Elevated Glucocorticoid Receptor Binding in Cultured Human Lymphoblasts following Hydroxyurea Treatment: Lack of Effect on Steroid Responsiveness

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ABSTRACT

While studying the effects of chemotherapy on glucocorticoid receptor (GR) binding levels in hematological malignancies, we observed a sizable increase in nuclear GR binding of [3H]dexamethasone in peripheral leukocytes from a chronic basophilic leukemia patient following treatment with hydroxyurea plus prednisone but not after prednisone alone. This apparent clinical effect of hydroxyurea led to an examination of hydroxyurea effects on GR binding and sensitivity in the glucocorticoid-sensitive human lymphoblast cell line GM4672A. GR binding levels in GM4672A cells were measured following a 3-day exposure to 50 μM hydroxyurea, a concentration chosen to have a minimal but measurable effect on cellular growth rates with little or no effect on cellular viability. Under these conditions, nuclear [3H]dexamethasone receptor binding measured by Scatchard analysis using a whole-cell assay was elevated 2.4-fold over control values (P < 0.05), while cytosolic residual receptor binding (measured at 37°C) remained unchanged. Thus, the total cellular content of measurable GR was increased, and this increase was totally accounted for by GR capable of nuclear binding. Hydroxyurea treatment of GM4672A cells had no effect on the affinity of nuclear or cytosolic GR for [3H]dexamethasone. The increase in measurable nuclear-bound receptors occurred in a time-dependent manner over a period of 3 days and was fully reversible within 3 days following removal of hydroxyurea. The increase in receptor binding could not be explained by the slight alterations in cell cycle kinetics which occur at this low level of hydroxyurea. Despite increased receptor binding, cellular glucocorticoid responsiveness was unaltered as assessed by dexamethasone inhibition of GM4672A cells. GR binding levels in leukocytes from a patient with the rare disease CBL, we observed an increase in nuclear [3H]dexamethasone (1,4-pregnadien-9-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione) receptor binding following chemotherapy with hydroxyurea plus prednisone. This increase in receptor binding had not been seen following a prior treatment with prednisone alone. In an attempt to determine whether this apparent effect of hydroxyurea in CBL was caused by direct effects of hydroxyurea on lymphoblasts, or by indirect mechanisms such as effects on cellular migration in the patient, we examined the effects of hydroxyurea on GM4672A human lymphoblasts, a cell line which we have previously shown to be glucocorticoid responsive and to contain high affinity receptors specific for active glucocorticoids. In this report, we demonstrate that hydroxyurea treatment markedly increases nuclear and total cellular GR binding levels in GM4672A cells without altering cellular glucocorticoid sensitivity. By way of introduction, we also present the results obtained in the CBL patient which led us to undertake this project in cultured cells.

MATERIALS AND METHODS

Preparation of Peripheral Leukocytes from CBL Patient. The CBL patient had a high WBC count (>20,000/mm3) and greater than 90% leukemia cells on differential at each assay point. Peripheral blood was collected in 20-ml heparinized volumes and placed immediately on ice. Blood was diluted with an equal volume of McCoy’s Medium 5A (Gibco) and layered over Ficoll-Paque (Pharmacia) in a 2:1 (v/v; sample/Ficoll-Paque) ratio. Leukocytes were removed from the interface after a 30-min centrifugation at 400 × g in a Beckman J6-B centrifuge at 4°C. Cells were washed once in McCoy’s medium and resuspended at a cell density of approximately 1 × 10⁹ cells/ml (50-ml volume). This cell suspension was then incubated at 37°C for 30 min to reduce the effects of endogenous steroid (20). Cell viability was not affected during this process. Cells were then collected by centrifugation and resuspended in 2-5 ml of McCoy’s Medium 5A (Gibco) and layered over Ficoll-Paque (Pharmacia) in a 2:1 (v/v; sample/Ficoll-Paque) ratio. Leukocytes were removed from the interface after a 30-min centrifugation at 400 × g in a Beckman J6-B centrifuge at 4°C. Cells were washed once in McCoy’s medium and resuspended at a cell density of approximately 1 × 10⁹ cells/ml (50-ml volume). This cell suspension was then incubated at 37°C for 30 min to reduce the effects of endogenous steroid (20). Cell viability was not affected during this process. Cells were then collected by centrifugation and resuspended in 2-5 ml of McCoy’s medium to yield a final cell density of 2 × 10⁶ to 1 × 10⁷ cells/ml. Cell number was determined by hemacytometer counting, and cell viabilities were scored using standard trypan blue exclusion techniques. Viabilities were generally greater than 90%.

