Lymphocytes obtained from patients with chronic lymphocytic leukemia (CLL) respond to the in vitro presence of cortisol by depressed incorporation of precursors into RNA and protein. The decreased incorporation of uridine into RNA is the sum of (1) an inhibition in the synthesis of RNA and (2) an enhanced destruction of newly synthesized RNA. Whereas cortisol was not dependent upon plasma for the manifestation of the above effects, the presence of plasma was an absolute requirement in order for cortisol to have an inhibitory effect on the synthesis of protein. A comparison of leukemic and normal lymphocytes demonstrated that the magnitude of inhibition of precursors into RNA and protein was greater in leukemic cells. Because it is believed that the plasma factor required is transcortin, determination of transcortin levels by cortisol-binding gel filtration techniques were performed. These indicated that transcortin levels of CLL plasma were about 50 per cent lower than that of the normal. Consequently, further experiments involving type-specific plasma substitutions were performed. The results obtained from these experiments indicated that the magnitude of the effect of cortisol on the synthesis of lymphocyte protein was directly related to the transcortin level of the plasma employed.

The use of steroids (cortisol or cortisol analogs) in the therapy of chronic lymphatic leukemia (CLL) has proven effective in decreasing the number of circulating lymphocytes. The precise mechanism by which cortisol promotes this effect in the CLL patient, as well as producing lymphopenia in the normal, is not yet clearly understood. However, cortisol has been demonstrated to affect the incorporation of precursors of DNA,1,2 RNA,3,5 and protein3,4,6,7 by lymphocytes and to decrease the utilization of glucose by rabbit lymphoid cells.8

Our previous studies demonstrated that the presence of cortisol in the medium yielded alterations in lymphocyte mitochondrial and Golgi structure,4 promoted the degradation of newly-labeled RNA4,5 (presumably by the induced removal of an inhibitor of RNase9 and that only when autologous plasma was present in the medium did it have an inhibitory effect on the synthesis of protein.8 In contradistinction to the requirement of autologous plasma for the manifestation of an inhibition on the synthesis of lymphocyte...
protein, plasma plays no role in the cortisol promoted decreased incorporation of uridine into normal lymphocyte RNA. Because autologous plasma was required for cortisol to have an effect on protein synthesis of the normal lymphocyte, it was postulated that transcortin, the cortisol-binding protein of human plasma, was required. This hypothesis is supported by the work of Hofert and White who demonstrated that the lymphopenia observed in an intact animal as the result of cortisol administration, could be prevented if the animal was heparctomized prior to cortisol treatment. Since the liver synthesizes transcortin, it can be assumed that heparctectomy reduces the level of plasma transcortin.

Chronic lymphocytic leukemia (CLL) is characterized by large numbers of circulating lymphocytes. The increase in circulating lymphocytes in CLL appears to result from an accumulation of lymphocytes whose longevity has been enhanced and not from an increase in their mitotic rate. This increased longevity might be the result of a repressed lympholytic mechanism. If transcortin is required for the lymphopenic effect of cortisol, low levels in CLL might be one factor responsible for increased longevity of lymphocytes.

As a consequence of this hypothesis, this study is primarily concerned with:

1. The response of cultured purified normal and CLL lymphocytes to the presence of cortisol in the medium, together with the possible role that autologous plasma plays in the affected parameters.
2. The regulation of the effect of cortisol in the CLL lymphocyte by type-specific normal plasma.
3. The levels of plasma transcortin of the normal and CLL individual.

The results presented in this study indicate that transcortin appears to regulate the effect of cortisol on the synthesis of lymphocyte (both CLL and normal) protein and appears to play no detectable role in the cortisol promoted degradation of newly synthesized RNA. Evidence is also presented that indicates that the CLL lymphocyte responds in a different manner than its normal counterpart with respect to the effect of cortisol on the incorporation of RNA and protein precursors. In certain respects these differences appear to be due to (1) amount of available RNase and (2) polyribosomal stability.

