptor is apparently much larger and may consist of a multimer of receptor subunits or a complex with other nonreceptor proteins (15, 17, 23).

In malignant lymphoid cells, an $M_\text{r} \approx 95,000$ moiety is labeled covalently with [3H]DM, and labeling is inhibited by excess TA. The limited quantities of patient material have not permitted analysis of the $M_\text{r} \approx 95,000$ moiety by activation and binding to DNA-cellulose. Four of the 5 human neoplastic cell samples show saturable bindings of smaller labeled moieties ($M_\text{r} \approx 75,000$, $M_\text{r} \approx 57,000$, $M_\text{r} \approx 45,000$, $M_\text{r} \approx 35,000$, and $M_\text{r} \approx 31,000$). These moieties may represent proteolytic fragments of the $M_\text{r} \approx 95,000$ receptor subunit. The glucocorticoid receptor is unstable in some human leukemia cell extracts (6) and may be subjected to proteolysis during the limited exposure to cell-free conditions in our present assay. When the covalently labeled rat liver receptor is treated with exogenous proteases such as trypsin, multiple smaller labeled forms are produced.4 Alternatively, these smaller moieties may be present in the intact cell and could be produced by processes that do not involve proteolysis (e.g., mRNA processing to smaller species that encode only a portion of the $M_\text{r} \approx 95,000$ subunit).

Covalent labeling of the glucocorticoid receptor in lymphoid cells may be performed in intact cells or cell extracts. However, fewer proteins are nonsaturably labeled in intact thymocytes than in cell extracts, and specifically labeled proteins with molecular weight less than $\approx 95,000$ are present in cell extracts which are not present when intact cells are labeled (Charts 2 and 4).

These initial studies demonstrate that glucocorticoid receptors in intact human malignant cells can be labeled covalently. The methods in the current study (rapid denaturing of cytosolic proteins with SDS) appear suitable for protection of thymocyte and some human lymphoid glucocorticoid receptors from proteases but may not provide sufficiently rapid denaturation of all human lymphoid cells. We are currently evaluating techniques to denature cells directly in an effort to minimize formation of smaller labeled proteins. Further, it will be of interest to evaluate cytosols of malignant cells to identify factors that either promote or protect against formation of receptor fragments.


ACKNOWLEDGMENTS

We would like to thank Linda A. Hamilton for her expert secretarial assistance.

REFERENCES

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