Cell Culture Techniques and Hydroxyurea Treatment of GM4672A Cells. GM4672A human lymphoblasts were grown, passaged, and counted as described in detail previously (19). Cells were treated with hydroxyurea by the addition of sterile, 10-fold concentrates of hydroxyurea in culture medium. Sterile concentrates of hydroxyurea were freshly prepared for each experiment and stored at 4°C until use. Release of hydroxyurea treatment was accomplished by collection of the cells by centrifugation at 400 × g for 10 min, resuspension, and washing in medium without the drug, collection by centrifugation, and final resuspension in hydroxyurea-free medium. At this point, cells

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3 The abbreviations used are: GR, glucocorticoid receptor(s); CBL, chronic basophilic leukemia; PA, plasminogen activator.
were counted using a hemocytometer and replated at cell concentrations chosen so that cell densities of the various released groups would be approximately equal at the time of GR measurement. The initial plating of cells, the 3-day exposure to hydroxyurea, and the release times were staggered so that all GR measurements were performed at the same time on the same day.

Whole Cell Nuclear and Cytosolic Glucocorticoid Receptor Assays. These are essentially the assays of Crabtree et al. (20), with minor variations as described by us previously (9, 19). When appropriate, hydroxyurea was maintained in all washes and incubations. For the saturation binding studies, whole cells were incubated in serum-free McCoy's Medium 5A at 37°C for 45 min in a series of 1.5-ml polypropylene microcentrifuge tubes containing 2.0 x 10^16 to 7.8 x 10^15 M [3H]dexamethasone (New England Nuclear; 48.9 Ci/mmol). Cells were present at 2.5 x 10^8 cells/ml in a final volume of 132 μl (nuclear assay only) or 264 μl (simultaneous nuclear and cytosolic assays). Nuclear [3H]dexamethasone was measured in duplicate 50-μl aliquots using a hypotonic lysis technique as previously described (9, 19). Cytosolic GR binding in duplicate 50-μl aliquots was quantitated by hypotonic lysis in a dextran-coated charcoal suspension (9). Levels of free [3H]dexamethasone were determined by centrifuging the remaining cell suspensions at 10,000 x g for 1 min and measuring radioactivity in duplicate 6-μl samples. All radioactivity measurements were made in a 2:1 (v/v) mixture of Phase Combining System (Amersham) and xylene. Data were plotted according to Scatchard (21), with correction for nonsaturable binding by the radial subtraction method of Rosenthal (22).

For the hydroxyurea time course and release experiments (Fig. 4), as well as the GR, protein, and DNA quantitation studies (Table 3), nuclear GR binding was measured using a "single-point" assay rather than the Scatchard approach just described. Whole cells were incubated at 37°C for 45 min with either 1 x 10^-8 M [3H]dexamethasone alone or in combination with 1 x 10^-8 M unlabeled dexamethasone (Steraloids, Wilton, NH). Nuclear-bound radioactivity was then quantitated as described above for the Scatchard approach. Saturable binding was considered to be the difference between [3H]dexamethasone binding in the absence and presence of the 100-fold excess of unlabeled dexamethasone.

Control and hydroxyurea-treated cells were found to be equally susceptible to hypotonic lysis (>90% lysis) as judged by high-power phase-contrast microscopy in the presence of trypan blue (data not shown). Thus, the observed effects of hydroxyurea on GR binding levels are not artifacts of incomplete hypotonic lysis of hydroxyurea-treated cells (see "Discussion").

Flow Cytometry Analysis of Cell Cycle Kinetics. GM4672A cells were grown in the presence or absence of 50 μM hydroxyurea for 3 days followed by centrifugal collection (400 x g), washing, and resuspension in 0.85% (w/v) NaCl (saline) solution. Cells were counted using a hemocytometer, and 1.25 x 10^8 cells were resuspended in 3 ml of saline in a 15-ml conical polystyrene tube (Corning). Nine ml of 95% ethanol were then added, and the tubes were mixed by inversion and allowed to chill on ice for 10 min. Tubes were stored overnight at 4°C. Fixed cells were then collected by centrifugation, the ethanol was aspirated, and 0.5 ml of RNase (0.2 mg/ml; preboiled 5 min) in 0.15 M NaCl:13 mM disodium EDTA:10 mM sodium phosphate buffer, pH 7.4, were added. Fixed cells were gently resuspended with a Pasteur pipet, transferred to 12- x 75-mm plastic culture tubes, and incubated at 37°C for 30 min. Five hundred μl of propidium iodide (160 μg/ml) were then added, followed by incubation on ice for 30 min. DNA content was then assessed using a Becton-Dickinson FACS IV fluorescence-activated cell sorter (514-nm excitation wavelength; >620-nm emission).