**Materials and Methods**

**Materials**

Hydrocortisone (compound F Sigma), uridine-3H (UL), specific activity of 5 Ci/mM, leucine-3H, specific activity of 2 Ci/mM, and hydrocortisone-1,2,3H, specific activity of 9 Ci/mM (New England Nuclear), TC 199 medium (Difco), actinomycin D (Mann Research Laboratory), and penicillin (Difco) were employed in this study.

**Methods**

Preparation of suspension of normal and CLL lymphocytes. All glassware used for preparation and incubation was siliconized and sterilized. Whole blood, donated by normal healthy employees of the Methodist Hospital of Brooklyn, and chronic lymphocytic leukemic ambulatory patients of Dr. Alan Morrison, was heparinized (20 units per ml of blood) and allowed to sediment for a period of 2 hours at 37°C in 5-ml pipettes. After the sedimentation period, suspensions of pure lymphocytes were obtained by methods...
previously described\(^6,16\) and the cell number was adjusted with plasma to \(16 \times 10^6\) cells per ml.

For those experiments involving the use of saline or homologous plasma-suspended lymphocytes the following procedures were used:

After the adjusted lymphocyte suspensions were obtained, 0.6 ml. (10\(^7\) cells) of the suspension was placed into culture tubes and 2 ml. of saline or type-specific homologous plasma was added. The culture tubes were then centrifuged at 200 \(\times\) g., the supernatant was discarded, and the procedure was repeated once. After the supernatant was discarded, the cells were gently suspended in 0.6 ml. of either saline or type-specific homologous plasma. Cell counts were performed, and under the conditions employed no detectable loss in cells was evident. The description of the composition of the cultures employed for the determination of the effect of cortisol on the incorporation of RNA and protein precursors has been described elsewhere.\(^5,6\)

**Determination of the effect of cortisol (10\(^{-4}\)) on the degradation of labeled lymphocyte RNA.** For those experiments involving the use of prelabeled lymphocyte RNA, 0.6 ml. of lymphocytes (10\(^7\) cells) suspended in autologous plasma were cultured in 2 ml. of TC 199 medium containing 4 \(\mu\)C of uridine for a period of 45 minutes, at 37\(^\circ\)C. After synthesis of RNA was stopped by the addition of 30 \(\mu\)g. of actinomycin D in 10 \(\mu\)l. saline, the culture was then incubated for a further 15 minutes and then received 0.4 ml. of either 2 per cent ethanol in saline (controls) or cortisol to a final concentration of 10\(^{-6}\) M.

**Determination of viability.** The use of trypan blue exclusion test for viability\(^18\) as previously described\(^6\) demonstrated that little lethal injury had occurred during each of the above steps. Cell counts after every incubation showed no loss of cells had occurred.

**Procedures used after the incubation of cultures.** At the end of the incubation periods the cultures were placed in an ice bath and cooled to 4\(^\circ\)C, centrifuged at 200 \(\times\) g. for 10 minutes, the supernatant discarded, and the cap of cells suspended in 1.0 ml. of cold saline. The cells were disrupted by means of sonication and the acid insoluble RNA and protein present were precipitated with an equal volume of cold 10 per cent TCA. After filtration through Millipore filters (45 \(\mu\) size) the filters were washed repeatedly with cold 5 per cent trichloroacetic acid (TC) then dried at 60\(^\circ\)C for 1 hour and placed into liquid scintillation vials (20 ml. capacity) containing 20 ml. of Liquiflor. The activities of \(^{3}H\)-uridine and \(^{3}H\)-leucine were determined with a Picker Ansitron II Liquid Scintillation Counter (efficiency of 51\% for tritium). The use of internal standards allowed corrections for quenching when deemed necessary. The data presented are the average counts per minute obtained from each triplicate culture (maximum deviation of 5\%) or the average per cent difference (control/experimental) whenever more than one experiment was conducted. The latter presentation is necessary in order to compare groups and to obviate biological variations among the cells from different donors. That is, since the absolute number of cpm per 10\(^7\) cells differs widely between donor cells cultured under identical conditions, data obtained from more than one experiment are presented as the average per cent difference between control and experimental sets.

