Glucocorticoid Receptor Determinations in Leukemia Patients Using Cytosol and Whole-Cell Assays

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ABSTRACT

Parallel determinations of glucocorticoid receptors in the cells of patients with various forms of leukemia were made by two assay methods, one using cell-free cytosolic extracts and the other using whole-cell preparations. Both assays revealed saturable binding of triamcinolone acetonide in all cases. The mean equilibrium dissociation constant for the interaction of triamcinolone acetonide with the cytoplasmic receptor at 2°C was 9.45 ± 6.33 (S.D.) nM while that for the whole-cell binding at 37°C was 6.13 ± 3.25 nM, suggesting an increase in receptor affinity at physiological temperatures. Competition experiments with various unlabeled steroids revealed a higher degree of glucocorticoid specificity at 37°C in whole-cell suspensions than at 2°C in cytosol. In a comparative analysis of 41 leukemic cell specimens, it was found that determinations carried out by whole-cell assay, calculated as number of sites per cell, correlated well with those performed by cytosol assay, calculated as fmol/mg protein, independent of the type of leukemia. However, for cells with low receptor content, the two assay methods were more difficult to compare. In agreement with previous reports, the cytosol assay consistently underestimated the number of receptors with respect to the whole-cell assay, particularly in cases of lymphatic leukemia. Furthermore, the underestimation decreased for increasing levels of total cellular receptor. These results suggest that, in addition to possible defects in the cytoplasm-to-nucleus translocation process, the acceptor-binding capacity of the nucleus may also represent one of the factors which determines the levels of assayable cytoplasmic receptors. Moreover, they indicate that the two assay methods furnish nonequivalent information.

INTRODUCTION

The possibility of using steroid receptor analysis to predict the response of a potentially hormone-dependent tumor to endocrine therapy has attracted considerable interest, especially in the area of breast cancer (20). On the basis of encouraging results obtained with this disease, many laboratories have attempted analogous studies on GR's in human leukemia cells in the hope of finding a correlation with the clinical response to glucocorticoids. So far, however, these studies have produced conflicting results. In early reports, Lippman et al. (13, 14) demonstrated that cells of 22 ALL patients with favorable prognoses contained high GR levels while the cells of 6 other patients in relapse who were refractory to treatment contained barely detectable GR levels. In a subsequent study, Lippman et al. (15) reported that the cells of AML patients had appreciable GR levels in only 3 of 16 cases. In independent studies, Gailani et al. (4) found that the cells of 4 of 4 lymphosarcoma patients, 3 of 3 ALL patients, 2 of 6 AML patients, 0 of 8 CLL patients, and 0 of 2 CML patients contained appreciable GR levels. Furthermore, Terenius et al. (28) reported that the lymphocytes of only 17 of 27 patients with CLL contained significant levels of GR's, while Homo et al. (8) were able to demonstrate the presence of specific receptors in all of the patients with CLL and ALL as well as in normal lymphocytes. Finally, more recent reports have shown the presence of GR's in virtually all patients with various forms of hematological cancer (1, 2, 11, 19).

Some of these discrepancies could be related to the different methods used to measure the receptors. Many of the studies cited above utilized either a whole-cell or a cytosol assay indifferently. Although cytosol preparations have been used to quantify a variety of steroid receptors, including those for glucocorticoids, several reports claim that this method is unreliable for the measurement of GR content in leukemia (1, 2, 7) since both normal and neoplastic lymphocytes contain a very thin rim of cytoplasm and one must be aware of the efficiency of cell breakage during homogenization. Besides, unbound GR's may be preferentially localized in the nuclei of lymphocytes (23); therefore, cytosolic assay may result in underestimation of cellular receptor content. By using a whole-cell procedure, some of these problems can be overcome and appropriate measurement of total (cytoplasmic plus nuclear) receptors can be obtained. However, a major technical problem associated with this assay technique is the difficulty of distinguishing the proportion of nonspecifically bound steroid, often quite elevated, from free steroid after cell washes. It appears obvious that a thorough comparison of GR measurement using both the whole-cell assay and the cytosol assay could be useful. This paper presents the results of such a comparison performed on the cells from a group of patients with various types of leukemia.