Plasminogen Activator Assay. Extracellular PA activity was measured in conditioned medium using the 125I-fibrinogen solid-phase radioassay of Roche et al. (23) with slight modifications as described by us previously (19).

Growth Inhibition Studies. The effects of hydroxyurea and dexamethasone on GM4672A cell growth were assessed during 3-day incubations in 24-well culture plates, using a Coulter Counter (Coulter Electronics) for cell counting as described previously (19).

Measurement of Protein and DNA. Protein was measured by the dye-binding method of Bradford (24). DNA was measured by the diphenylamine reaction of Burton (25).

RESULTS

A serial study of nuclear [3H]dexamethasone binding in leukocytes from a patient with CBL suggested that hydroxyurea might increase nuclear GR binding levels in this disease (Fig. 1). This initially untreated patient first received prednisone alone, with little or no effect on subsequent nuclear GR binding levels. One wk later the patient received combination therapy with hydroxyurea and prednisone. Nuclear binding of [3H]dexamethasone measured during the next 10 days was elevated over the prehydroxyurea levels. This observation led us to examine effects of hydroxyurea on GR binding in cultured lymphoblasts to determine if the effect observed in the CBL patient resulted from direct effects on cellular receptor levels, or indirect effects, such as differential cell migration patterns or other whole-body complications.

We recently demonstrated that GM4672A human lymphoblasts respond to glucocorticoids with decreased cell growth and decreased extracellular activity of a urokinase-like PA. These cells contain high affinity receptors specific for active glucocorticoids (19). To examine the effects of hydroxyurea on measurable GR levels and glucocorticoid responsiveness in GM4672A cells, we first selected a concentration of hydroxyurea that had a small but measurable effect on cell growth but did not decrease cell viability. Fig. 2 demonstrates that GM4672A cells are relatively sensitive to effects of hydroxyurea on cell growth and viability during a 3-day exposure period. Measurable inhibition of cell growth and viability was observed by 50 and 100 μM concentrations of hydroxyurea, respectively. Thus, the dose-dependent effects on growth preceded effects on viability, such that at 50 μM, cell growth was inhibited by about 33% with no reduction in viability. Although some variability has been noted from experiment to experiment, we have found that a hydroxyurea concentration of 50 μM gives the most consistent balance between observable growth inhibition and maintenance of high viability. Using this concentration, we then tested the effects of a 3-day exposure to hydroxyurea on nuclear GR binding of [3H]dexamethasone. As shown in the Scatchard analysis of Fig. 3, treatment of the cells with 50 μM hydroxyurea increased nuclear GR binding 2.9-fold without...
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Table 1 Effects of hydroxyurea on nuclear and cytosolic glucocorticoid receptor binding levels in GM4672A cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment status</th>
<th>Control</th>
<th>Hydroxyurea</th>
<th>$\Delta$</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurable glucocorticoid receptor no.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear bound</td>
<td>6</td>
<td>4.680 ± 727*</td>
<td>11,292 ± 2.277</td>
<td>2.4</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>4</td>
<td>3.896 ± 1.063</td>
<td>3.919 ± 594</td>
<td>1.0</td>
<td>NS*</td>
</tr>
<tr>
<td>Total cellular</td>
<td>8,576</td>
<td>15,211</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptor affinity ($K_a$, nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear</td>
<td>6</td>
<td>6.8 ± 0.8</td>
<td>5.7 ± 0.8</td>
<td>0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>4</td>
<td>5.0 ± 0.6</td>
<td>5.4 ± 0.6</td>
<td>1.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Cells were untreated or treated with 50 µM hydroxyurea for 3 days as indicated.
* Significance determined by two-sided paired t test.
* GR binding was measured by the whole cell Scatchard technique as described in "Materials and Methods."
* Mean ± SE of sites of [3H]dexamethasone binding per viable cell from n separate experiments.
* The term "cytosolic" refers to GR measured in the cytosol fraction following incubation of whole cells with [3H]dexamethasone at 37°C. See "Materials and Methods."
* NS, not significant.
* Refers to the sum of the mean nuclear and cytosolic GR values listed immediately above.

significantly altering the affinity of the receptor for [3H]dexamethasone. This effect was consistently observed in a number of experiments. Control experiments using untreated cells showed no effect of hydroxyurea on measured GR levels when the drug was included only during the 45-min incubation with [3H]dexamethasone (data not shown), indicating that the effects of hydroxyurea on GR binding levels are dependent on a prolonged exposure of the cells to the drug.

