Identification and Partial Purification of "Transcortin"-like Protein within Human Lymphocytes*

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SUMMARY

We have detected and localized in situ lymphocyte proteins with antigenic determinants similar to those of the cortisol-binding globulin of human plasma. All lymphocyte fractions, obtained by differential centrifugation and ammonium sulfate fractionations exhibited cortisol-binding capacities and were recognized by transcortin-specific antibody. Inasmuch as the 40 to 65% ammonium sulfate fraction of the 105,000 x g lymphocyte cytosol accounted for over 90% of the total binding capacity of the cell, this fraction was further purified by the procedure employed for the purification of plasma transcortin. These procedures yielded a protein with about 20% the cortisol-binding capacity of transcortin, migrated more rapidly than transcortin in disc acrylamide electrophoretic systems, and formed a line of identity with transcortin when treated with transcortin-specific antibody. Fluorescein-labeled transcortin antibody applied to sectioned lymphocytes showed almost exclusive cytoplasmic localization of transcortin-like protein. These results show for the first time the existence of cortisol-binding proteins within the human lymphocyte antigenically identical with transcortin.

The presence of specific intracellular glucocorticoid receptors in lymphoid cells is well documented and their specificity (1), nature (2), localization (2-5), and possible physiological functions (6, 7) have been briefly studied. Inasmuch as the binding of cortisol by these receptors is similar to that exhibited by transcortin it therefore seemed of great interest to ascertain whether transcortin and intracellular cortisol receptor proteins are related or possibly even identical.

In the present study, we identified a protein, localized primarily within the cytoplasm of human lymphocytes, with demonstrable antigenic determinants, cortisol-binding characteristics, and sedimentation behavior in sucrose gradients similar to that of plasma transcortin.

MATERIALS AND METHODS

Preparation of Purified Transcortin—Human transcortin was purified from 15 liters of pooled human plasma from healthy individuals by the method of Muldoon and Westphal (8). The degree of purity was determined by disc acrylamide electrophoresis (9, 10). The identity of our preparation as transcortin was established by analysis of amino acid composition and by cortisol-binding affinities at 4 and 37° according to the method of Muldoon and Westphal (8).

Determination of Sedimentation Coefficient of Purified Transcortin—Purified transcortin, 0.5 mg in 0.5 ml of 1 mM phosphate, pH 7.4, was layered over 5.0 ml of a 5 to 20% sucrose gradient and centrifuged in a Beckman SW 65 rotor at 60,000 rpm in an L-2-75 Beckman ultracentrifuge for 21 hours at 5°. Calibration of our instrument and procedure was performed by repeated and simultaneous centrifugations of the following pure markers: bovine pancreatic RNAse, human albumin, and peroxidase (all from Worthington Biochemicals) under the above conditions. After centrifugation 0.1-ml fractions were taken from the tubes with the aid of an Isco sucrose gradient fractionator and monitoring system. A total of 55 fractions was thus obtained. Since transcortin after its purification still contains some tightly bound radioactive cortisol that had been added at the onset of the purification process, 50-μl aliquots of the gradient were assayed for [1,2-3H]cortisol activity.

Preparation of Transcortin Antibody—Transcortin-specific antibodies were prepared as follows. One milligram of freshly purified transcortin was injected intravenously into each of two rabbits every other day for a period of 2 weeks. Two weeks later a booster containing 2 mg of transcortin was injected into each rabbit and 3 days later, 20 ml of blood were withdrawn from the marginal ear vein of each immunized rabbit for 7 consecutive days. Further such periodic injections resulted in the accumulation of high antibody titer. Serum was fractionated by bringing it up to 42% ammonium sulfate saturation. The precipitate obtained after centrifugation was dissolved in a small volume of 0.9% NaCl solution and dialyzed exhaustively against saline. Anti-transcortin activity of rabbit sera was assayed by the double diffusion method of Ouchterlony (11). Sera of rabbits prior to immunization did not contain antibodies against human transcortin. Sera with high anti-transcortin activities from the same rabbit were pooled, fractionated, and aliquots were labeled with fluorescein by the method of Coons (12).

Localization of Fluorescein-labeled Transcortin-specific Antibody—Forty milliliters of whole blood from individuals with chronic lymphocytic leukemia were collected and sedimented in 20-ml heparinized vacutainer tubes (1.5 x 16.5 cm) at 10° for a period of 2 hours. The buffy coat rich in lymphocytes was gently
removed and was centrifuged at 1000 \times g \text{ for } 10 \text{ min}; \text{ the pelleted lymphocytes were suspended in } 15 \text{ ml of ice-cold saline, and were washed five times. The last pellet of washed lymphocytes was suspended in } 2 \text{ ml of saline and a smear of this suspension was made on glass slides and air-dried. Fluorescein-labeled transcor tin antibody (diluted 1:40 with saline) was layered over the smear, the slide incubated at } 10^\circ \text{C for } 15 \text{ min, rinsed with three changes for } 20 \text{ min in ice-cold phosphate-buffered saline, pH 7.4, and cover slips mounted with } 50\% \text{ glycerol-buffered saline. In another set of procedures an aliquot of ly mphocytes in saline was incubated for } 15 \text{ min with equal volumes of fluorescein-labeled transcor tin antibody (1:20), centrifuged at } 1000 \times g \text{ for } 10 \text{ min, suspended in saline-phosphate buffer and divided into two portions. One portion was smeared onto glass slides and mounted. No fluorescence was detected. The other portion was smeared and layered with the fluorescein-labeled transcor tin antibody (1:40) and processed as described; these latter slides exhibited fluorescence. These results together suggest that fluorescence is not due to antigens that are bound to the membrane but occurs only after the disruption of the plasma membrane, thus allowing penetration of the antibody.}

