Glucocorticoid Receptors in Immunological Subtypes of Childhood Acute Lymphocytic Leukemia Cells: A Pediatric Oncology Group Study

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ABSTRACT

Glucocorticoid receptors were quantitated by a whole cell method in cells from 593 children with acute leukemia at the time of diagnosis. Leukemia cells were also immunologically typed and divided into early pre-B- (not reactive with antibodies to T-lymphocyte antigens, surface immunoglobulin-negative, cytoplasmic immunoglobulin-negative), pre-B- (not reactive with antibodies to T-lymphocyte antigens, surface immunoglobulin-negative, cytoplasmic immunoglobulin-positive), B- (not reactive with antibodies to T-lymphocyte antigens, surface immunoglobulin-positive), and T- (reactive with antibodies to T-lymphocyte antigens) subtypes. There was a median of 9.7 x 10^3 sites per cell in the 359 with early pre-B-acute lymphocytic leukemia, a median of 8.1 x 10^3 sites per cell from 103 patients with pre-B- cell leukemia, and a median of 4.0 x 10^3 sites per cell from 116 patients with T-cell leukemia. The distributions per cell were significantly different among these 3 groups (P < 0.0001). The 15 patients with B-cell disease had a median of 3.2 x 10^3 sites per cell.

At the time of analysis, remission induction data were available for most of these patients. Within the early pre-B- group 291 patients with a median receptor number of 9.9 x 10^3 achieved remission, while 13 with a median receptor number of 4.8 x 10^3 did not. These distributions were significantly different (P = 0.034). Within the pre-B- and T-cell groups the distributions of receptor numbers for responders and non-responders were not significantly different. We conclude that each immunological subtype has characteristic receptor distribution. High receptor number within the null group is associated with the ability of the patient to achieve remission; however, the range of values within each patient group is too broad to use this assay as a predictor of response for any individual patient.

INTRODUCTION

In studies of GRs in patients with acute lymphocytic leukemia several questions have been raised. First, is there a characteristic receptor number associated with a specific immunological subtype of the leukemia cell (1-4)? Second, is there a correlation between receptor number and the patient’s ability to achieve a remission with steroids or a steroid-containing combination (5)? Third, does the receptor number operate as an independent variable to predict overall response to combination chemotherapy (5, 6)? In 1981, the Pediatric Oncology Group decided to measure glucocorticoid receptor number in blasts from all patients being entered on the front line acute lymphocytic leukemia protocols. The following report represents the initial description of the results of this study in 593 children in whom satisfactory immunological typing and glucocorticoid receptor quantitation were performed. The first two issues will be addressed here, but the third will require longer follow-up and will be reported later.

MATERIALS AND METHODS

Materials. Preservative-free heparin was obtained from Upjohn (Washington, DC); Hypaque was from Winthrop Laboratories (New York, NY); Ficoll-type 400 was from Sigma Chemical Company (St. Louis, MO); the penicillin-streptomycin mixture was from Microbiological Associates (Walkersville, MD); fetal calf serum was from Grand Island Biological Co. (Grand Island, NY); 1,2-3H]-dexamethasone (specific activity, 20 Ci/mmol) was from Amersham (Arlington Heights, IL); and unlabeled dexamethasone was from Steraloids (Wilton, NH).

Patient Population and Immunological Subtyping. All patients were under 22 years of age. The diagnosis of acute lymphocytic leukemia was established by morphology and cell staining characteristics. Only those leukemia samples that contained an adequate number of viable blasts (>5 x 10^7) were studied. Immunological subtyping was performed as reported previously (6) and included serological reactivity with anti-T-cell sera present; B-cell leukemia if surface immunoglobulin is present; pre-B- if cytoplasmic immunoglobulin is present; and early pre-B- if these markers are lacking. This designation of the category formerly called “null” is given because these cells are known to show immune-associated antigen and immunoglobulin rearrangement (10).