**Determination of the cortisol-binding capacity of normal and CLL plasma.** Each of the remaining normal and CLL platelet free plasmas, obtained by the above procedures, was assayed for \(^{3}H\)-cortisol binding capacity. Triplicate aliquots of 0.1 ml. of each plasma were incubated with 5 \(\mu\)l. of \(^{3}H\)-cortisol (100 \(\mu\)Ci/ml.) at room temperature (26\(^\circ\)C) for a period of 60 minutes. At the end of this time, 0.9 ml. of 0.001 M phosphate, pH 7.4, was added and the contents of each incubation were loaded onto a Sephadex G-25 column (0.5 cm. \(\times\) 20 cm.) previously equilibrated with the same buffer. Elution proceeded with the same buffer and fractions of 0.5 ml. were collected into scintillation vials containing 15 ml. of Bray's scintillant. These vials were left in the Ansitron II liquid scintillation counter for a period of 2 hours at 10\(^\circ\)C and the radioactivities subsequently determined. Two peaks of activity were obtained for each plasma. The first peak (Fig. 1) was always contained within the first 14 ml. of eluent and represents protein bound \(^{3}H\)-cortisol. The second peak represents unbound \(^{3}H\)-cortisol. The total cpm of the first 14 ml. of eluent was obtained and averaged for each triplicate run. It has been previously demonstrated\(^19,20\) that with the use of Sephadex G-25 columns, transcortin, the cortisol-
Fig. 1.—The elution of plasma-bound $^3$H-cortisol from Sephadex G-25 columns (0.5 × 20 cm.). Applied materials eluted with 0.001 M phosphate, pH 7.4 at a flow rate of 1 ml. per minute. Elution of $^3$H-cortisol that had been incubated with plasma; note that the front moving peak occurs only with elution of applied cortisol-plasma. Rising peak represents elution of free cortisol.

binding plasma protein, is wholly responsible for the binding activities of the front running peak. Undialyzed and dialyzed plasma yielded identical results, indicating that the presence of endogenous cortisol did not affect the amount of labeled cortisol bound. In the cases of CLL plasma, only those plasmas obtained from individuals who were untreated or who had not been treated with steroids were employed in this study.

RESULTS

Part I

Table 1 indicates that cortisol decreases the incorporation of $^3$H-uridine into RNA by the lymphocytes from patients with CLL more than by the lymphocytes from normal individuals. Equal enhancement of this effect of cortisol is found even in CLL patients treated with prednisone (Table 2). It is not affected by plasma, as it can be demonstrated to the same degree whether

Table 1.—The Effect of Cortisol Concentration on the Incorporation of Tritiated Uridine Into the RNA of CLL and Normal Lymphocytes Cultured for 2 Hours

<table>
<thead>
<tr>
<th>Culture</th>
<th>CPM</th>
<th>Per Cent Difference</th>
<th>CPM</th>
<th>Per Cent Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31,000</td>
<td>—</td>
<td>4300</td>
<td>—</td>
</tr>
<tr>
<td>Cortisol $10^{-5}$</td>
<td>9000</td>
<td>— 71</td>
<td>2300</td>
<td>— 47</td>
</tr>
<tr>
<td>Cortisol $10^{-6}$</td>
<td>23,000</td>
<td>— 26</td>
<td>3200</td>
<td>— 26</td>
</tr>
<tr>
<td>Cortisol $10^{-7}$</td>
<td>24,500</td>
<td>— 21</td>
<td>3900</td>
<td>— 9</td>
</tr>
<tr>
<td>Cortisol $10^{-8}$</td>
<td>26,000</td>
<td>— 16</td>
<td>4300</td>
<td>0</td>
</tr>
</tbody>
</table>

* This experiment was performed with the lymphocytes of an untreated CLL patient.
† For molar concentrations of $10^{-5}$ to $10^{-6}$, the per cent differences stated are significant to $p < 0.005$. 

