**Estrogen Receptor Analysis in Chronic Lymphocytic Leukemia**

By Steven T. Rosen, Zosia Maciorowski, Frederick Wittlin, Alan L. Epstein, Leo I. Gordon, Merrill S. Kies, Omer Kucuk, Hau C. Kwaan, Huibert Vriesendorp, Jane N. Winter, Ellen Fors, and Agostino Molteni

Estrogen receptor (ER) determinations were performed on cytosol preparations of Ficoll-Hypaque density separated mononuclear cells from 11 patients with chronic lymphocytic leukemia (CLL). The presence of ER was noted in 8 of 11 specimens (73%). ER ranged from 431 fmole/mg to 4.3 fmole/mg cytosol protein. Two types of receptor subunits were observed at the 8S and 4S region of the sucrose gradient. In addition, 1 of 3 Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines from healthy donors had a measurable amount of ER. Patient R.L., who was refractory to standard chemotherapy and radiation and was ER positive, experienced a minor response to Tamoxifen therapy, with subsequent loss of ER. The demonstration of ER in CLL suggests that this malignancy may have a hormone-dependent subpopulation of cells.

**Chronic Lymphocytic Leukemia (CLL)** represents a clonal proliferation of neoplastic lymphocytes, usually of B-lymphocyte derivation. The disease initially has an indolent course; however, an expected median survival of less than 2 yr is witnessed when bone marrow function is compromised. Conventional chemotherapy and radiation therapy, though providing effective palliation, rarely result in complete remissions. Results of salvage regimens for the patient refractory to standard therapy have not been encouraging.5

Narasimhan recently reported a patient with CLL that was refractory to conventional combination chemotherapy who responded to treatment with diethylstilbestrol. This suggested the presence of estrogen receptor (ER) and the possible hormone dependence of at least some lymphocytes involved in CLL. ER has been detected in rat thymus cells, and investigators have demonstrated thymocyte lysis and thymus involution in estrogen-treated animals. It has been reported that many neoplasms other than those of the breast and of the female genitalia have estrogen-receptor proteins, including renal cell carcinoma, melanoma, colon carcinoma, prostatic cell carcinoma, carcinoid, pancreatic carcinoma, pleomorphic adenocarcinoma of the parotid gland, squamous cell carcinoma of anterior portion of the floor of the mouth, and Hodgkin's disease, although for some of these tumors, the specificity of the receptors is still under question. In this study, peripheral tumor cells from patients with CLL were examined for the presence of ER.

**MATERIALS AND METHODS**

**Estrogen Receptor Binding Activity Assay**

Fifty milliliters of heparinized (100 U/ml preservative-free) peripheral blood was obtained from 11 patients with CLL. The cells were washed twice with phosphate-buffered saline (PBS) and the mononuclear cells were separated by Ficoll-Hypaque density centrifugation. Cell counts ranged from $1.6 \times 10^9$ to $5.0 \times 10^9$. The mononuclear cell population was then washed twice in PBS and resuspended in 1 mM dithiothreitol (Sigma, St. Louis, MO) in a Tris buffer (pH 7.4). Suspended cells were lysed by sonication, spun in a microfuge for 2 min, and the supernatant was prepared by ultracentrifugation (34 K for 30 min). All procedures were performed at 4°C.

The ER binding activity was measured using the method of Jensen et al. which identifies the estradiol receptor and separates it from nonspecific binding proteins.

The supernatant collected after sonication and ultracentrifugation of the specimen is designated the cytosol fraction. A 150-μl portion of this fraction is incubated with buffer and another 150-μl portion is incubated with Parke-Davis CI-628, an inhibitor to estradiol. After the addition of tritium-labeled 17β-bound estradiol, each portion is layered on a discontinuous sucrose density gradient and ultracentrifuged for 16 hr. Successive 100-μl fractions are collected from each tube, and the radioactivity is determined in each fraction utilizing a commercial scintillation medium. Specific binding in both 8S and 4S regions is calculated by the difference between the sedimentation patterns in the absence and the presence of the estradiol inhibitor (Parke-Davis CI-628). The irradiated estradiol saturates the cytosol receptor, and this protein complex can be estimated from the area of the 8S sedimentation peak. A protein complex can be measured in the 4S area of some cytosols. Often, much of this complex can be washed off with a dextran charcoal incubation step just before the 16 hr of centrifugation.