Patients were considered to be non-responders if they had failed to achieve complete remission (<5% lymphoblasts in a normocellular marrow) after 6 weeks of vincristine and prednisone treatment in patients with early pre-B- or pre-B leukemia or after 6 weeks of vincristine, prednisone, daunorubicin, and cytoxan in patients with T-cell leukemia.

Isolation of Human Leukemia Cells. Prior to initiation of chemotherapy, 3-5 ml of bone marrow were aspirated into a heparinized syringe from individual pediatric patients and added to a 50-ml tube containing 40 ml RPMI 1640 supplemented with 20% fetal calf serum, penicillin G (50 units/ml), and streptomycin (500 µg/ml). Samples were shipped via express mail to the reference laboratory at Johns Hopkins Hospital at ambient temperature from participating member institutions of the Pediatric Oncology Group.

Lymphoblasts were separated from bone marrow by the Ficoll-Hypaque method of Boyum (11), washed three times in room-temperature express mail to the reference laboratory at Johns Hopkins Hospital at ambient temperature from participating member institutions of the Pediatric Oncology Group.

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The abbreviations used are: GR, glucocorticoid receptor; ALL, acute lymphocytic leukemia.
Buffer A, and resuspended in the same at a concentration of $2 \times 10^7$/ml. All of the bone marrow specimens contained >90% abnormal blasts. Differential counts after separation usually revealed <5% erythrocyte contamination. Specimens were studied if cell numbers were adequate and if greater than 90% of the separated lymphoblasts excluded trypan blue.

Normal Human Peripheral Blood Mononuclear Cells. For use as control cell samples, lymphocyte-enriched mononuclear cell fractions were obtained from normal donors during plateletpheresis by standard techniques (12). Mononuclear cells were further purified by the Ficoll-Hypaque method, washed 3 times with room-temperature RPMI 1640, and resuspended in the same at a concentration of $2 \times 10^7$/ml. Differential counts revealed >90% lymphocytes on Wright’s stain and <5% erythrocytes. Greater than 95% of the cells excluded trypan blue. These cells were stored in the laboratory at ambient temperature in the transport medium used for leukemia samples for varying periods of time during the experiments to study the effects of storage.

Quantitation of Glucocorticoid Binding. Whole cell glucocorticoid receptors were quantitated as described previously with minor changes (4). Briefly, triplicate or duplicate aliquots of cells were incubated for 180 min in RPMI 1640 at 21°C with 5 concentrations of [3H]dexamethasone (from 0.25 to 30 nM). Nonsaturable binding was measured by incubating cells with 100-fold excess unlabeled dexamethasone in the presence of 10 and 30 nM [3H]dexamethasone. At the completion of incubation, cell samples for determination of whole cell binding were diluted 30-fold with ice-cold RPMI 1640, sedimented for 10 min at 400 x g in a refrigerated centrifuge, and washed 3 times for 10 min with ice-cold RPMI 1640.

When the number of cells from an individual sample was limited, measurement was made at a single saturating concentration (30 nM) of [3H]dexamethasone in the absence and presence of 100-fold excess unlabeled dexamethasone. Over this range of cell number, the number of receptors detected per cell was therefore constant. All further experiments were performed with cell numbers within this range.

After the final sedimentation, washed cells were extracted with 1 ml absolute ethanol at 21°C for a minimum of 30 min. The supernatants were diluted in 10 ml Aquasol (New England Nuclear) for scintillation counting at an efficiency of 35%.

Data Presentation and Statistical Methods. For each analysis the amount of specific binding was calculated by subtracting the amount of non-saturable binding from the total amount bound. The criterion for the determination of receptor number per cell, aliquots containing 1-10 x 10^6 cells were incubated in triplicate at 22°C for 180 min with 30 nM [3H]dexamethasone. At the completion of incubation, cell samples for determination of whole cell binding were diluted 30-fold with ice-cold RPMI 1640, sedimented for 10 min at 400 x g in a refrigerated centrifuge, and washed 3 times for 10 min with ice-cold RPMI 1640. When the number of cells from an individual sample was limited, measurement was made at a single saturating concentration (30 nM) of [3H]dexamethasone in the absence and presence of 100-fold excess unlabeled dexamethasone.