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Table 2.—The Effect of a 10⁻⁵ Molar Concentration of Cortisol on the Incorporation of ³H-Uridine into the RNA of Lymphocytes Cultured for 2 Hours

<table>
<thead>
<tr>
<th>Number of Experiments</th>
<th>Per Cent Decrease in Incorporation ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL untreated</td>
<td>- 75 ± 7.2</td>
</tr>
<tr>
<td>CLL steroid-treated</td>
<td>- 74 ± 3.4</td>
</tr>
<tr>
<td>All CLL</td>
<td>- 74.5 ± 5.7 †</td>
</tr>
<tr>
<td>Normal</td>
<td>- 47 ± 4.6 †</td>
</tr>
</tbody>
</table>

* Steroid treatment employed was prednisone.
† The comparison of the means of the normal and all the CLL experiments by the use of student t yield p < 0.005.

Table 3.—The Effect of 10⁻⁵ M Cortisol on the Incorporation of Uridine Into the RNA of CLL Lymphocytes*

<table>
<thead>
<tr>
<th>Number of Individuals</th>
<th>Per Cent Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous plasma</td>
<td>- 74.5 ± 5.7</td>
</tr>
<tr>
<td>Absence of plasma</td>
<td>- 81 ± 5.4</td>
</tr>
<tr>
<td>Normal plasma</td>
<td>- 71 ± 1.9</td>
</tr>
</tbody>
</table>

* Cultured for 2 hours in the presence and absence of autologous plasma and in the presence of normal (type-specific) plasma.

homologous or autologous plasma is added or whether the lymphocytes are incubated in a plasma-free medium (Table 3).

A study of the time relationship of cortisol in the culture shows that a significant reduction of ³H-uridine incorporation into RNA occurs within 30 minutes (Fig. 2). The drop in absolute cpm after 90 minutes of culture must be at least partly due to breakdown of newly synthesized RNA.

Fig. 2.—The effect of cortisol on the incorporation of ³H-uridine into CLL lymphocyte acid insoluble RNA in accordance to time. Control cultures: cortisol-containing cultures; Bars indicate ranges within triplicate cultures; cpm are given per culture of 10⁶ cells. Note that after 90 minutes of incubation there is a decrease in the absolute number of cpm in the cortisol containing cultures. The control cultures after this time (90 minutes) continue to incorporate the uridine linearly.
The average per cent decrease that cortisol promoted in nine cultures containing normal lymphocytes was 21 ± 7.3 per cent.6

Fig. 3.—(A) The effect of cortisol on the degradation of newly labeled RNA of the normal lymphocyte. Normal lymphocytes (10⁷ cells) were incubated for 1 hour in medium containing ³H-uridine. At the end of this time actinomycin D was added. After 15 minutes of additional incubation, the experimental cultures received cortisol to yield a final molar concentration of 10⁻⁵ and the control cultures received the equivalent volume of the cortisol vehicle. All incubations were conducted in triplicate. The maximum variation within triplicates never exceeded 5 per cent. The cortisol containing cultures at the end of 120 minutes of incubation contained 33 per cent fewer cpm than did their respective controls. The significant difference between the CPM of these two sets was a value of p < 0.005. The effect of cortisol on the degradation of newly labeled RNA of the CLL lymphocyte. Conditions employed are identical to those of experiment of 3(A). Observe that the cpm of "zero time" culture is about ten times that of the comparable cultures of 3(A).