Table 1 demonstrates that the increase in nuclear GR binding of [3H]dexamethasone following hydroxyurea treatment was not simply due to the nuclear localization of large quantities of previously cytosolic GR. The hydroxyurea effect was completely accounted for by an increase in nuclear GR binding (mean 2.4-fold increase). In contrast, treatment with hydroxyurea did not affect cytosolic receptor binding measured in whole cells at 37°C. Thus, the mean cellular increase of approximately 6600 measurable GR was comprised of receptors capable of nuclear binding at 37°C. As shown in Table 1, hydroxyurea treatment did not significantly alter the affinity of either nuclear-bound or cytosolic GR for [3H]dexamethasone.

Hydroxyurea increased nuclear GR binding of [3H]dexamethasone in GM4672A cells in a time-dependent manner, as shown in Fig. 4, left. Increased receptor binding was seen as early as 1 day after exposure to 50 µM hydroxyurea. By 2 and 3 days, the effect was much larger and represented at least a doubling or tripling of nuclear GR binding. Cells pretreated with hydroxyurea for 3 days and then released showed a time-dependent return to control levels (Fig. 4, right). Thus, the hydroxyurea stimulation of nuclear GR binding is fully reversible over a 3-day period.

In HeLa S3 cells, measurable GR levels double during the S phase (26). Thus, G2-phase HeLa S3 cells have twice the GR
binding capacity of G1-phase cells (26). Since the concentration of 50 μM hydroxyurea was chosen so that inhibition of cell growth was just detectable (Fig. 2), its effects on the GM4672A cell cycle were probably minimal. However, considering the known effects of hydroxyurea on cell cycle kinetics in other cell types (27), it was necessary to consider altered cell cycle kinetics as a possible cause for the increased receptor binding seen following hydroxyurea treatment. GM4672A cells were therefore treated with 50 μM hydroxyurea for 3 days and analyzed for DNA content and cell cycle phase by flow cytometry using propidium iodide as a stain. As expected, a 3-day treatment with 50 μM hydroxyurea had only very small effects on the DNA content and cell cycle distribution of GM4672A cells (Fig. 5). These slight changes appeared to include a small increase in the proportion of S-phase cells and a slight decrease in the proportion of G2-phase cells. These small effects are more easily seen in Table 2, where the data of Fig. 5 and a similar experiment have been combined and summarized to reflect the percentages of cells in each of the cell cycle phases. Hydroxyurea treatment led to only a 1.7% decrease in the percentage of cells in the G1 phase, the phase in which about half of both control and treated cells resided. Hydroxyurea effects on the S and G2-M phases were also small in magnitude (4.3% increase and 2.6% decrease, respectively). It is clear from these data that the mean 2.4-fold increase in nuclear GR binding of [3H]dexamethasone seen in hydroxyurea-treated GM4672A cells (Fig. 3; Table 1) is not explained by a large increase in S- or G2-phase cells. Thus, changes in cell cycle kinetics do not directly account for the increased nuclear GR binding levels observed after hydroxyurea treatment.

We wished to determine if the increases in GR binding levels following hydroxyurea treatment were associated with increased cellular sensitivity to glucocorticoids. GM4672A cells respond to glucocorticoids with both decreased growth rates and decreased levels of extracellular, urokinase-like PA activity (19). We therefore examined these two effects of glucocorticoids as a function of hydroxyurea treatment. As previously shown in Fig. 2, 50 μM hydroxyurea itself had a small but measurable inhibitory effect on GM4672A cell growth. However, when normalized for this small inhibitory effect of hydroxyurea (in the absence of dexamethasone treatment), the hydroxyurea-treated cells showed a degree of growth inhibition by dexamethasone which was essentially identical to that observed in control cells (Fig. 6). Cell growth over a 3-day period in both groups was maximally inhibited with 1 x 10^-6 M dexamethasone, with half-maximal inhibition occurring at 1 x 10^-8 M, a value which agrees well with the Kd of the receptor for [3H]dexamethasone (Fig. 3; Table 1; Ref. 19). Thus, despite the increases in measurable nuclear-bound GR found in hydroxyurea-treated cells, there was no alteration in the magnitude of glucocorticoid inhibition of cell growth.