MATERIALS AND METHODS

Patients

The study was done on 41 patients with various forms of leukemia (14 patients with CLL, 15 patients with ALL, and 12 patients with ANLL). Diagnoses were established by means of conventional morphological and cytochemical criteria. None of the ALL and ANLL patients...
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had received antileukemic therapy of any kind at the time of study. None of the CLL patients under treatment had received any glucocorticoid therapy within a week of their inclusion in the study.

Isolation of Leukocytes

Heparinized peripheral blood was diluted 2 to 3 times with 0.9% NaCl solution. Dextran T500 (Pharmacia, Uppsala, Sweden) was added to a final concentration of 1% (w/v). After gravity sedimentation for 30 min at room temperature, the supernatant containing leukocytes was centrifuged at 400 x g for 6 min. Hypotonic lysis of contaminating erythrocytes was achieved by treating the cells with 0.84% ammonium chloride for 15 min. After 2 washes with phosphate-buffered saline (0.136 M NaCl: 2.6 mM KCl: 6.4 mM Na2HPO4: 1.4 mM KH2PO4), the cell pellets were resuspended in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer (Sigma Chemical Co., St. Louis, Mo.), penicillin (100 units/ml), and streptomycin (100 µg/ml). Viability was always greater than 90%, as determined by the trypan blue exclusion procedure (24). A portion of the cell suspension was used immediately for GR determination by whole-cell assay while the remaining part was centrifuged again and the cell pellet stored frozen at -85°C for subsequent analysis of GR content by cytosol assay. Under these storage conditions, no loss of receptor activity was noted for up to 3 months.

Binding Studies

Whole-Cell Assay. Duplicate 0.1-ml aliquots of the leukocyte suspensions (10 to 20 x 10^6/ml) were pipetted into 10 x 85 plastic tubes which already contained 0.15 ml of Roswell Park Memorial Institute Tissue Culture Medium 1640 with [3H]TA (25 CI/ml; the Radiochemical Centre, Amersham, England) at 10 different concentrations varying from 0.8 to 25 nM, with or without a 100-fold excess of radioinert TA. Tubes were incubated under 5% CO2 in air at 37°C for 60 min except for experiments examining the time course of binding. After incubation, 1.5 ml of phosphate-buffered saline were added to each tube; the tubes were kept in ice for 5 min and then centrifuged at 200 x g for 6 min. The washing process was repeated twice. The extent of non-specific bound counts was reduced to a minimum, and less than 7% of specifically bound counts were lost in this washing procedure. Cell numbers recovered at the end of the washing procedure, 4 x 10^6, were measured in all the patients studied. Furthermore, in some patients with a high peripheral white blood count, receptor determinations were made by using dextran-coated charcoal and filter assays simultaneously. The 2 procedures gave comparable results (data not shown).

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RESULTS

GR Assays. Since the amount of cells recovered from most of the patients was not sufficient to furnish high enough cytosol protein concentrations to make a complete binding curve, we decided to use the DE 81 ion-exchange filter assay which accurately measures GR levels at very low protein concentrations (25). Using this procedure, appreciable levels of receptor were measured in all the patients studied. Furthermore, in some patients with a high peripheral white blood count, receptor determinations were made by using dextran-coated charcoal and filter assays simultaneously. The 2 procedures gave comparable results (data not shown).

Chart 1 illustrates the time course of [3H]TA binding to receptor in whole cells as well as in cytosol preparations. In the cells, the highest level of specific binding is achieved between 20 and 30 min of incubation at 37°C and remains stable for at least 60 min thereafter. At 2°C, specific steroid binding in cytosol reaches a maximum after 120 min of incubation and remains constant between 120 and 240 min.

The affinity of various steroids for GR sites in the cells of some patients was evaluated by both assay methods. A representative experiment carried out in one case of CLL is illustrated in Table 1 where the results are expressed as relative binding affinity. In both assays, the most efficient competitor was TA itself (relative binding affinity defined as 100) followed by dexamethasone, prednisolone, and cortisol. Corticosterone and aldosterone displayed an intermediate level of competition which was not dissimilar in both intact cells and cytosol preparations. 17ß-Estradiol, testosterone, and biologically inactive glucocorticoids such as 21-deoxycorticosterone and prednisolone did not compete. However, there are definite differences in the