**Purification of Lymphocytes**—The remainder of the buffy coat was centrifuged at 1000 \times g and the plasma obtained was transferred and stored at \(-20^\circ \text{C} \text{ until further use. The pellet of lymphocytes (with some neutrophils and other white blood cells) was resuspended in autologous plasma and subsequently processed by the lymphocyte purification procedures of Rabonowitz (13) which yield upward of 99\% pure preparations of lymphocytes. Purified lymphocytes suspended in autologous plasma were washed six times with saline at 4^\circ \text{C} by centrifugations at 1000 \times g. The washed pellets were rapidly frozen in isopentane at \(-90^\circ \text{C} \text{ and stored at this temperature until further use. Frozen pellets of lymphocytes were mounted in Tissue Tek 30, brought up to \(-30^\circ \text{C}, \text{ and sectioned in a cryostat at a thickness of } 4 \mu\text{m}; \text{ the sections subsequently treated with fluorescein-labeled antibody according to the methods and schedules described above.}

**Preparation of Nuclei**—Frozen pellets of lymphocytes were slowly thawed in an ice bath, homogenized in 0.25 \text{ M sucrose with a tight-fitting, motor-driven glass homogenizer (300 rmp, using four up and down strokes, each for } 20 \text{ s), centrifuged at 600 \times g; smears from these “nuclear pellets” were made on glass slides, air-dried, and treated with fluorescein-labeled transcor tin antibody diluted 1:20 with 0.25 \text{ M sucrose-saline. After an incubation of } 15 \text{ min at } 10^\circ \text{C the slides were washed in buffered saline containing } 0.25 \text{ M sucrose, and cover slips mounted with } 50\% \text{ glycerol } 0.25 \text{ M sucrose-saline.}

**Fractionation of Lymphocytes**—Stored pellets of lymphocytes were slowly thawed in an ice bath, pooled (pooled material contained a total of 16.5 \text{ mg of DNA, as determined by the method of Burton (14)}), homogenized in 0.25 \text{ M sucrose-1 mM phosphate, pH 7.4, in a glass homogenizer as previously stated, and centrifuged at 2000 \times g \text{ for } 10 \text{ min at } 4^\circ \text{C. The pellet (2000 } \times g) \text{ containing nuclei and large membranes was suspended in } 1 \text{ ml of } 1 \text{ mM phosphate, pH 7.4, and temporarily set aside. The supernatant was centrifuged at 27,000 \times g, the pellet set aside, and the supernatant dialyzed exhaustively against three changes of 4 liters of } 1 \text{ mM phosphate for } 48 \text{ hours at } 10^\circ \text{C, and centrifuged at 105,000 } \times g \text{ for } 3 \text{ hours at } 4^\circ \text{C. This last centrifugation yielded the cytosol fraction of the lymphocyte. The pellets of the } 2000, 27,000, \text{ and } 105,000 \times g \text{ centrifugations were homogenized in } 1 \text{ ml of } 0.35 \text{ M NaCl, centrifuged at } 105,000 \times g \text{ for } 3 \text{ hours, the pellets discarded, and the supernatants exhaustively dialyzed against } 1 \text{ mM phosphate. Lymphocyte preparation and fractionation procedures are presented in Scheme 1. Each of the final fractions as well as our preparation of pure transcortin were: (a) assayed for cortisol-binding activities by competitive adsorption assays of De Moor (15), by Sephadex G-25 filtration (16), and by equilibrium dialysis (17); (b) tested for the effect of p-MB \text{ and HgCl}_2 \text{ on cortisol binding; (c) assayed by means of the Ouchterlony procedure for the presence of transcortin-like protein; and (d) centrifuged in sucrose gradients of 5 to } 20\% \text{ in order to determine sedimentation characteristics of the cortisol-binding proteins.}