RESULTS

Since cells are shipped by participating institutions from all over the country, it was important to assess the reproducibility of the assay with time. For this purpose, cells from single normal donors were stored in sterile tissue culture medium supplemented with fetal calf serum and antibiotics at room temperature for varying periods of time, prior to being assayed for whole cell GR. Data from cells of several donors are shown in Table 1. Over several days, control cells kept under these conditions showed stable levels of whole cell receptor number.

A few leukemic samples contained enough cells to permit duplicate assays on two consecutive days. Data on stability of cells from seven patients received at Johns Hopkins University from participating institutions is shown in Table 2.

Normal donor lymphocytes were used as controls each day to assess reproducibility of experimental conditions. In 73 donor specimens the mean GR receptor number was $4.8 \pm 1.5$ (SD) ($\times 10^3$) with a median of $5.0 \times 10^3$, with a normal distribution as shown in Chart 1.

Patient samples had varying numbers of cells available for assay. To verify that the number of cells assayed did not affect the determination of receptor number per cell, aliquots containing 1-10 x 10^6 cells were incubated in triplicate at 22°C for 180 min with 30 nM [3H]dexamethasone in the absence and presence of unlabeled dexamethasone. Over this range of cell number, the specific binding was directly proportional to the number of cells, and the number of receptors detected per cell was therefore constant. All further experiments were performed with cell numbers within this range.

Since the incubation period used was longer than that used by some other investigators, time course experiments were conducted with both normal donor lymphocytes and patient
lymphoblasts at 22°C for 180 min at a single saturating concentration of [3H]dexamethasone (30 mw) as described in "Materials and Methods." Specific binding was almost maximal at 60 min, and it was constant from 90–180 min. All further experiments were therefore performed using a binding time of 180 min.

As mentioned in "Materials and Methods," cells were incubated at 5 concentrations of [3H]dexamethasone, and binding isotherms were obtained whenever an adequate number of cells were available. Chart 2 shows binding plots and Scatchard analysis of the data obtained when assaying blast cells from a leukemia patient (14). Ks obtained from these experiments in 14 donor lymphocytes from Scatchard analysis of binding data was 7.6 ± 4 x 10^{-9} M. In experiments with cells from 112 different leukemic patients, the Ks was 3.6 ± 2.5 x 10^{-9} M, signifying no noteworthy difference in the affinity of the GR for its ligand in leukemic blasts versus normal peripheral blood mononuclear cells.

Overall, 87% of patients registered on study for treatment had cells sent to Johns Hopkins University, and 69% of those had sufficient viable blast cells to permit satisfactory analysis.

Immunological Subtypes. When the patient samples were divided according to immunological cell subtype (Chart 3), the whole cell receptor number for patients with early pre-B-ALL (i.e., non-T-, non-B-, and non-pre-B) showed a mean of 11.2 ± 8.5 sites/cell for 359 patients. This was significantly different from the distribution of receptor number in 103 patients with pre-B-lymphoblasts [mean, 9.5 ± 6.9 x 10^3 GR sites/cell (P < 0.02)]. T-lymphoblasts from 116 patient samples had a mean GR number of 5.4 ± 4.4 x 10^3, and the distribution of receptor number was significantly different statistically from that of the other cell types (P < 0.0001). There are too few patients with B-cell disease for statistical comparison; however, they had the lowest number of GRs (mean, 5.0 ± 5.5 x 10^3).