Glucocorticoid inhibition of extracellular PA activity was then used as a second marker to examine the relationship between hydroxyurea stimulation of GR binding levels and glucocorticoid responsiveness. Consistent with our previous results (19), treatment of GM4672A cells with 1 x 10^-6 M dexamethasone for 3 days led to inhibition of extracellular PA activity (Fig. 7). PA inhibition by dexamethasone in hydroxyurea-treated cells was of the same relative magnitude as in cells not treated with hydroxyurea. Unexpectedly, hydroxyurea treatment alone resulted in almost a doubling of mean PA levels over untreated cells (Fig. 7, inset). Nevertheless, hydroxyurea treatment did not alter the relative degree of PA inhibition by dexamethasone.

Since both nuclear GR binding levels and extracellular PA activities were increased after hydroxyurea treatment, it was possible that these increases were not specific, but rather part of an overall increase in protein content or protein biosynthesis in the treated cells. Also, if undetected problems existed in our quantitation of hydroxyurea-treated cells (leading to erroneously low cell numbers), the calculated levels of nuclear GR binding and PA activity would be artificially high when normalized for cell number. To test these two possibilities, we analyzed the protein, DNA, and measurable GR content of untreated and hydroxyurea-treated cells (Table 3). Nuclear GR binding of [3H]dexamethasone was measured as a positive control to demonstrate the previously observed effects of hydroxy-

**Table 2 Effects of hydroxyurea on cell cycle kinetics of GM4672A cells**

<table>
<thead>
<tr>
<th>Treatment status</th>
<th>G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52.9</td>
<td>29.8</td>
<td>17.3</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>51.2</td>
<td>34.1</td>
<td>14.7</td>
</tr>
</tbody>
</table>

* Cells were untreated or treated with 50 μM hydroxyurea for 3 days as indicated.

* Percentages determined by flow cytometry using propidium iodide as described in “Materials and Methods.” Results represent the means of two determinations from separate experiments which yielded similar results. One of the two experiments is presented graphically in Fig. 5.
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Fig. 7. Lack of effect of hydroxyurea on dexamethasone inhibition of GM4672A plasminogen activator activity. Cells were grown for 3 days without additions (CON), or in the presence of 1 μM dexamethasone (DEX), 50 μM hydroxyurea (HU), or both (HU + DEX). The last 18 h were in serum-free medium. PA activities in the conditioned medium were then measured. Results are expressed as percentages of either control or hydroxyurea-treated cell PA levels, in the absence of dexamethasone. *Bars, mean of percentages derived from four separately normalized experiments, each experiment having been performed using four replicates at each point; bars, SE. Inset shows the mean (± SE) of actual PA values from the four experiments for control and hydroxyurea-treated conditions. Details of cell growth conditions and PA measurement are presented in “Materials and Methods.” mPU, MilliPloug Units.

Table 3  Effects of hydroxyurea on protein and DNA content and nuclear glucocorticoid receptor binding levels in GM4672A cells

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>Protein (μg/10^6 cells)^2</th>
<th>DNA (μg/10^6 cells)^2</th>
<th>Nuclear glucocorticoid receptor binding (sites/cell)^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>221</td>
<td>7.9</td>
<td>1,586 ± 479*</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>251</td>
<td>8.5</td>
<td>11,700 ± 974*</td>
</tr>
</tbody>
</table>

*GM4672A cells were grown for 3 days in the absence or presence of 50 μM hydroxyurea.
^2Protein was quantitated as described in “Materials and Methods.” Hydroxyurea/control = 1.1.
^3DNA was quantitated as described in “Materials and Methods.” Hydroxyurea/control = 1.1.
^4Nuclear GR binding of [3H]dexamethasone was quantitated using the “single-point” assay as described in “Materials and Methods.” Hydroxyurea/control = 7.4.

Table 3.5  Mean ± SE for quadruplicate determinations.