**Cortisol Binding Assays and Effects of Buffers and Sulphydryl Reagents**—Determination of cortisol-binding activity by the competitive adsorption method of De Moor was performed as follows. Of each fraction, 0.5 mg was diluted to 1 ml with 1 \text{ mM phosphate, pH 7.4, and incubated with } 10^6 \text{ cpm of } [1,2^-\text{H}]\text{cortisol (Schwarz-Mann, specific activity of } 35 \text{ Ci per mole). The mixture was incubated for } 1 \text{ hour in a shaking bath at } 4^\circ \text{C. At the end of the incubation period, } 100 \text{ mg of Norit A were added to each mixture and incubation continued for an additional hour at the same temperature. The mixtures were then centrifuged at } 4^\circ \text{C at } 20,000 \times g \text{ for } 10 \text{ min in a swinging bucket rotor and aliquots of } 0.5 \text{ ml of the supernatant were placed into scintillation vials containing } 15 \text{ ml of Bray's scintillator solution, equilibrated overnight at } 10^\circ \text{C}, \text{ and activities obtained with the aid of a Picker Anisotron II liquid scintillation counter (efficiency of } 42\% \text{ for tritium); appropriate controls without protein, afforded corrections.}

Determination of cortisol-binding activity by Sephadex G-25 filtration was performed as follows. One milligram of each fraction was dissolved in 1 ml of 1 \text{ mM phosphate, pH 7.4, and incubated with } 10^6 \text{ cpm of } [1,2^-\text{H}]\text{cortisol for } 1 \text{ hour at } 4^\circ \text{C and applied to a Sephadex G-25 column (1.5 } \times 35 \text{ cm}) \text{ previously equilibrated with } 1 \text{ mM phosphate buffer, pH 7.4. The column was eluted at a flow rate of } 2 \text{ ml per min with the same buffer and fractions of } 1 \text{ ml collected. All procedures were performed at } 4^\circ \text{C.}

Determination of cortisol binding by the method of equilibrium dialysis was as follows. Tubular dialysis membranes, 0.5 \text{ cm in diameter, were washed, rinsed, and equilibrated with } 1 \text{ mM phosphate, pH 7.4, at } 4^\circ \text{C. One milligram of protein of each fraction in } 1 \text{ ml of same buffer was placed into the dialysis bag and each bag immersed in } 1 \text{ liter of } 1 \text{ mM phosphate buffer, pH 7.4, containing } 1000 \text{ cpm of } [1,2^-\text{H}]\text{cortisol per ml of buffer. Continuous mixing was carried out. Equilibrium was reached by } 48 \text{ hours as indicated by periodic sampling. Corrections required for volume changes due to periodic sampling of the contents inside and out of the bags were performed. Corrections for the counts per min due to free cortisol were accomplished by the subtraction of those activities of the dialysis bags containing no protein from those that contained the fractions.}

For those studies involving the effects of the sulphydryl binders, p-MB \text{ and mercuric chloride, on the extent of binding of cortisol in equilibrium dialysis systems and the effect of dialysis on such binding (ascertained by Sephadex G-25 filtration methods), the precise conditions employed will be found in the respective legends of the tables. Determination of protein was performed by the method of Lowry et al. (18), and the specific activities of cortisol binding are given as counts per min of } [1,2^-\text{H}]\text{cortisol per mg of protein.}

**Determination of Sedimentation Coefficient**—Of each fraction 0.5 mg in 0.5 ml of phosphate buffer was incubated with } 10^6 \text{ cpm of } [1,2^-\text{H}]\text{cortisol for } 1 \text{ hour at } 4^\circ \text{C and subsequently layered onto }

\text{1 The abbreviation used is: p-MB, p-mercuribenzoate.}
Whole blood sedimented at 4° for 2 hours

Shrift coat (rich suspension of W. B. C.)

Purification of lymphocytes via glass bead column of Habermann (12)

Purified lymphocytes 1000xg

Lymphocyte pellet—resuspend in saline 1000xg

Pellet washed lymphocytes Repeat 5 times

Purified washed lymphocytes—Suspension in 0.001 M Phosphate, pH 7.4—0.05 M Sucrose

Homogenize

Dialyze against 0.001 M Phosphate, pH 7.4

20,000 xg/30 minutes

Supernate

Homogenize in 0.35 M NaCl

105,000 xg/3 hours

Pellet of crude membranes

Dialyze against 0.001 M Phosphate, pH 7.4

27,000 xg Extract

Supernate

0.40% Ammonium Sulfate Saturation

20,000 xg/30 minutes

Pellet discarded

Pellet—homogenize in 0.35 M NaCl

105,000 xg/3 hours

Dialyze against 0.001 M Phosphate

105,000 xg Extract

Supernate

Dialyze against 0.001 M Phosphate, pH 7.4

20,000 xg/30 minutes

Pellet discarded

Supernate adjusted to 65% A5

40% A5 Cet

Pellet suspended in 0.001 M Phosphate
dialyzed against 0.001 M Phosphate

40-85% A5 Cet

Purified protein according to procedure of Mlodow and Westphal

Lymphocyte contract binding protein

Scheme 1. Lymphocyte preparation and fractionation.
5 to 20% sucrose gradient and centrifuged under those conditions employed for the determination of the sedimentation coefficient of transcortin.

