The In Vitro Sensitivity of Leukemic and Normal Leukocytes to Hydrocortisone Induced Cytolysis

By Uri Galili, Miron Prokocimer, and Gabriel Izak*

A marked sensitivity of CLL lymphocytes to hydrocortisone in vitro was demonstrated in each of the 25 patients tested. The sensitivity was manifested by the eventual lysis of the affected cells. Malignant lymphocytes from 8 out of 14 ALL patients were found also to be in vitro sensitive, whereas CML cells, AML cells, normal BM cells, thymocytes, peripheral blood lymphocytes, and polymorphonuclear cells were resistant. Within a tested CLL lymphoid suspension it is proven that the hydrocortisone causes the specific lysis of the malignant cells leaving the normal lymphocytes undamaged. The cytolysis is not an immediate action, but is expressed within 7–8 hr of incubation. However, 30 min incubation with the hormone is sufficient for the cytolytic effect to occur 20 hr later. The possible mechanisms involved in the specific glucocorticoid induced cytolysis are discussed.

The widespread therapeutic application of glucocorticoids in hematologic malignancies is based on the observed ability of these hormones to destroy certain, poorly differentiated malignant hemopoietic cells in experimental animals and in man, leaving the normal hemopoietic cells with the aim to establish a correlation between the cytoplasmatic receptor level and the sensitivity of any malignant cell clone to glucocorticoids.5,7–8

As a result of these observations, much effort has been invested recently into the quantitative estimation of steroid receptors in malignant and normal hemopoietic cells with the aim to establish a correlation between the cytoplasmatic receptor level and the sensitivity of any malignant cell clone to glucocorticoids.5,7–8

In the present study the in vitro sensitivity of well defined malignant hemopoietic cells to the cytolytic effect of glucocorticoids is scored and compared to that of normal blood, bone marrow, and thymic cells. Normal peripheral blood and bone marrow mononuclear cells, polymorphonuclear leukocytes (PMN), normal thymocytes and blast cells from patients with acute myeloblastic leukemia (AML), and chronic myelocytic leukemia (CML) were found to be resistant. Cells from patients with chronic lymphocytic leukemia (CLL) were sensitive to the cytotoxic action of hydrocortisone succinate and cortisol while blast cells from patients with acute lymphoblastic leukemia (ALL) showed a variable sensitivity to the hormone.

MATERIALS AND METHODS

One hundred and eight subjects, 53 of them normal controls and 55 patients with various malignant hemato logic diseases, served as sources for the cell populations studied. Their age, sex distribution, diagnoses, and pertinent hematologic data are summarized in Table 1. Twenty-five patients with B-cell CLL, 9 patients with chronic myelogenous leukemia (CML), 14 with ALL, and 7 with AML were studied.

Cell Suspensions

Peripheral blood buffy coats and bone marrow cell suspensions were fractionated on 9% Ficoll-Hypaque (F/H) (supplied by Pharmacia/Uppsala) and the mononuclear cells were collected from the interphase, while the PMN leucocytes of the peripheral blood were harvested from the bottom layer. Thymocytes were obtained from thymic tissue of patients undergoing coronary bypass surgery. All cell suspensions were washed three times with physiologic saline, and were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO).

E-rosette Formation

Aliquots of 0.1 ml mononuclear cell suspensions (10⁶ cells/ml) were mixed with 0.1 ml of sheep red blood cells (1%) suspended in RPMI 1640; the mixture was centrifuged at 200 g for 5 min and the sediment was kept at room temperature (22–24°C), for 1 hr. Thereafter the proportion of rosetting cells was scored in a hemocytometer.9,10

Cell Sensitivity to Glucocorticoids

The method was described previously.16,17 Aliquots of 0.2 ml cells (10⁶ cells/ml) were incubated in flat bottom microwells (Cooke) for 20 hr at 37°C in a humidified CO₂-Air (5%-95%) atmosphere with varying concentrations of hydrocortisone succinate (Solu cortef, Uipohn Co., Belgium), or with cortisol (Ikapharm, Israel). Since a variable proportion of the cells incubated with the glucocorticoids was lysed within 20 hr, the amount of cells, irreversibly damaged was assessed by determining the concentration of the remaining viable cells (Trypan-blue exclusion test), in a hemocytometer by magnification of × 400. Their percentage (%) lysis was calculated according to the formula: (a – b)/a × 100, where “a” is the concentration of viable cells in wells containing medium without steroids, and “b” equals the concentration of viable cells in wells containing the drug. Simultaneously with pathologic sample, cells obtained from normal controls were tested. Further incubation of the cells indicated that all cells stained by Trypan-blue were completely lysed i.e., cell disintegration.

