A Single Common Electrophoretic Abnormality of Glucocorticoid Receptors in Human Leukemia Cells

By Clark W. Distelhorst, Barbara M. Benutto, and Rogers C. Griffith

We determined the mol wt of glucocorticoid receptors in human leukemia cells in order to detect glucocorticoid receptor defects that might cause glucocorticoid resistance. Glucocorticoid receptors in intact cells were affinity labeled with [3H]dexamethasone-21-mesylate and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Receptors in normal human peripheral blood mononuclear cells and six human leukemia cell lines had mol wt of 97,000. Malignant cells from ten of 25 patients with leukemia contained electrophoretically abnormal glucocorticoid receptors having mol wt of 55,000 in addition to normal size receptors (M, = 97,000). The receptor abnormality was not restricted to a particular type of leukemia and was seen in cells from both newly diagnosed patients and patients who had received prior chemotherapy, including prednisone. The abnormal receptor was not generated when cells having only normal size receptors were assayed under conditions that favor proteolysis or when cytosol from cells containing the abnormal receptor form was mixed with cytosol from cells containing only normal size receptors. The mol wt of the abnormal receptors in human leukemia cells was the same as the mol wt of receptors in mutant mouse lymphoma cell lines, S49 143R and S49 55R, which have the nuclear transfer-increased phenotype of glucocorticoid resistance.

This work describes for the first time a single common electrophoretic abnormality of glucocorticoid receptors in human leukemia cells. Further investigation of glucocorticoid receptor defects in human leukemia cells could lead to an improved understanding of the mechanisms of glucocorticoid resistance in leukemia as well as a method of predicting which patients are likely to be resistant to glucocorticoid therapy.

GLUCOCORTICOIDS, such as prednisone, are among the most effective agents for the treatment of acute lymphoblastic leukemia and, when used in combination with vincristine, induce a complete remission in more than 90% of children with this disease. Prednisone is also used frequently to treat chronic lymphocytic leukemia, acute nonlymphoblastic leukemia, Hodgkin’s disease, and non-Hodgkin’s lymphomas. However, up to 50% of patients with newly diagnosed acute lymphoblastic leukemia fail to respond to prednisone when used as a single agent,1,2 and an even greater proportion fail to respond to prednisone after relapse.1 A similar proportion of patients with non-Hodgkin’s lymphoma fail to respond to dexamethasone used as a single agent.3 Therefore, the effectiveness of glucocorticoid therapy in human malignancies is frequently limited by the development of glucocorticoid resistance.

Mechanisms by which malignant cells become resistant to glucocorticoids have been elucidated in studies of glucocorticoid-resistant mutants of the S49 mouse lymphoma cell line.4 These studies demonstrated that glucocorticoid killing of mouse lymphoma cells is mediated by glucocorticoid receptors and that defects in glucocorticoid receptor function result in glucocorticoid resistance.5

Mechanisms by which human malignant cells develop glucocorticoid resistance are less well defined. Lippman et al6 first demonstrated the presence of glucocorticoid receptors in human acute lymphoblastic leukemia cells and suggested a correlation between a loss of receptors and the development of glucocorticoid resistance. Subsequent studies have demonstrated a correlation between the number of glucocorticoid receptors and glucocorticoid responsiveness in human leukemias and lymphomas.9,10 In addition, a recent study demonstrated that low glucocorticoid receptor levels correlate with an increased frequency of remission induction failure and relapse in childhood acute lymphoblastic leukemia treated with a combination of chemotherapeutic agents, including prednisone.8 However, the presence of substantial numbers of glucocorticoid receptors in human leukemia and lymphoma cells does not necessarily confer glucocorticoid responsiveness,9,10 indicating that defects in glucocorticoid receptor function or defects in the cell-killing process subsequent to glucocorticoid receptor action account for the development of glucocorticoid resistance in many patients with leukemia and lymphoma. A defect in glucocorticoid receptor activation has been identified in a human leukemia cell line,11 and recent studies have found that glucocorticoid receptors in a large proportion of human acute leukemias are abnormal in terms of their physical properties and their ability to bind to DNA-cellulose or DEAE-cellulose.12,13 These results suggest that glucocorticoid receptor defects might be common in human leukemias and lymphomas. Better definition of the defects at the molecular level should result in an improved understanding of the mechanisms by which malignant cells develop glucocorticoid resistance and could lead to a means of predicting which patients are likely to fail glucocorticoid therapy at the time of diagnosis or relapse.