**General Procedures**—The lymphocyte fractions showing precipitin lines and highest cortisol binding were purified by the method employed for the purification of transcortin. Disc acrylamide gel electrophoresis of purified transcortin and the purified cell extract was performed according to the methods of Ornstein (9) and Davis (10). Fluorescence microscopy of those preparations involving the fluorescein-labeled antibody was performed with a Leitz ultraviolet microscope.

**RESULTS**

**Polymerization of Transcortin**—Results of amino acid analysis of the transcortin preparation are presented in Table I and compared to the calculated averages of those analyses performed by three other laboratories (8, 19, 20). It is readily evident that our values are in agreement with those of others. The molar binding affinities of our transcortin preparation for cortisol at 4 and 37° were similar to those previously published (8). Disc acrylamide gel electrophoresis of a solution of freshly purified (180 μg) human transcortin yielded a single band. However, transcortin stored in solution at 4° for 1 week yielded two bands. The front running band of the “aged” transcortin had a mobility identical with that of the single band of the fresh preparation. The “aged” transcortin when subjected to electrophoresis in the presence of 8 M urea yielded a single band (Fig. 1). Ultracentrifugation in sucrose gradients of RNase, human albumin, and peroxidase, all of known S values, allows the calculation of a sedimentation coefficient for the transcortin preparation. The calibration of our system is presented in Fig. 2. The fractionation of the transcortin preparation (Fig. 3) after ultracentrifugation indicated a single homogenous peak corresponding to a sedimentation value of 3.75, a value consistent with that obtained by others for human transcortin (8). The fractions containing transcortin were pooled and exhaustively dialyzed at 4° (three changes in 3 days) against 1 mM phosphate, pH 7.4, in order to remove sucrose. The dialyzed portion (0.6 ml) was layered onto 5 to 20% sucrose gradients and ultracentrifugation repeated. Fig. 4 illustrates the presence of two protein peaks, one corresponding to an S value of about 3.8 and the other peak, of unknown S value, was found at the bottom of the gradient. Only that absorbance peak representing unadulterated transcortin contained radioactive cortisol. Fractions under each protein peak were pooled into two respective fractions and each subsequently dialyzed and lyophilized. Electrophoresis of each of these fractions revealed two stained bands for each peak. When urea was added to each, a single band resulted. These results collectively indicate that contrary to previously published reports (8, 19, 20), human transcortin, as does that of the rat (21) and

**TABLE I**

Amino acid composition of purified transcortin

Comparison of calculated average values obtained from data of Muldoon et al. (8), Schneider et al. (20), and Seal et al. (19) (Column A) to those obtained by us (Column B).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Column A</th>
<th>Range of values</th>
<th>Column B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>4.33</td>
<td>3.91-5.03</td>
<td>4.85</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.39</td>
<td>3.14-3.75</td>
<td>3.27</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.65</td>
<td>5.00-6.05</td>
<td>6.00</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.81</td>
<td>8.91-10.81</td>
<td>10.15</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.73</td>
<td>5.41-6.05</td>
<td>5.41</td>
</tr>
<tr>
<td>Serine</td>
<td>6.06</td>
<td>5.62-6.42</td>
<td>5.72</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.84</td>
<td>12.36-13.38</td>
<td>12.60</td>
</tr>
<tr>
<td>Proline</td>
<td>6.12</td>
<td>5.12-7.40</td>
<td>6.40</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.56</td>
<td>2.89-4.20</td>
<td>4.14</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.92</td>
<td>4.34-5.49</td>
<td>4.99</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.43 a</td>
<td>0.32-0.54</td>
<td>0.49</td>
</tr>
<tr>
<td>Valine</td>
<td>6.27</td>
<td>5.00-7.15</td>
<td>5.51</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.38</td>
<td>1.20-3.52</td>
<td>2.41</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.20</td>
<td>3.27-4.70</td>
<td>4.00</td>
</tr>
<tr>
<td>Leucine</td>
<td>12.63</td>
<td>11.98-12.97</td>
<td>11.95</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.67</td>
<td>2.91-4.22</td>
<td>2.44</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.35</td>
<td>6.86-7.91</td>
<td>6.21</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.87 a</td>
<td>1.49-2.18</td>
<td>1.85</td>
</tr>
</tbody>
</table>

a Only two values are available.

**FIG. 1.** Disc acrylamide gel electrophoresis of “aged” purified human transcortin in absence (a) and presence (b) of urea. Disc acrylamide electrophoretic gels were prepared as described by Davis (10) with the exception that the separation gels were composed of 10% acrylamide. The gel on the right (b) has the buffer made up in 8 M urea. A total of 280 μg of “aged” purified transcortin suspended in 100 μl of 0.5 M NaH2PO4 and 0.0001 M ascorbate, pH 7.4, or the same buffer containing 8 M urea was subjected to electrophoresis for 60 min at 5 ma per column, stained for 30 min in 0.1% Amido black in 7% acetic acid, and destained electrophoretically (10).
guinea pig (20) undergoes urea-reversible polymerization. During
the revision of this manuscript, Dr. William Rosner presented
evidence that human transcortin does indeed undergo polymer-
ization (22).