The degradation of RNA newly synthesized by lymphocytes was studied by stopping synthesis with actinomycin D after 45 minutes of culture in autologous plasma with ³H-uridine, and comparing the fall in labeled RNA over the ensuing 120 minutes in cultures with and without cortisol. Figures 3(A) and 3(B) show the results obtained with normal and CLL lymphocytes, respectively. RNA from normal lymphocytes is degraded more rapidly and, although the total effect of cortisol is the same after 2 hours on CLL lymphocyte RNA, it is demonstrable much earlier. These results confirm that the observed reduction of ³H-uridine incorporation into RNA by CLL lymphocytes (Fig. 2) during the first 30 minutes is genuine, and that the experimental findings cannot be due to increased RNA degradation, which does not occur until after 90 minutes.

Part II

In contrast to the experiments on ³H-uridine incorporation into RNA, it is shown (Table 4) that plasma is a necessary requirement for the reduced incorporation into total protein of CLL lymphocytes cultured for 2 hours.

Table 4.—The Effect of Cortisol on Leucine Incorporation Into Total Protein of CLL Lymphocytes Cultured for 2 Hours

<table>
<thead>
<tr>
<th>No. of Experiments</th>
<th>Type of Plasma Present</th>
<th>Per Cent Decrease in Amino Acid Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Autologous</td>
<td>29 ± 8.3 * (p &lt; 0.001)</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Normal type-specific</td>
<td>49 ± 10 (p &lt; 0.005)</td>
</tr>
</tbody>
</table>

* The average per cent decrease that cortisol promoted in nine cultures containing normal lymphocytes was 21 ± 7.3 per cent.6
Table 5.—Cortisol-binding Capacity of Normal and CLL Plasma

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Average cpm of Cortisol Bound</th>
<th>Per Cent Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>53,000 ± 10,000</td>
<td>-</td>
</tr>
<tr>
<td>CLL</td>
<td>28,000 ± 10,500</td>
<td>- 47</td>
</tr>
</tbody>
</table>

* The leukemic patients were all ambulatory outpatients.
† The binding capacity of CLL plasma is significantly different (p < 0.005) from that of the normal.

corporation of ³H-leucine by the CLL lymphocyte into total protein. Normal plasma resulted in almost twice the reduction found with autologous plasma. The cortisol-binding capacity of normal plasma was also found to be about twice that of CLL plasma (Table 5).

Table 6 shows the results of the incorporation of ³H-leucine by normal lymphocytes into protein, depending on the plasma used with the culture. In this experiment CLL plasma with only 10 per cent of normal plasma cortisol-binding activity was ineffective.

DISCUSSION

The results presented in Part I of this study demonstrate that the presence of cortisol in the culture for 2 hours results in a decreased incorporation of uridine into the RNA of normal and CLL lymphocytes. The lowest concentration of cortisol promoting a significant decrease in uridine incorporation of the CLL lymphocyte is 10⁻⁸ M, whereas at this concentration no effect is produced in the normal. This indication of greater susceptibility of the CLL cell to the presence of cortisol is further reinforced by the even greater decrease of uridine incorporation at 10⁻⁶ M. These results confirm the observations of Schrek and Batra,²¹ who found that CLL cells are more susceptible to the action of cortisol analogs than are normal lymphocytes. The decrease of 75 per cent in the incorporation of uridine by the CLL cell, promoted by the presence of 10⁻⁵ cortisol in the culture is the summation of both the degradation of newly synthesized RNA as well as the inhibition of its synthesis. However, the final decrease observed in the normal lymphocyte appears to be almost entirely due to the degradation of newly synthesized RNA. This final per cent degradation is similar to that observed in the CLL system but appears much earlier in time. This suggests that if cortisol promotes the degradation of RNA by the removal of an inhibitor of RNase activity it occurs more readily in the normal than in the CLL cell. Furthermore, since the

Table 6.—The Effect of Cortisol on Amino Acid Incorporation by Normal Lymphocytes*

<table>
<thead>
<tr>
<th>Plasma Used</th>
<th>Per Cent Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous</td>
<td>- 21 (p &lt; 0.001)</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>CLL type-specific</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cultured from 2 hours in the absence and presence of autologous and CLL type-specific plasma. The CLL plasma used had only 10 per cent of the binding capacity of the normal plasma used.
final per cent degradation in the CLL and the normal is similar, and this appears to be the result of increased activity, it is concluded that the total amount of inactive RNAse might be similar.