The present work was undertaken in order to identify glucocorticoid receptor abnormalities in human leukemia cells. We affinity-labeled glucocorticoid receptors in leukemia cells with [3H]dexamethasone-21-mesylate and then analyzed the receptors by gel electrophoresis. Our results demonstrate a single common electrophoretic abnormality of the glucocorticoid receptors in leukemia cells from ten of 25...
patients studied. The electrophoretic abnormality is similar to that observed in certain glucocorticoid-resistant mouse lymphoma cell lines.

MATERIALS AND METHODS

**Materials.** All chemicals and unlabeled dexamethasone were purchased from Sigma Chemical Company, St Louis. Acrylamide, N,N,N',N'-tetramethylenediamine (TEMED), and Coomassie brilliant blue R-250 were from Bio-Rad Laboratories, Richmond, Calif. The low mol wt standards kit for gel electrophoresis containing phosphorylase a, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and a-lactalbumin was from Pharmacia Fine Chemicals, Piscataway, NJ. Glucocorticoid receptors were labeled with [3H]dexamethasone-21-mesylate essentially as described by Foster et al with a few modifications. Cells were used on the same day in which they were obtained from patients or donors or after overnight incubation in culture medium at 4°C. Cells were washed three times in PBS at 4°C and were resuspended in PBS at 4°C after the final wash. Aliquots of 0.48 mL of the cell suspensions were transferred to 1.5 mL Eppendorf microcentrifuge tubes so that each tube contained at least 20 million cells and not more than 40 million cells. Double samples both without and with 20 nmol/L unlabeled dexamethasone were incubated with 200 nmol/L [3H]dexamethasone-21-mesylate for four hours at 4°C with periodic gentle resuspension. At the end of the incubation period the cell suspension was gently pelleted and the supernatant was discarded. The cell pellet was frozen at –80°C for 15 minutes and then 0.02 mL of hypotonic buffer was added. The cell pellet was allowed to thaw at 4°C for 15 minutes with gentle resuspension in the hypotonic buffer and then was centrifuged in an Eppendorf centrifuge at 4°C for 15 minutes. The supernatant, (0.02 mL), which is referred to as cytosol, was immediately added to the sample buffer for gel electrophoresis, followed by the addition of β-mercaptoethanol. The sample was immediately boiled for two minutes and then applied directly to the polyacrylamide gel. In certain experiments, samples were handled in an identical manner.

**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>Source</th>
<th>Age</th>
<th>Prior Chemotherapy</th>
<th>Prior Steroid Therapy</th>
<th>Receptor Mol Wt</th>
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<tr>
<td>2</td>
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<td>BM</td>
<td>4 yr</td>
<td>–</td>
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<td>BM</td>
<td>5 yr</td>
<td>–</td>
<td>–</td>
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<tr>
<td>4</td>
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<td>BM</td>
<td>39 yr</td>
<td>–</td>
<td>–</td>
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<tr>
<td>5</td>
<td>ALL</td>
<td>PB</td>
<td>4 yr</td>
<td>–</td>
<td>+</td>
<td>97,000 55,000</td>
</tr>
<tr>
<td>6</td>
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<td>BM</td>
<td>7 yr</td>
<td>–</td>
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<tr>
<td>7</td>
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<td>–</td>
<td>–</td>
<td>97,000 55,000</td>
</tr>
<tr>
<td>8</td>
<td>T-ALL</td>
<td>BM</td>
<td>22 yr</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>+</td>
<td>–</td>
<td>97,000 55,000</td>
</tr>
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<td>BM</td>
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<td>–</td>
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<td>BM</td>
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<td>–</td>
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</tr>
<tr>
<td>19</td>
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</tr>
<tr>
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<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>21</td>
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<td>PB</td>
<td>40 yr</td>
<td>+</td>
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<tr>
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<td>BM</td>
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<td>+</td>
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<td>97,000 55,000</td>
</tr>
<tr>
<td>23</td>
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<td>BM</td>
<td>70 yr</td>
<td>–</td>
<td>–</td>
<td>97,000 55,000</td>
</tr>
<tr>
<td>24</td>
<td>CLL</td>
<td>BM</td>
<td>70 yr</td>
<td>–</td>
<td>–</td>
<td>97,000 55,000</td>
</tr>
<tr>
<td>25</td>
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<td>BM</td>
<td>70 yr</td>
<td>–</td>
<td>–</td>
<td>97,000 55,000</td>
</tr>
</tbody>
</table>