Reaction of Anti-transcortin with Transcortin and Plasmas in
Ouchterlony System—The result of the reaction between anti-
transcortin and dilutions of up to 1:16 of transcortin in Ouchter-
lony plates is depicted in Fig. 5. The presence of two distinct
precipitin bands is evident between the center well (antibody) and
Wells 1, 2, and 3. Although not readily seen in this photo-
graph, Well 4 also has two precipitin bands. At higher dilutions
(up to 1:256) only the inner band remains visible (not shown).
Absorption of the antibody with pure transcortin precludes the
formation of any precipitin band. Clearly then a fresh prepara-
tion of purified transcortin which appears pure by each criterion
employed, yields two well defined precipitin bands in the Ouch-
terlony system. It is our interpretation that the precipitin line
closest to the antibody well and appearing earliest in time is the
monomeric form of the transcortin molecule and corresponds to
the front running band in disc acrylamide electrophoresis of the
“aged” transcortin. The outer precipitin band (i.e., farthest
from the antibody well) we interpret as a polymer (possibly a
dimer) of transcortin, and this would obviously correspond to

Fig. 2. Ultracentrifugation of markers of known S values and
purified human transcortin. Bovine pancreatic RNase (0.5 mg),
human albumin, and peroxidase in 0.5 ml of 10⁻¹ M phosphate
buffer, pH 7.4, were layered onto 5 to 20% sucrose gradients
(prepared with same buffer), and centrifugations performed as
described in text.

Fig. 3 (left). Sucrose gradient ultracentrifugation and frac-
tionation profile of purified transcortin, freshly prepared. Solid
line, [3H]cortisol activity; dotted line, absorbance at 280 nm.
Note that peak absorbance and [3H] activity correspond to Fraction
32 of the gradient. T, top of gradient; B, bottom of gradient.

Fig. 4 (right). Sucrose gradient ultracentrifugation of trans-
cortin previously ultracentrifuged in sucrose (single component),
fractionated, and subsequently dialyzed for 3 days. Solid line,
[3H]cortisol activity; dotted line, absorbance at 280 nm. [3H]corti-
sol peak at top of gradient represents free cortisol. Main peak
of activity is found at Fraction 32. Note that at the bottom of
the gradient there is protein of unknown sedimentation value not
found during the initial centrifugation.

Fig. 5 (top). Double diffusion in Ouchterlony plates of serial
dilutions of freshly purified human transcortin against rabbit
anti-transcortin. Wells 1 to 6 contain 6, 3, 1.5, 0.75, and 0.375
µg of transcortin, respectively. After the development of precipi-
tin lines, the gels were carefully removed and rinsed in saline
(three changes/24 hours) for 72 hours, stained with Ponceau S
in 3% acetic acid, and free stain removed by extensive washing
in distilled water. Note the presence of continuous inner precipi-
tin line. The outer precipitin line (from central well) is readily
evident in Wells 1 to 3 and barely visible in Well 4.

Fig. 6 (bottom). Double diffusion in Ouchterlony plates of
purified human transcortin (1), normal plasma (2 and 4), chronic
lymphocytic leukemic plasma (3), normal plasma (6), against
rabbit anti-transcortin (central well). Note continuous lines of
identity of transcortin with all plasmas employed.
the slower, "urea-eliminated" band after electrophoresis of the "aged" transcortin.

The possibility that either precipitin band is due to the presence of an antibody produced as a result of a minor contaminant in the transcortin preparation employed for eliciting an antibody in rabbits may be dismissed by the following argument. The outer precipitin band (Fig. 5) is heavily stained at Well 1 and gradually undergoes a decrease in intensity as well as a greater degree of separation from the inner band with serial dilution. The inner band, however, increases its relative intensity with dilution such that even with very high dilutions it is still very evident whereas the outer band continues to diminish in its stained intensity. Thus, if one then envisages the formation of a polymer as a function of concentration and time, then it follows that if time is kept constant, the formation of a polymer decreases with decreased concentration. As a result of this, more monomer will be available in the Ouchterlony system at the zone of precipitin formation. Therefore, the inner band remains and in fact increases its intensity of staining (in certain dilutions) with dilution. If on the other hand an impurity were to be present, then both bands would decrease concomitantly in staining intensity with dilution.

Plasma from normal humans and patients with chronic lymphocytic leukemia form two precipitin bands showing identity with those of transcortin (Fig. 6). Here again we see evidence that either one of the precipitin lines cannot be due to a trace impurity, insuspect as it is highly unlikely that such a hypothetical impurity would be maintained at its relative plasma concentration during extensive purification of transcortin. Since previous results (23) indicated that the cortisol binding capacity of plasma from leukemic patients was less than one-half that of plasma from the normal, and results presented indicate complete antigenic identity between the two types of plasma, diminished plasma-binding capacity may therefore indicate (a) lower content of plasma transcortin in chronic lymphocytic leukemia, or (b) modification of transcortin not involving the antigenic determinants.