![Chart 1. Time course of specific [3H]TA binding to GR in intact cells (a) or in cytosol (b) in one case of ALL. Aliquots of cell suspensions (0.1 ml; 18 x 10^6 cells/ml) or cytosol (0.06 ml) were incubated at 37°C and 2°C, respectively, for the times indicated with 20 nM [3H]TA alone (total binding) or in the presence of 2 µM radioinert TA (nonspecific binding). Specific binding was calculated as the difference between total binding and nonspecific binding. The maximum binding attainable during the time of the kinetic experiment was taken as 100% and the other values were expressed as a percentage thereof. The maximum binding for whole cells and cytosol was 4200 sites/cell and 318 fmol/mg protein, respectively.](chart1.png)
The results of GR determinations by cytosol assay using whole-cell assay are given in Chart 3. The values of receptors are: mean, 570 ± 540 fmol/mg protein; median, 325 fmol/mg protein \((N = 14)\) in CLL; mean, 1492 ± 1521 fmol/mg protein; median, 706 fmol/mg protein \((N = 15)\) in ALL; mean, 1239 ± 1156 fmol/mg protein; median, 720 fmol/mg protein \((N = 12)\) in ANLL. The \(K_d\) values are: mean, 12.8 ± 8.2 nM; median, 9.18 nM \((N = 14)\) in CLL; mean, 8.34 ± 4.65 nM; median, 7.2 nM \((N = 15)\) in ALL; mean, 9.1 ± 7.44 nM; median, 7.3 nM \((N = 12)\) in ANLL. The values of receptor sites are significantly different among the various types of leukemia \((P < 0.05, \text{Kruskal-Wallis test})\). Analogous to previous findings (18), the \(K_d\) value measured by cytosol assay in all types of leukemia \(9.45 ± 6.33 \text{ nM}\) is significantly higher \((p < 0.01, \text{Student’s t test})\) compared to that found in intact cells \(6.13 ± 3.25 \text{ nM}\).

The results of GR determinations by cytosol assay using cytosol obtained from the same leukocyte specimens used for whole-cell assay are given in Chart 3. The values of receptors are: mean, 570 ± 540 fmol/mg protein; median, 325 fmol/mg protein \((N = 14)\) in CLL; mean, 1492 ± 1521 fmol/mg protein; median, 706 fmol/mg protein \((N = 15)\) in ALL; mean, 1239 ± 1156 fmol/mg protein; median, 720 fmol/mg protein \((N = 12)\) in ANLL. The \(K_d\) values are: mean, 12.8 ± 8.2 nM; median, 9.18 nM \((N = 14)\) in CLL; mean, 8.34 ± 4.65 nM; median, 7.2 nM \((N = 15)\) in ALL; mean, 9.1 ± 7.44 nM; median, 7.3 nM \((N = 12)\) in ANLL. The values of receptor sites are significantly different among the various types of leukemia \((P < 0.05, \text{Kruskal-Wallis test})\). Analogous to previous findings (18), the \(K_d\) value measured by cytosol assay in all types of leukemia \(9.45 ± 6.33 \text{ nM}\) is significantly higher \((p < 0.01, \text{Student’s t test})\) compared to that found in intact cells \(6.13 ± 3.25 \text{ nM}\).

Comparison of GR Measurement: Whole-Cell versus Cytosol Assay. In Chart 4, the numbers of GR sites per cell measured by whole-cell assay are plotted against the amounts of fmol per mg protein determined by cytosol assay. There appears to be a good linear correlation between the 2 series of measurements \(r = 0.91\) independent of the type of leukemia \(\text{CLL}, r = 0.78; \text{ALL}, r = 0.92; \text{ANLL}, r = 0.90\). However, for low binding site concentrations (less than 10,000 sites/cell or 1000 fmol/mg protein), the points are less aligned along the regression line.