ALL, non-T, non-B acute lymphoblastic leukemia; T-ALL, T cell acute lymphoblastic leukemia; ANLL, acute nonlymphoblastic leukemia; CML, chronic myelogenous leukemia in myeloid blast crisis; CLL, chronic lymphocytic leukemia; PB, peripheral blood; BM, bone marrow.
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except that the buffer did not contain molybdate and the temperature at which the cells were thawed was increased to 37°C as described in the text. In mixing experiments, unlabeled cytosol was prepared from cells that contained the abnormal receptor form. The unlabeled cytosol was then added to an equal volume of affinity-labeled cytosol from cells that had only normal mol wt receptors. After incubation at 4°C for 15 minutes, the cytosol mixture was added to sample buffer and analyzed by gel electrophoresis as described above. Labeling efficiency was calculated by the method of Simons et al except that gel slices were digested overnight at 37°C in a 2.5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene (PPO-POPOP)-toluene scintillation mixture containing NCS reagent and was 80 ± 12% (mean ± SEM).

Polyacrylamide gel electrophoresis. Polyacrylamide gels (9.2% acrylamide and 0.24% N,N'-methylenebisacrylamide with 0.1% SDS) were prepared as described by Laemmli using a slab gel apparatus. Electrophoresis was performed at 7.5 mA per gel, and gels were stained with 0.05% (wt/vol) Coomassie brilliant blue in H2O:methanol:acetic acid, 4:5:1 (by volume) and destained with H2O:ethanol:acetic acid, 20:1:1 (by volume). After the destaining procedure, gels were analyzed by fluorography. Gels were rinsed for one hour with water and then incubated with 1 mol/L sodium salicylate for one hour at room temperature and dried under a vacuum. Gels were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for seven to 21 days at −80°C. Mol wt standards were β-galactosidase, Mr = 116,00017; phosphorylase b, Mr = 97,40022; bovine serum albumin, Mr = 67,00021; ovalbumin, Mr = 45,00022; carbonic anhydrase, Mr = 30,00022; trypsin inhibitor, Mr = 20,10022; and α-lactalbumin, Mr = 14,400. The mol wt of receptor proteins were determined by comparing the mobility of the receptor protein with the mobility of the standard proteins according to the method of Weber and Osborn.

RESULTS

In order to determine the mol wt of glucocorticoid receptors in normal human peripheral blood mononuclear cells, intact cells were incubated with [3H] dexamethasone-21-mesylate in the absence (−) or presence (+) of unlabeled excess dexamethasone, and cytosol samples were analyzed by SDS-polyacrylamide gel electrophoresis. Mol wt (Mr) of standard proteins are indicated by arrows. Multiple proteins bind the radiolabeled steroid, but the glucocorticoid receptor band, which is opposite the phosphorylase b standard, can be distinguished from other protein bands because it is eliminated by excess unlabeled dexamethasone.

![Fig 1. Autoradiogram of affinity-labeled receptors from normal human mononuclear cells.](image-url)

RESULTS

100,000 and 110,000. In addition, smaller forms of the receptor having mol wt of 52,000, 20,000, and 17,000 are observed. A glucocorticoid-resistant nuclear transfer increased mutant, S49 143R, contains a single receptor form that has a mol wt of approximately 54,000 (Fig 2B). The same result was obtained with another nuclear transfer increased mutant, S49 55R (not shown). A glucocorticoid-resistant nuclear transfer decreased mutant, S49 22R, contains a single receptor that has a mol wt of approximately 97,000 (Fig 2C).