**In Situ Localization of Transcortin-like Protein of Human Lymphocyte by Immunofluorescence**—The use of fluorescein-labeled transcortin-specific antibody affords a method by which one may detect the presence of transcortin within the cell. To this end, smears of washed lymphocytes were made and the fluorescein-labeled antibody applied. Fig. 7 illustrates the presence of proteins antigenically similar to transcortin as denoted by fluorescence. Fluorescence is not due to surface-bound transcortin since control experiments with lymphocytes incubated in fluorescein-labeled antibody, washed, and subsequently used for smears, failed to show any fluorescence. The localization of fluorescence appears to be almost entirely in the cytoplasm and not on the nuclear membrane since similarly treated smears of purified lymphocyte nuclei lacked any membrane fluorescence. Frozen sections of pellets of purified lymphocytes gave identical results. However, in some cases, pinpoint fluorescence was localized within the chromatin (Fig. 8, see arrows). In all cases, fluorescence could be precluded by the prior application of unlabeled anti-transcortin or absorption of antibody by pure transcortin. Other cells such as red blood cells, gastric epithelial cells, and smooth muscle cells gave negative results.

**Reaction of Anti-transcortin with Subcellular Fractions of Human Lymphocyte**—From the above results it appears that there is a protein with antigenic determinants of transcortin in the cytoplasm of the lymphocyte. Crude homogenates of lymphocytes from patients with chronic lymphocytic leukemia showed two main precipitin lines forming lines of identity with those of transcortin (Fig. 9). Furthermore, at least two additional very faint precipitin lines appear. However, these additional lines require more than a week's time for their formation, whereas the others develop much earlier. We interpret these findings as an indication that in the lymphocyte, the protein (or proteins) recognized by our antibody is (are) capable of undergoing aggregation or polymerization.

Fractionations of the lymphocyte crude homogenate by multiple centrifugations and with ammonium sulfate yielded an inner precipitin band splitting at wells labeled 2000 (2000 × g), 27,000 (27,000 × g), and 105,000 (105,000 × g), and 40% ammonium sulfate (A.S.) and continuous with that at Well 65% A.S. (Fig. 10). The outer band present only in Wells 40 and 65% A.S. occurs later in time and hence is not expected, in accordance to our previous interpretation, to form lines of identity with the inner bands.

**Determination of Cortisol Binding by Subcellular Fractions of Human Lymphocyte and Effects of Sulfhydryl Reagents**—We have determined the cortisol-binding capacities for each of the above subcellular fractions by the competitive absorption method of De Moor, Sephadex G-25 filtration, and equilibrium dialysis. The results shown in Table II show that all fractions were capable of binding cortisol to varying degrees, the highest specific activity being that of the 40 to 65% ammonium sulfate fraction in all procedures. It should be stated, that data obtained by equilibrium dialysis represent the summation of both specific and nonspecific binding whereas those obtained by the other two methods is representative of specific binding alone. When we
employed the method of equilibrium dialysis as a means of determining the effect of EDTA, ascorbate, and mercaptoethanol on the binding of cortisol by transcortin and lymphocyte fractions. It is evident that all of the proteins exhibited greater degrees of cortisol binding (Table III) in the presence of mercaptoethanol. In order to determine whether mercaptoethanol activated or simply protected the sites involved in binding, Sephadex C-25 filtration assays for cortisol-binding capacities were conducted on all fractions prior to and after dialysis in both phosphate buffer alone, and buffer containing mercaptoethanol. It is clear from the data presented in Table IV that dialysis itself reduces the ability of all fractions to bind cortisol specifically. With the exception of transcortin all other fractions are more active in the system dialyzed with buffer containing mercaptoethanol and indeed surpass the activities prior to dialysis. Since mercaptoethanol appears to affect binding of the steroid an attempt was

**Figure 8.** Immunofluorescence localization of transcortin-like protein within sectioned lymphocytes. Pellets of extensively washed lymphocytes were rapidly frozen in isopentane at -90°, mounted, and sectioned at a thickness of 4 µm. The sections were transferred to slides, air-dried, and processed for immunofluorescence microscopy as previously stated in Fig. 7. Sectioned lymphocytes denote various sized rims of fluorescence as well as sporadic granules of fluorescence within the nuclei. These granules are not readily evident in whole lymphocytes, being possibly due to the masking of such granules by the fluorescence of underlying and overlying cytoplasm present in the whole lymphocyte.

**Figure 9 (left).** Double diffusion in Ouchterlony plates of purified transcortin (T) and cell extract (CE, and CE₂) against anti-transcortin. Extensively washed lymphocytes were homogenized as stated in the text, and centrifuged at 2000 x g. The supernatant fluids were retained for antigenic activity. Note the presence in the transcortin wells of two well defined precipitin lines forming continuous lines of identity with those of the cell extracts. CE₁ shows two additional precipitin lines. These are interpreted as evidence for states of higher levels of polymerization of the transcortin-like protein of the human lymphocyte. **Figure 10 (right).** Double diffusion in Ouchterlony plates of lymphocyte fractions against anti-transcortin antibody. Note that all fractions assayed exhibit precipitin activities. A.S., ammonium sulfate.