RESULTS

At a hydrocortisone concentration of 1 mg/ml medium the mononuclear cells isolated from normal bone marrow, peripheral blood, normal polymorpho-
Table 1. Data on Normal Control Subjects and Leukemic Patients Studied

<table>
<thead>
<tr>
<th>Number of Samples</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Hemoglobin g/dl</th>
<th>WBC $10^3$/mm$^3$</th>
<th>Platelet $10^5$/mm$^3$</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>21-69</td>
<td>45</td>
<td>Normal</td>
<td>13.9-16.6</td>
<td>5.5-10</td>
<td>180-280</td>
<td>Normal cell distribution in bone-marrow, peripheral blood, and thymus</td>
</tr>
<tr>
<td>25</td>
<td>43-80</td>
<td>17</td>
<td>CLL</td>
<td>6.8-16.6</td>
<td>14.2-150</td>
<td>36-240</td>
<td>85%-95% - B cells 5%-15% - T cells</td>
</tr>
<tr>
<td>14</td>
<td>4-79</td>
<td>8</td>
<td>ALL</td>
<td>5.7-8</td>
<td>15.7-500</td>
<td>8-36</td>
<td>Non-T, Non-B pts T-type - 2 pts</td>
</tr>
<tr>
<td>9</td>
<td>19-56</td>
<td>9</td>
<td>CML</td>
<td>6.1-10.2</td>
<td>28-270</td>
<td>10-584</td>
<td>CML Philadelphia positive - 1 pt Philadelphia negative - 2 pts Blastic crisis - 5 pts</td>
</tr>
<tr>
<td>7</td>
<td>8-63</td>
<td>2</td>
<td>AML</td>
<td>7.2-11.5</td>
<td>25-80</td>
<td>10-55</td>
<td>AML relapse. Identified by cytochemistry</td>
</tr>
</tbody>
</table>

nuclear (PMN) leukocytes and thymocytes were resistant to the cytotoxic action of the hormone, as less than 5% of the cells were lysed during the 20 hr of incubation (Fig. 1B). Augmenting the hydrocortisone succinate concentration above 1 mg/ml led to the indiscriminate destruction of cells from whatever source. Thus PBL, BM cells, and PMN incubated with 2 mg/ml hydrocortisone succinate were lysed upon 20 hr incubation period. In the cell suspensions isolated from patients with hematologic malignancies, the normal T-cell population never exceeded 15% of the cells as indicated by the E-rosette formation. The cytolytic effect of hydrocortisone succinate was most pronounced in each of the 25 cell suspensions obtained from CLL patients with the percent of cells killed ranging from 60% to 97% (Fig. 1A). A similar, though somewhat less striking cytolytic effect of the hormone was observed among mononuclear cells of 8 out of 14 patients suffering from ALL with the percent of cells killed ranging from 56 to 96 (Fig. 1A). The cells from patients with CML or AML were found to be resistant to the hormone.

The cytotoxic effect of increasing hydrocortisone concentrations on normal and malignant cells is shown in Fig. 2A. A gradual rise in the percent of the CLL cells killed was produced by increasing concentrations of hydrocortisone from 0.06 mg/ml up to 1.0 mg/ml medium, leaving the resistant cells intact. Cortisol also was found to induce in vitro cytolysis in CLL cells in pharmacologic concentration ($10^{-5}M$). However, no cytolysis of normal PBL, PMN, thymocytes, and bone marrow cells was detected even at a tenfold higher concentration of cortisol. (Fig. 2B). The physiologic

Fig. 1. Cytolytic effect of hydrocortisone succinate (1 mg/ml) on various malignant (A) and normal (B) lymphoid and myeloid cell populations. Each circle represents the result obtained from one patient.
level of cortisol (10^{-7} M) does not affect the viability of the CLL cells.

CLL lymphocytes exposed to hydrocortisone for periods varying from 2 to 180 min, followed by removal of the hormone by washing the exposed cell suspension 3 times, and continued incubation thereafter up to a total of 20 hr in the absence of the hormone revealed the following: A 2–5 min exposure of the CLL lymphocytes to hydrocortisone imparted a sufficient injury to the malignant cells to reduce their number by almost 50% by the end of the 20-hr incubation (Fig. 3). The exposure of these cells to hydrocortisone for 3 hr, produced a cell destruction comparable to that observed in CLL lymphocytes incubated with hydrocortisone for the whole of 20-hr incubation period. That the cytotoxic effect of the hormone was directed against the malignant cells in the suspension was borne out by the gradual rise in the proportion of E-rosette forming cells during the incubation (Fig. 3). The cytolytic effect of hydrocortisone is not an immediate one. Cell lysis was observed usually following 6–8 hr incubation with the drug (Fig. 4).