In some of the previous reports on the levels of GR sites in leukemic cells, the binding studies were done on cytosol preparations at 0-4°C (4, 13). It has been shown that this procedure leads to an underestimation of the total number of binding sites present in the cell. To verify this, the number of GR sites per cell determined by whole-cell assay has been compared with the number of sites per cell obtained by cytosol assay. This latter parameter was calculated by dividing the fmol bound/mg of cytosol protein by the number of cells per mg protein \([\text{based on cell count and protein determination by the method of Lowry et al. (17)}]\) and multiplying by Avogadro’s number. The results of this comparison indicate that the relationship between these 2 estimates is not linear and may be well represented by a parabolic equation (Chart 5). Specifically, in cells with low GR content (less than 10,000 sites/cell), the amount of receptor measured by the cytosol assay does not vary greatly as a function of the variations in the number of sites per cell, whereas it increases rapidly in cells with higher receptor levels. Furthermore, it is interesting to note that, even though all 3 types of leukemia follow the same behavior, ANLL is predominantly distributed above the theoretical equation curve with respect to the others. This behavior pattern is further illustrated
Chart 4. Correlation between GR levels measured by whole-cell assay and cytosol assay in leukocytes from patients with CLL (●), ALL (▲), and ANLL (○). Receptor determinations were carried out as described in “Materials and Methods.”

Chart 5. Correlation between the number of GR sites measured by whole-cell assay and cytosol assay in leukocytes from patients with CLL (●), ALL (▲), and ANLL (○). The number of sites per cell relative to the cytosol assay were calculated from the binding data as described in “Results.” The best fit for the regression curve was represented by a parabolic equation \( y = a + bx + cx^2 \); \( r \), the correlation coefficient for this curve.

by the data in Table 2 which compares the means of values for receptor sites obtained by the 2 assay methods. The percentage of underestimation (cytosol assay/whole-cell assay) is significantly lower in ANLL than in CLL and ALL (\( p < 0.05 \) and 0.02, respectively).

Loss of Binding Capacity. For these experiments, the cytosol was prepared and maintained at 2°C, and at hourly intervals thereafter up to 6 hr, aliquots were incubated with 20 nM \(^{[3H]TA} \) alone (total binding) or in the presence of 2 μM radioinert TA (nonspecific binding) for 3 hr at 2°C. The loss of binding capacity referred to specific binding which was calculated as the difference between total binding and nonspecific binding. One-half of the maximum binding capacity was calculated as 50% of the initial (zero time) amount of specific binding.

Table 2

<table>
<thead>
<tr>
<th>Leukemia type</th>
<th>Whole cell</th>
<th>Cytosol</th>
<th>Underestimation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL (14)*</td>
<td>8,061 ± 4,491</td>
<td>1,551 ± 1,201</td>
<td>78.7 ± 18.4</td>
</tr>
<tr>
<td>ALL (15)</td>
<td>17,106 ± 13,737</td>
<td>5,434 ± 6,817</td>
<td>73.1 ± 14.4</td>
</tr>
<tr>
<td>ANLL (12)</td>
<td>14,954 ± 10,083</td>
<td>6,536 ± 6,822</td>
<td>57.8 ± 22.3</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of cases.
\( a \) Mean ± S.D.
\( c \) * Mean difference (Student’s t test): c versus e, \( p < 0.05 \); d versus e, \( p < 0.02 \); c versus d, not significant.

Table 3

<table>
<thead>
<tr>
<th>Loss of one-half of the maximum binding capacity</th>
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<tr>
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</tr>
<tr>
<td>CLL</td>
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<tr>
<td>ANLL</td>
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* Numbers in parentheses, percentage.

were arbitrarily established as less or more than 3 hr as shown in Table 3. A rapid loss (less than 3 hr) was observed in 87% (7 of 8) of CLL, 67% (4 of 6) of ALL, and 50% (3 of 6) of ANLL.

DISCUSSION

The results of this study demonstrate that the 2 GR assay methods furnish correlated values, independent of the type of leukemia studied. The correlation is less evident for cells with low receptor content in which the 2 series of measurements are more difficult to compare. Moreover, our data indicate that the cytosol assay consistently leads to an underestimation of the number of binding sites as compared to the values obtained by whole-cell assay. There may be several factors accounting for this.