**Table II**

<table>
<thead>
<tr>
<th>Competitive Adsorption</th>
<th>Equilibrium Dialysis</th>
<th>Sephadex G-25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcortin</td>
<td>5,250</td>
<td>21,000</td>
</tr>
<tr>
<td>2000 x g extract</td>
<td>142</td>
<td>475</td>
</tr>
<tr>
<td>0 to 40% ammonium sulfate</td>
<td>79</td>
<td>10</td>
</tr>
<tr>
<td>40 to 65% ammonium sulfate</td>
<td>421</td>
<td>570</td>
</tr>
</tbody>
</table>

**Table III**

Cortisol binding by transcortin and subcellular lymphocyte fractions in equilibrium dialysis systems employing additions to buffer

<table>
<thead>
<tr>
<th></th>
<th>1 mM Na₃PO₄</th>
<th>1 mM Na₃PO₄ + 0.1 mM EDTA</th>
<th>1 mM Na₃PO₄ + 0.1 mM ascorbate</th>
<th>1 mM Na₃PO₄ + 1 mM mercaptoethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcortin</td>
<td>21,000</td>
<td>24,000</td>
<td>27,000</td>
<td>30,000</td>
</tr>
<tr>
<td>2000 x g extract</td>
<td>475</td>
<td>1,270</td>
<td>2,970</td>
<td>2,980</td>
</tr>
<tr>
<td>0 to 40% ammonium sulfate</td>
<td>10</td>
<td>250</td>
<td>510</td>
<td>650</td>
</tr>
<tr>
<td>40 to 65% ammonium sulfate</td>
<td>570</td>
<td>5,000</td>
<td>11,260</td>
<td>12,890</td>
</tr>
<tr>
<td>105,000 x g extract</td>
<td>0</td>
<td>550</td>
<td>1,010</td>
<td>1,430</td>
</tr>
</tbody>
</table>
made to preclude or decrease binding by the addition of sulphydryl-binding agents. Table V illustrates that p-mercuribenzoate as well as HgCl₂ eliminates binding of cortisol by transcortin as well as by lymphocyte fractions.

### Table IV

**Effect of dialysis on binding of cortisol by transcortin and lymphocyte subcellular fractions determined by Sephadex G-25**

One milligram of each fraction was dialyzed for 48 hours at 4°C in 1 mM phosphate, pH 7.4, alone or with 1 mM mercaptoethanol. After dialysis the fractions were incubated with 10⁶ cpm of [1,2-³H]cortisol for 1 hour at 4°C and determination of cortisol binding obtained by the method of Sephadex G-25 filtration.

| Predialysis | Postdialysis in 1 mM NaPO₄ | Postdialysis in 1 mM NaPO₄ and 1 mM mercaptoethanol
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcortin</td>
<td>57,000</td>
<td>12,100 (−79)</td>
</tr>
<tr>
<td>2000 × g extract</td>
<td>565</td>
<td>380 (−33)</td>
</tr>
<tr>
<td>0 to 40% ammonium sulfate</td>
<td>690</td>
<td>000 (−13)</td>
</tr>
<tr>
<td>40 to 65% ammonium sulfate</td>
<td>2400</td>
<td>720 (−67)</td>
</tr>
</tbody>
</table>

### Table V

**Effect of HgCl₂ and p-mercuribenzoate on binding of cortisol by transcortin and subcellular fractions of human lymphocyte**

Determination of cortisol binding was performed by the stated method of equilibrium dialysis.

<table>
<thead>
<tr>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM HgCl₂</td>
</tr>
<tr>
<td>Transcortin</td>
</tr>
<tr>
<td>2000 × g extract</td>
</tr>
<tr>
<td>0 to 40% ammonium sulfate</td>
</tr>
<tr>
<td>40 to 65% ammonium sulfate</td>
</tr>
</tbody>
</table>

**Sedimentation Values of Cortisol-binding Proteins of Human Lymphocyte—**

Sedimentation gradient ultracentrifugation of the lymphocyte fractions after incubation with radioactive steroid revealed that in both the nucleus and the 40 to 65% ammonium sulfate fraction there exists a cortisol-binding protein with sedimentation behavior similar to that of transcortin (Fig. 11).

**Characteristics of Semipurified Protein of Human Lymphocyte Exhibiting Greatest Cortisol-binding Ability—**

Inasmuch as the 40 to 65% fraction contained up to 90% of the total binding capacity of the cell, it was purified (see “Materials and Methods”). Ninety per cent of the purified preparation (by densitometry) ran as a single band in disc acrylamide electrophoresis with a mobility greater than that of purified transcortin (Fig. 12). It formed a single precipitin line of identity continuous with the inner line of both transcortin and plasma (Fig. 13). Its cortisol-binding activity was about 20% that of purified transcortin (Table VI). Repeated freezing and thawing of this preparation yielded two precipitin lines identical with those of transcortin.