A number of studies of GR binding levels in hematological malignancies have demonstrated a relationship between chemotherapy and lower total cellular or cytosolic GR binding levels (13–18). In contrast, we have now demonstrated that hydroxyurea treatment can lead to increased nuclear GR binding levels in leukemic lymphoblasts. This effect was initially observed in a CBL patient and subsequently demonstrated in cultured GM4672A lymphoblasts under controlled conditions. Hydroxyurea had no effect on the affinity of either cytosolic or nuclear GR for [3H]dexamethasone. Alternations in cell cycle kinetics were minimal at the low hydroxyurea concentrations used and did not account for the sizable increase in measured receptor levels in treated cells. Using dexamethasone inhibition of cell growth and PA activity, hydroxyurea-treated GM4672A cells showed no increase in sensitivity to glucocorticoids despite the increase in nuclear binding of steroid. Thus, similar to our previous studies using sodium butyrate-treated HeLa S3 cells (9, 28), correlations between altered GR binding levels following hydroxyurea treatment and glucocorticoid responsiveness do not exist for GM4672A cells. Our work indicates that GR measurements in leukocytes from hydroxyurea-treated patients should be interpreted with caution.

Although our results might have been explained by a decreased susceptibility of hydroxyurea-treated cells to hypotonic lysis (leading to inclusion of whole cells in the nuclear pellets), we feel that this possibility can be ruled out for the following reasons. (a) Any artificial inclusion of cytosolic GR binding from unlysed hydroxyurea-treated cells in the nuclear GR assay would be counterbalanced by artificial decreases in GR binding measured in cytosol, since the cytosolic assay also relies on hypotonic lysis followed by centrifugal removal of nuclei. However, data presented in Table 1 indicate that cytosolic GR binding levels remained identical following hydroxyurea treatment. (b) Even if all of the cytosolic GR binding in control cells were to artificially become “nuclear-bound” following hydroxyurea treatment, the resultant nuclear GR binding levels would still be too low to account for the nuclear-binding levels actually seen following hydroxyurea treatment (Table 1). (c) Any extra nonsaturably bound [3H]dexamethasone which might have been associated with unlysed hydroxyurea-treated cells in the nuclear pellets would have been corrected for in either the Scatchard analyses (Figs. 1 and 3; Table 1) or the single point assays (Fig. 4; Table 3), using the radial subtraction method of Rosenthal (22) or the 100-fold excess unlabeled steroid technique, respectively. (d) High power phase contrast microscopy in the presence of trypan blue indicated that control and hydroxyurea-treated cells were both equally susceptible to hypotonic lysis (>90%, not shown). Because of the above reasons, we feel that an artificial explanation of our data based on differences in susceptibility to hypotonic lysis cannot be reasonably entertained.

The mechanisms by which hydroxyurea increases measurable GR binding levels in GM4672A cells are currently unknown. While increased de novo synthesis of receptors is a possibility, it is also possible that regulation of functionality plays a role. Thus, the increase in measured receptor levels might result from activation of preexisting receptors from forms incapable of binding steroid to a steroid-binding form. In this regard, there are a number of possibilities by which hydroxyurea might lead to increased measurable GR levels. The primary effect of hydroxyurea on cellular metabolism is an inhibition of ribonucleotide reductase (29–31). Since thioredoxin provides the re-
ducing power for the generation of deoxyribonucleoside-5'-diphosphates by this enzyme. inhibition of this process could lead to accumulation of reduced thioredoxin. Reduced thioredoxin maintains GR in a steroid binding form (32, 33), apparently by reducing sulfhydryl groups on GR (32–34). Thus, inhibition of ribonucleotide reductase by hydroxyurea could ultimately lead to increased levels of steroid-binding GR via increased availability of reduced thioredoxin. Alternatively, blockage of ribonucleotide reductase by hydroxyurea could lead to accumulation of ribonucleotide diphosphates, including ADP. Accumulation of ADP might then lead to increases in functional GR by either of two mechanisms. (a) Compensation for increased ADP levels could lead to increased ATP levels, which might then activate preexisting GR to forms capable of binding steroid (34–36). (b) Increased ADP levels might have direct stabilizing effects on unoccupied GR (37), again leading to increased measurement of steroid-binding forms of GR. Hydroxyurea might have other unknown effects on ribonucleotide pools or the overall energy balance of GM4672A cells which could lead to the increased GR binding levels which we have observed. The actual mechanisms involved in this process are currently being considered in our laboratory. Elucidation of such mechanisms may ultimately shed light on the normal processes involved in regulating the levels of steroid-binding forms of GR in lymphoid cells.

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