The range of GRs in each cell type was broad. There was no significant correlation of GR number with race or age of patient, WBC count, hemoglobin or platelet count at diagnosis, blast cell morphology (French-American-British), or extent of extramedullary disease at the time of initial presentation within each immunological subtype of ALL. In T-cell ALL there was a significant difference in distribution of receptor numbers between the sexes. There were 64 males with a median of 4.5 x 10^3 and 38 females with a median of 3.7 x 10^3 GR sites/cell (P = 0.05). Within the patient group with T-cell ALL, 62 patients had a mediastinal mass, while 54 did not. The median GR number was not significantly different in cells from both subgroups (4.0 x 10^3 versus 4.5 x 10^3 GR sites/cell respectively). In the patients with pre-B- and early pre-B-ALL, only 4 had a mediastinal mass. Similarly the presence or absence of immune-associated antigen or common acute leukemia antigen in all types of ALL was unrelated to the GR number of the cells.

Remission Induction. Patients with early pre-B- and pre-B-ALL were treated with a combination of vincristine and prednisone, and remission was achieved after 4–6 weeks of therapy. Five children who sustained early death were excluded from this analysis. There were 304 patients with early pre-B-ALL available for analysis of response. The distribution of receptor number in cells from patients who fail to achieve remission had a significantly lower mean (P = 0.034) than that from patients who did achieve remission (Table 3). However, in the B-subgroup there was no significant difference between the GR number for the patients who did and did not achieve remission. The patients with T-cell disease received therapy with vincristine, prednisone, daunomycin (60 mg/m^2 × 2), and cytoxan (1200 mg/m^2). In these patients there was also no significant difference in the receptor number between those patients who did and did not achieve remission. The patients with B-cell disease were such a small number that response analysis was not performed.

In looking at clinical characteristics of the patients in whom GR analysis was and was not technically satisfactorily performed, there was no difference in the distribution of immune phenotypes or in the ability of the patient groups to achieve remission. There was a statistically significant association between white cell count at presentation and successful GR analysis, with those patients with higher counts being more likely to have the analysis successfully performed.
GR IN IMMUNOLOGICAL SUBTYPES OF CHILDHOOD ALL CELLS

<table>
<thead>
<tr>
<th>GR sites/cell x10^3</th>
<th>Cell type</th>
<th>n</th>
<th>Mean</th>
<th>Median</th>
<th>P</th>
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<tr>
<td></td>
<td>Early pre-B (clg-^-)</td>
<td>291</td>
<td>11.4 ± 8.8</td>
<td>9.9 (0.8-25)</td>
<td>0.034</td>
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<tr>
<td></td>
<td>CR</td>
<td>13</td>
<td>7.4 ± 5.9</td>
<td>4.8 (2.5-21)</td>
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<tr>
<td></td>
<td>NR</td>
<td>73</td>
<td>6.1 ± 5.2</td>
<td>3.6 (2.5-21)</td>
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<tr>
<td></td>
<td>Pre-B (clg+)</td>
<td>58</td>
<td>9.1 ± 6.4</td>
<td>7.4 (0.8-27.8)</td>
<td>0.71</td>
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<tr>
<td></td>
<td>CR</td>
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<td>14.7 ± 16.2</td>
<td>7.8 (1.8-38.3)</td>
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<td></td>
<td>NR</td>
<td>27</td>
<td>4.7 ± 3.6</td>
<td>4.7 (2.5-21)</td>
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<tr>
<td></td>
<td>T-cell</td>
<td>83</td>
<td>5.5 ± 4.2</td>
<td>4.3 (3.2-20)</td>
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<td>NR</td>
<td>75</td>
<td>4.9 ± 2.5</td>
<td>3.7 (2.5-21)</td>
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</tr>
</tbody>
</table>

a clg-, cytoplasmic immunoglobulin-negative; CR, complete remission; NR, no remission; clg+, cytoplasmic immunoglobulin-positive.

b Mean ± SD.
c Two patients with early death were excluded from response data.
d One patient with early death was excluded from response data.