First, the whole-cell assay procedure might ensure the determination of the total (cytoplasmic and nuclear) receptor content. It has long been reported that in rat thymocytes exposed to glucocorticoids at 37°C the majority (approximately 80%) of the receptor is found to be associated with the nucleus in the form of receptor-hormone complex (22). Similar observations have recently been made on human thymocytes (10). Interestingly enough, a similar percentage of receptor underestimation was seen in the cytosol of leukemic cells of lymphatic origin (Table 2). Thus, a simple explanation which could account for the low number of binding sites measured by the cytosol assay is that in intact cells at physiological temperatures the majority of receptor is localized in the nucleus. On the other hand, the higher number of binding sites detected in the cytoplasm of ANLL could reflect an altered distribution of receptor between the cytoplasmic and nuclear compartments possibly due to a variable rate of receptor translocation as reported elsewhere (1).
Second, possible breakage of lysosomes and release of proteolytic enzymes that partially inactivate the cytoplasmic receptor must also be considered. Our data show that the receptor-binding capacity in cytosol from certain types of leukemic cells, notably those of CLL, decreases rather rapidly, suggesting that substances capable of inactivating the receptor molecule (which could account for the generally lower binding capacity of these particular cells) are active in these cells. Although these substances (possibly enzymes) have not yet been identified, it is interesting that Sloman and Bell (27) have shown that the cytosol of CML contains receptor-inactivating factor(s) which could be activated during the homogenization process. The presence of similar factors in leukemic myeloblasts of AML has also been proposed by other authors (1).

Third, it is possible that some cells are particularly resistant to the breaking process during homogenization. Although this possibility has been postulated to explain the negative results obtained by the cytosol assay (1), it seems to be unlikely since, at least in our hands, complete cell breakage was observed in all cases.

The concentrations of GR sites in the different types of leukemic cells used in our study are analogous to those observed elsewhere (1, 10). The receptor content of these leukemic cells greatly exceeds that found in normal leukocytes of both lymphatic and myelomonocytic origin (12, 23). This capacity of the leukemic cell to express a greater quantity of receptor protein with respect to its normal counterpart seems to be a more general characteristic of the neoplastic phenotype. Thus, for example, the normal mammary cell does not seem to possess appreciable amounts of estrogen receptor while as a result of the neoplastic transformation process the quantity of receptor molecules can reach abnormally high levels (3). Although the mechanisms which regulate receptor levels in the cell are only now beginning to be clarified, it is possible to hypothesize that the redundancy of GR’s in leukemic cells, especially in ALL and ANLL as indicated in this study, represents a class of heterogeneous receptor molecules with functional defects such as wide ranges of steroid-binding affinity (this paper and Ref. 1) as well as variable capacities of cytoplasm—to-nucleus translocation and of interaction with acceptor sites in the chromatin (5). In the presence of this redundancy, the nuclear acceptor-binding capacity could become the limiting step in the process, independent of any translocation defect. Therefore, in cells with low receptor content (i.e., closer to normal cell values), the quantity of binding sites measured in the cytoplasm could represent a minimal part of the total cellular receptor sites which in the presence of endogenous hormone would be preferentially localized in the nucleus. This could explain the high frequency of negative results in GR determinations made by cytosol assay in leukemic cells with low receptor levels, particularly in CLL (4, 28). However, when the cellular receptor levels exceed the nuclear acceptor capacity, as in the case of cells with extremely high receptor content, high proportions of binding sites are found in the cytoplasmic compartment. This hypothesis could explain the nonlinear correlation between the quantity of receptor sites measured by the 2 assay methods.

It has been suggested that steroid receptors are not restricted to the cytoplasm alone and that in some cases they can be localized exclusively in the nucleus (9). As a consequence, the measurement of cytoplasmic binding sites alone may lead to falsely negative results if nuclear receptors are not considered as well. On the other hand, demonstration of total cellular receptor sites in whole-cell preparations does not give information about their localization, i.e., if in the cytoplasm and/or in the nucleus. Moreover, binding to receptor in intact cells may depend on such factors as the metabolic state of the cells (21) and the rate of steroid transport through the cell membrane.

While the conclusions drawn from this study may be used to partly explain the contrasting data on GR determinations in leukemic cells so far reported, at least in cases of low receptor content, they indicate that receptor assessments by whole-cell and cytosol assays provide nonequivalent but possibly complementary information. Interestingly, each of the only 2 studies correlating GR levels in leukemic cells with clinical aspects furnished entirely different prognostic indications and each was based on one of the 2 assay procedures herein discussed (13, 16). Therefore, it seems reasonable not to depend exclusively on only one of these procedures, at least until their precise prognostic significance is more fully clarified by further correlative clinical studies.

REFERENCES


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