The mol wt of glucocorticoid receptors in leukemia cells from 25 patients who were either newly diagnosed or in relapse is shown in Table 1. Most of the patients who were in relapse had received glucocorticoids as part of their previous chemotherapy. None of the patients had received glucocorticoids or other chemotherapy within two weeks of the present study. Because patients were treated with multiple agents, it was not possible in each case to determine responsiveness to glucocorticoids. More than 80% of the cells in each sample were malignant as judged by morphologic criteria or serologic markers. In all of the patient samples a receptor band was observed opposite the phosphorylase b standard, thus corresponding to the location of the receptor band in normal human peripheral blood mononuclear cells. In 15 of the 25 patient samples this was the only receptor band observed, as illustrated in Fig 3A. In ten of the 25 patients, samples an additional receptor band corresponding to a mol wt of about 55,000 was observed. The abnormal receptor band is shown in Fig 3 (B through E) and Fig 4 (A and B). The mol wt of the abnormal receptor in the human leukemia cells is the same as the mol wt of the abnormal receptor in the
S49 143R and S49 55R mouse lymphoma cells (Fig 2). As illustrated in Figs 3 and 4, the relative intensity of normal and abnormal receptor bands varied among the patient samples. The abnormal receptor form was observed both in leukemia cells that were labeled immediately after isolation and after overnight storage at 4 °C.

Experiments were performed in which duplicate samples of cells from patients 4 and 12 (Table 1) were handled in such a manner as to encourage proteolysis of the glucocorticoid receptor. Cells were labeled with [3H]dexamethasone-21-mesylate and then thawed in hypotonic buffer at 37 °C instead of at 4 °C and in the absence of molybdate, which has been found to inhibit certain proteases. In each of these experiments only the receptor form corresponding to a mol wt of 97,000 was observed. In the experiments shown in Fig 4, unlabeled cytosol from leukemia cells that had the abnormal receptor form was mixed with affinity-labeled cytosol from leukemia cells that did not contain the abnormal receptor form. An abnormal receptor form was not generated by this procedure, indicating that the abnormal receptor form is unlikely to be generated from the normal receptor form by proteolysis. This observation was confirmed in one other experiment.

DISCUSSION

This study demonstrates a single common electrophoretic abnormality of glucocorticoid receptors in malignant cells from patients with leukemia. The abnormality was identified in the leukemia cells of ten of 25 patients and consisted of a receptor band corresponding to a mol wt of 55,000. The electrophoretic abnormality did not appear to be specific for
a particular type of leukemia and was observed in both newly diagnosed patients and patients who had received prior chemotherapy.

There are several explanations for the abnormal receptor described in this study. First, the abnormal receptor could be an artifact generated by proteolysis of the normal receptor during cytosol preparation. However, the abnormal receptor was not observed in control experiments using normal peripheral blood mononuclear cells and six human cell lines. Also, the abnormal receptor was not produced when leukemia cells having only the normal receptor were assayed under conditions that favor proteolysis or when cytosol from cells containing the abnormal receptor form was mixed with cytosol from cells containing only normal-size receptors. A second possible explanation is that the abnormal receptor is the result of a genetic defect. A deletion mutation in the receptor gene might lead to synthesis of a receptor molecule from which one region is missing, or a nonsense mutation might cause premature protein chain termination. Alternatively, an amino acid substitution might render the receptor particularly susceptible to proteolysis. A third possible explanation is that the leukemia cells that demonstrate the abnormal receptor contain a protease that digests the receptor molecule at a particular site and that is not present in normal peripheral blood mononuclear cells, the cell lines that were studied, or the majority of human leukemia cells. The mixing studies described above appear to make this possibility remote.

In each of the autoradiograms showing the abnormal receptor, a normal-size receptor was also present. In some of the autoradiograms the abnormal receptor band appeared less intense than the normal receptor band. This could be interpreted as indicating that the abnormal receptor was derived from the normal receptor. However, differences in intensity could be secondary to differences in steroid-binding affinity and labeling efficiency or to a decreased stability of the abnormal receptor. If a genetic defect is responsible for the abnormal receptor in human leukemia cells, the defect is likely to be heterozygous since both normal and abnormal receptors were present simultaneously. A heterozygous receptor defect might not result in glucocorticoid resistance if the normal receptor component is present in sufficient amounts to mediate glucocorticoid killing. Alternatively, the abnormal receptor could be present in a subpopulation of leukemia cells. A leukemia cell subpopulation that contains only abnormal receptors could be potentially important since...
the eventual emergence of this subpopulation could result in relapse with a glucocorticoid-resistant population of cells. The detection of such a subpopulation of leukemia cells at the time of diagnosis might predict poor responses to prednisone and a decreased prognosis.