DISCUSSION

The following conclusions can be drawn from these observations. First, each of the immunological subtypes of ALL blasts has a significantly different distribution of GR number. Early pre-B-ALL has the highest number, with pre-B- significantly lower. This is consistent with the initial observations from our laboratory (4) and in contrast to the preliminary study of Vogler et al. (3) from the Pediatric Oncology Group; however, we feel that the large number of patients studied in the present report gives it greater weight. It is of interest that an occasional pre-B- patient may have very high values. On the other hand, the patients with B-cell leukemia have extremely low values for GR receptors, which is consistent with previous reports and in accord with the general poor response of these patients to steroid therapy. Patients with T-cell leukemia have a significantly lower value than those with common or pre-B- leukemia, and this number is similar to that seen with normal mature lymphocytes in the peripheral blood. Thus, it would seem that the GR content of an ALL blast is related to the immunological subtype. The range found within each group, however, precludes individual prediction of the immunophenotype.

The treatment regimen for remission induction was identical for the early pre-B- and pre-B- patients and consisted of vincristine and prednisone. Overall, if the entire group with non-B-, non-T- leukemia is combined, the GR number in lymphoblasts from those who achieved remission was significantly lower than for those who failed to do so (Table 3). However, this is not true for the pre-B- group alone.

Other investigators have shown a correlation between receptor number in the cell and response to glucocorticoid alone in short term treatment of children with ALL (15, 16). In other small series of patients with ALL treated with glucocorticoid alone, the median number of receptors was always lower in the group which failed to respond; however the differences either were not significant or were not analyzed for significance (17-19). The largest total number of patients in these series was 11.

In addition, several papers have reported a response to combinations which are heavily dependent on steroids for their effectiveness. In a previous paper Lippman et al. (6) saw a correlation between receptor number and response to vincristine-prednisone-containing combinations. This study included a number of patients who were treated at the time of relapse.

Costlow et al. (1) studied 174 patients who were given vincristine, prednisone, and daunorubicin and found that a lower receptor number was associated with induction failure (P = 0.014), and Iacobelli et al. (20) studied 71 evaluable patients treated with a four-drug combination consisting of vincristine, methotrexate, mercaptopurine, and prednisone and also found a significantly lower GR number in patients failing to achieve complete remission. However, in a further group of patients studied by Pui et al. (21) at St. Jude Children's Hospital whose induction included 4'-dimethyl-epipodophyllotoxin 9-(4,6-O-2-thenyldene β-glucopyranoside) and 1-β-arabinofuranosylcytosine which preceded steroid therapy, the correlation between receptor number and achieving induction no longer remained. Thus, it appears from previous reports that in steroid-responsive tumors there is an association between receptor number and the ability of the patients to achieve remission as long as the regimen is heavily dependent on steroids. As regimens become more complex with additional drugs, it seems that the correlation of steroid receptor number with response to therapy disappears.

It also appears from the current study that this correlation only holds true for cells from patients with early pre-B- (cytoplasmic immunoglobulin-negative) ALL, since it was not seen in the pre-B- or the T-cell group of patients. Inconsistencies between results in previous studies may have been the result of varying proportions of patients within the different immunological phenotypes.

Although the correlations for the group are statistically significant, within any group of patients the range of receptor numbers is so broad that it is impossible to use the test as an individual assay for response. It is obvious that further work on the qualitative nature of the receptor and on events beyond the receptor level are required to understand the factors which lead to a response to steroids in these tumors. Bell et al. (22), for example, have noted that some steroid-resistant animal tumors have an apparently abnormal glucocorticoid receptor complex which can be distinguished on DEAE chromatograms. It is clear that not only quantitative but qualitative analysis of glucocorticoid receptors will be important in future studies if we wish to use in vitro tests to predict individual responses.

The important question of whether glucocorticoid receptor number also correlates with remission duration remains to be assessed. In our original study (6) we found such a correlation, and this finding was confirmed in the paper of Costlow et al. (1) from the first group of patients treated at St. Jude Children's Hospital. It seems logical to suppose that here again the complexity of the drug regimen used may be inversely correlated with the importance of glucocorticoid receptor number as a predictor. The data in the current protocol are too premature to analyze for this question, but the answer will be of great interest, since the maintenance therapy includes steroid pulses.

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