Paired mononuclear cell samples isolated from the bone marrow and peripheral blood of the same patients with CLL and ALL were tested simultaneously for their sensitivity to hydrocortisone. The mortality of the bone marrow derived cells far exceeded that of the cells isolated from the peripheral blood of the same patient (Fig. 5). However, when the sensitivity of CLL cells from the blood and the lymph node is compared in one patient studied, the blood malignant cells are more sensitive (Fig. 6).

**DISCUSSION**

A marked cytolytic effect of hydrocortisone was observed on Ficoll-Hypaque isolated mononuclear cells from all patients with CLL, and from some patients with ALL. Cells from normal bone marrow
Glucocorticoids have been shown to damage lymphoid cells by a number of pathways: including inhibition of DNA synthesis changing the membrane structure and cytolyis.\(^{10,14}\) It is not clear at present whether the differential in vitro glucocorticoid induced cytolyis reported in this study reflects differences in the level of cytoplasmatic receptors. Such a possibility was proposed by several investigators who demonstrated that the clinical effect obtained by glucocorticoid treatment correlate with the receptor level in the malignant cell.\(^{5,6}\) The second possibility that should be considered in light of reports that the level of cytoplasmatic receptors of CLL, ALL, CML, and normal PBL cells do not differ significantly,\(^{15}\) is that the nuclear binding of the hormone-receptor complex differ in sensitive and resistant cells.\(^{3}\) Both possibilities are under study at present.

Myeloid cells were found to be resistant to steroid toxicity, which was true, irrespective of whether the cells tested were benign or malignant poorly differentiated or mature. Marked susceptibility to hydrocortisone induced damage was exhibited by cells of lymphoid origin only. It seems safe to conclude, therefore, that the origin and type of the cell comprising a given cell population is of substantial importance in the determination of its vulnerability to steroids.

Our previous studies\(^{16,17}\) demonstrating the glucocorticoid induced cytolysis of in vivo and in vitro activated T lymphocytes, as compared to the resistance of resting lymphocytes, suggest that the sensitivity of the lymphocyte is a reflection of the differentiation stage. Recent findings in our laboratory indicate that unlike the cortical or medullary thymocytes, the human prothymocytes that are located in the extracortical area are highly sensitive in vitro to the glucocorticoid cytolytic effect (in preparation). This may support the assumption that the sensitivity is confined to distinct differentiation stages. It should be noted that in the few cases studied, the sensitivity of the malignant lymphocytes from the blood differ from that of the cells in the bone marrow or in the lymph node. It is not clear at present whether this reflects also difference in the differentiation stage of the malignant cells.

The experiments reported here, designed to investigate the kinetics of the glucocorticoid-induced cell injury yielded results in line with present concepts concerning the pathways of intracellular steroid action.\(^{3}\) According to these, the hormone having penetrated the cell membrane rapidly, forms a complex with its cytoplasmic binder. This complex finds its way through an unknown pathway into the nucleus, where it activates a DNA segment from which messenger RNA is transcribed subsequently. While occurrence of these steps has been sufficiently documented, their mechanism and their regulation have not been clarified. It has been assumed that the mRNA produced in the affected nuclei is translated by the protein synthesizing system of the cell, and the peptides or protein(s) that emerge, have a deleterious effect on the cell. It should be noted, however, that such suicidal products have not been isolated and until such a time, their existence remains doubtful.

In the present study, the short exposure (2–5 min) of the susceptible target cells to hydrocortisone lead to the death of over 50% of the cells within another 20 hr incubation period, the latter in the absence of steroids. This finding is in conformity with the rapid entrance of the hormone into the cells. The demonstration of cell destruction only after 7–8 hr exposure to hydrocortisone, is compatible with the period required for
the chain of events outlined above to take place. Inhibition of cytolysis by blocking RNA or protein synthesis may help to clarify the mechanism of killing. The mechanism of cytolysis is at present under study.

The high hydrocortisone concentration required to achieve cytolysis in vitro may make the possible implications of these observations doubtful. It should be noted, however, that the glucocorticoid preparation employed is coupled with sodium succinate, to render it water-soluble hence the pharmacologic action of an unknown proportion of the compound is neutralized. The active portion seems to be in the pharmacologic range since the cytolysis was demonstrated also with the pharmacological concentration of cortisol—$10^{-5} M$, whereas tenfold increase in the cortisol concentration had no cytolytic effect on the cells that in parallel tests were resistant to 1 mg/ml hydrocortisone succinate.

The specific lytic effect of hydrocortisone on CLL or ALL cells may serve as a proper model for studying the glucocorticoid induced lympholysis in man which is considered so far to be a glucocorticoid "resistant species." Correlation between the in vitro sensitivity and the therapeutic effect of these drugs may help to evaluate the clinical and in vivo relevance of the phenomenon reported in this study.

REFERENCES

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