Henry et al.\textsuperscript{17} have suggested that the CLL cell synthesizes more RNA than its normal counterpart. Although no data have been presented bearing directly on this question, our experience that the total amount of uridine incorporation has always been much greater in the CLL than in the normal may be relevant. This increase in incorporation may not be the result of a greater rate of synthesis, but rather of the slower rate of degradation and thus, increased accumulation of the labeled species. This contention is supported by the work of Cooper\textsuperscript{22,23} who has recently demonstrated that in resting normal human lymphocytes, half of the ribosomal RNA synthesized is rapidly degraded. Purines obtained from this degradation would be converted to uric acid. Since the uric acid/lymphocyte content of CLL plasma is much lower than that of the normal,\textsuperscript{24} the extent of degradation of the RNA of the CLL may be much less than that observed in the normal.

The results presented in the Part II of our study demonstrate that the inhibitions in the synthesis of both normal and CLL protein promoted by cortisol are dependent upon the presence of plasma in the culture. The inhibitions found in the CLL cell are slightly (though not significantly) greater than those obtained in normal lymphocytes. When CLL cells are incubated with normal type-specific plasma, protein synthesis is inhibited to a significantly greater degree than when incubated in autologous plasma. Conversely, no inhibition of protein synthesis occurs when normal cells are incubated in CLL plasma. Coupled with the observation of the almost complete depression of cortisol-binding capacity (90\%) of the CLL plasma, these findings strongly suggest that the factor required for cortisol to inhibit the synthesis of lymphocyte protein is transcortin. Since the effect of cortisol on the synthesis and degradation of normal and CLL lymphocyte RNA (based on our results) is not dependent upon that postulated plasma factor (transcortin), the mechanism of the observed inhibition in the synthesis of lymphocyte protein must be independent of the effect of cortisol on lymphocyte RNA, at least during the periods of incubation studied. As the inhibition of protein synthesis produced in the normal lymphocyte is less than that of the CLL, in spite of higher levels of transcortin present in normal plasma, it appears that the CLL protein-synthesis apparatus is more susceptible to cortisol-transcortin action. This is reinforced by the results of those experiments involving the substitution of CLL plasma by normal plasma. The postulated fragility may lie in a faulty organization of polyribosomal units due to an inherent fault in the nature and number of the ribosomes present.\textsuperscript{17,26-27} If this is the case, then it can be reasoned that the mechanism by which cortisol-transcortin promotes an effect on the synthesis of normal lymphocyte protein may be similar to that found in bacteriophage systems;\textsuperscript{28} that is, the presence of the cortisol-transcortin complex in the cytoplasm leads to the disruption of the cell's polyribosomal units.

Whether or not the above mechanism exists, the results presented in this study support the contention that a cortisol-transcortin complex inhibits the
RESPONSE OF LYMPHOCYTES TO CORTISOL

There appears to be a greater susceptibility of the CLL lymphocyte to cortisol-transcortin in spite of depressed levels of circulating transcortin. It is noteworthy to point out that temporary reductions of lymphocyte counts in a CLL patient can be elicited by the administration of fresh whole blood.29-32

ACKNOWLEDGMENTS

We wish to extend our most grateful thanks to Dr. Alan Morrison, whose cooperation in supplying the clinical material made this study possible. We would also like to thank Miss Jo Wolen for her expert secretarial work and general counseling and Dr. Leonard Ornstein and Dr. E. J. Watson-Williams for helping us with the manuscript. Particularly, we would like to thank Miss Joanne Gallagher for her excellent technical assistance.

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The Response of Leukemic Lymphocytes to Cortisol: A Suggested Role of Transcortin

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