Our findings also demonstrate that the mol wt of the abnormal receptors in human leukemia cells was the same as that of the abnormal receptors in the S49 143R and S49 55R mutant mouse lymphoma cell lines. In contrast to the human leukemia cells that contained both normal and abnormal receptors, the S49 143R and S49 55R cells contained only the abnormal receptor. This observation was made previously by other investigators who analyzed photoaffinity-labeling in these cell lines by gel electrophoresis and is explained by recent studies that indicate the S49 mouse lymphoma cells have only one functional gene for the glucocorticoid receptor. The S49 143R and S49 55R mutants both have the nuclear transfer-increased phenotype of glucocorticoid resistance. Because of the limited number of cells available, we were unable to determine whether the human leukemia cells that had the abnormal receptor had the same functional abnormality. The receptor defect in mouse lymphoma cell lines with the nuclear transfer-increased phenotype of glucocorticoid resistance has been well defined. Normal receptors can be separated into three functional regions by limited tryptic digestion: a steroid-binding region, a DNA-binding region, and a third region that has an unknown function but that binds antireceptor antibodies. Receptors in the nuclear transfer-increased mutants are missing the third receptor region, resulting in a smaller-than-normal receptor molecule. Recent studies suggest that the glucocorticoid receptor in intact S49 143R cells has a normal mol wt and that the lower mol wt form appears after cells are lysed to produce cytosol. Thus the receptors in these cells may be intact but have an increased susceptibility to proteolysis.

Our results are different from those of Foster et al, who used similar methods to investigate glucocorticoid receptors in malignant cells from two patients with acute lymphoblastic leukemia and three patients with non-Hodgkin's lymphoma. They observed multiple different mol wt forms of the glucocorticoid receptor in each of the samples tested instead of a single common electrophoretic abnormality. The reason for this difference is not readily apparent but could conceivably be due to differences in the patient populations studied or in the techniques used. In our study, malignant cells were separated from peripheral blood or bone marrow immediately after being obtained from patients and were assayed on the same day or after overnight incubation in culture medium at 4°C. In the study by Foster et al, cells were obtained at one institution and shipped to another institution at ambient temperature before cell separation and performance of assays. Also, gels were analyzed by different methods in the two studies. We analyzed gels by autoradiography, and the experiment shown in Fig 2 demonstrates that the technique was sensitive enough to detect lower mol wt forms of the receptor. Foster et al sliced their gels and counted a relatively low level of radioactivity after digesting the gel slices. We have also used this same method to analyze receptors in S49 mouse lymphoma cell lines and found it to be technically much more difficult and less reproducible than autoradiography.

Because patients in the present study were treated with multiple agents, it is not possible in each case to determine glucocorticoid responsiveness. Of the patients demonstrating the abnormal receptor form on gel electrophoresis, most were not treated with glucocorticoids (Table 1). Patient 2 entered complete remission after treatment with vincristine and prednisone. Patient 5 was unresponsive to the combination of vincristine and prednisone. A larger study with long-term follow-up will be required in order to determine the actual incidence of abnormal glucocorticoid receptors in leukemia and to test potential correlations with glucocorticoid responsiveness and overall prognosis. Studies using genetic and immunologic probes will be needed in order to define the molecular defect responsible for the electrophoretic abnormality described here.

Electrophoretically normal receptors are not necessarily functionally normal. This is shown in Fig 2, which shows that receptors from the S49 22R mouse lymphoma cell line, which has the nuclear transfer-decreased phenotype of glucocorticoid resistance, are electrophoretically normal. Thus the technique used in the present study will detect only those receptor defects that are associated with a change in the mol wt of the receptor molecule and may underestimate the actual incidence of glucocorticoid receptor defects in human leukemia cells.

ACKNOWLEDGMENT

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A single common electrophoretic abnormality of glucocorticoid receptors in human leukemia cells

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