**DISCUSSION**

In this study evidence is provided showing that human lymphocytes contain in their cytoplasm, and, to some extent in their nucleus, a protein or proteins with antigenic determinants closely similar to those of transcortin and which show a series of characteristics similar if not identical with transcortin. These are: (a) strong cortisol-binding capacities demonstrable by a variety of cortisol-binding assays, (b) their ability to bind cortisol is affected by buffers and sulfhydryl binders in a fashion similar to that of transcortin; and (c) it exhibits sedimentation characteristics in 5 to 20% sucrose gradients that are identical with transcortin.

Further purification of the lymphocyte fraction with the greatest cortisol-binding activity (40 to 65% ammonium sulfate precipitation of the cytosol) yielded primarily a single protein with some physicochemical characteristics slightly different from those of transcortin. These differences are: (a) less binding activity, (b) greater mobility in disc acrylamide gel electrophoresis, and (c) less tendency to form aggregates.

![Fig. 11. Ultracentrifugation in 5 to 20% sucrose gradients of subcellular lymphocyte fractions previously bound with [³H]cortisol. Solid line, [³H]cortisol activities; dotted lines, absorbance at 280 nm. A, fractionation of nuclear fraction (0.35 M NaCl extract of the 2,000 × g fraction). B, fractionation of the 0 to 40% ammonium sulfate of the 105,000 × g cytosol. C, fractionation of the 40 to 65% ammonium sulfate of the 105,000 × g cytosol. T, top of gradient; B, bottom of gradient.](http://www.jbc.org/content/265/17/6405)
The lack of complete identity of the lymphocyte protein to transcortin might be due to the following possibilities.

1. Homogenization and further handling of crude lymphocyte fractions artificially yields a partially enzymatically degraded transcortin.

2. Transcortin-cortisol complex enters the cell whereupon it is enzymatically cleaved, conceivably at the binding site terminus, thereby resulting in a smaller protein molecule with decreased cortisol-binding capacity.

3. The lymphocyte, as does the liver cell, synthesizes a transcortin-like protein.

The first possibility is feasible inasmuch as other investigators have encountered difficulties in the purification of steroid-binding proteins due to the nonspecific enzymatic degradation of such proteins during preliminary fractionation (24). It has also been recently stated that this difficulty might be overcome by the introduction of triamcinolone in the homogenization medium (7). It is interesting to note that removal of steroid from transcortin results in loss of binding ability in some cases (25). Similar observations have recently been stated in the case of partial purification of cortisol-binding proteins of rat liver (26).

The second alternative is attractive since previous preliminary studies by us demonstrated that the uptake of cortisol was influenced by serum transcortin (27) and that this process, as well as the extrusion of the hormone, was energy-dependent. It might be that the process of hormone extrusion is initiated by the postulated action of an enzyme, thus releasing cortisol to be extruded, while the altered transcortin remains to act on the protein-synthesizing system. If this is the case, then it might be that this process not only results in a decrease of cortisol binding by the altered transcortin molecule, but creates as well the ability to bind dexamethasone, a synthetic steroid reportedly not bound by rat transcortin (28). Although others utilize this difference to infer differences between the two proteins, they are perhaps in reality examining proteins before and after such postulated conversion. In any event, in studies to be reported elsewhere, our transcortin antibody also recognized those cytosol proteins capable of binding the synthetic glucocorticoid. Furthermore, we have found that during the purification of transcortin, the ability of this protein to bind dexamethasone is gradually lost.


Manuscript in preparation.
Studies by others have clearly shown that the killing action of cortisol on lymphoid cells is directly dependent upon the presence and number of specific intracellular cortisol receptor proteins (6, 28). Our studies suggest that such receptor proteins are very similar, if not identical, to plasma transcortin. The origin of such proteins is of vital concern and interest inasmuch as it is currently thought that plasma transcortin is exclusively synthesized by the liver (29–32). The lymphocyte might also synthesize transcortin. In order to investigate this possibility, we have applied our fluorescein-labeled transcortin-specific antibody to cultured human lymphocytes that have never been exposed to human serum. The presence of relatively weak fluorescence was noted. However, we have found that our transcortin-specific antibody formed weak precipitin lines with the fetal calf serum employed in the above cultures. We still do not know whether the intracellular transcortin-like protein of the human lymphocyte is synthesized by this cell or is of plasma origin. In any event, the evidence presented in this study, indicates that the human lymphocyte contains a protein (perhaps proteins) with many of the well defined characteristics of plasma transcortin. It remains for further studies, some of which are in progress, to ascertain the origin of this protein.

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Identification and Partial Purification of "Transcortin"-like Protein within Human Lymphocytes
Seymour Werthamer, Arthur J. Samuels and Leonard Amaral


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