The binding activity is reported as fmole/mg/ml cytosol protein. The protein content of these cytosols is determined by the micro method of Lowry. A value of 3 fmole/mg cytosol protein or greater was used as an indication of a measurable amount of estradiol receptor.

Three well-characterized EBV-transformed B-lymphoblastoid cell lines were processed in an identical fashion.

**Cytologic Evaluation**

Wright's stained cytospin preparations of the Ficoll-Hypaque density separated mononuclear cell populations were reviewed.
RESULTS

Estrogen Receptor Binding Activity

An outline of the ER determinations and patient characteristics is listed in Table 1. Eight of 11 patients (73%) showed ER binding activity that ranged from 431 fmole/mg cytosol protein to 4.3 fmole/mg cytosol protein. ER binding activity in both the 8S and 45 sedimentation peaks was noted in each instance. No correlation was noted among lymphocyte count, sex, race, time from diagnosis, clinical status, and ER binding activity. Of interest, the three oldest patients in our series (ages 69, 73, 74) were those without evidence of ER binding activity. One of the three

Table 1. Estrogen Receptor Analysis in Chronic Lymphocytic Leukemia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Diagnosis</th>
<th>Previous Treatment</th>
<th>Clinical Status</th>
<th>Lymphocyte Count Total</th>
<th>8S</th>
<th>4S</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.F.</td>
<td>52</td>
<td>M</td>
<td>B</td>
<td>1978 CH</td>
<td></td>
<td>A/S</td>
<td>14,000</td>
<td>431</td>
<td>0.1*</td>
<td>211/220</td>
</tr>
<tr>
<td>M.M.</td>
<td>52</td>
<td>F</td>
<td>W</td>
<td>1975 CH, COP</td>
<td></td>
<td>A/S</td>
<td>8,000</td>
<td>67.9</td>
<td>1.1</td>
<td>21.0/47.4</td>
</tr>
<tr>
<td>D.C.</td>
<td>51</td>
<td>M</td>
<td>W</td>
<td>1979 No Rx</td>
<td></td>
<td>A/S</td>
<td>76,000</td>
<td>28.3</td>
<td>2.3</td>
<td>6.6/21.7</td>
</tr>
<tr>
<td>E.B.</td>
<td>58</td>
<td>M</td>
<td>B</td>
<td>1975 CH, COP, RAD</td>
<td></td>
<td>A</td>
<td>130,000</td>
<td>21.9</td>
<td>5.2</td>
<td>6.8/15.1</td>
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<tr>
<td>R.L.</td>
<td>65</td>
<td>M</td>
<td>B</td>
<td>1977 CH, COP, RAD</td>
<td></td>
<td>A/S</td>
<td>178,000</td>
<td>16.4</td>
<td>4.1</td>
<td>14.4/2.0</td>
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<tr>
<td>R.S.</td>
<td>64</td>
<td>M</td>
<td>B</td>
<td>1977 COP</td>
<td></td>
<td>A/S</td>
<td>114,000</td>
<td>7.0</td>
<td>8.1</td>
<td>4.6/2.4</td>
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<tr>
<td>M.T.</td>
<td>60</td>
<td>M</td>
<td>W</td>
<td>1978 COP, RAD</td>
<td></td>
<td>A/S</td>
<td>172,000</td>
<td>5.5</td>
<td>5.7</td>
<td>4.6/0.9</td>
</tr>
<tr>
<td>L.R.</td>
<td>62</td>
<td>M</td>
<td>W</td>
<td>1976 No Rx</td>
<td></td>
<td>A/S</td>
<td>70,000</td>
<td>4.3</td>
<td>9.4</td>
<td>1.3/3.0</td>
</tr>
<tr>
<td>A.S.</td>
<td>73</td>
<td>M</td>
<td>W</td>
<td>1977 COP, Ad</td>
<td></td>
<td>A/S</td>
<td>31,000</td>
<td>No binding</td>
<td></td>
<td></td>
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<tr>
<td>H.B.</td>
<td>69</td>
<td>F</td>
<td>W</td>
<td>1981 COP</td>
<td></td>
<td>A/S</td>
<td>150,000</td>
<td>No binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R.W.</td>
<td>74</td>
<td>M</td>
<td>B</td>
<td>1981 CH, P</td>
<td></td>
<td>A/S</td>
<td>6,500</td>
<td>No binding</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B-lymphoblastoid lines

ERIC LB-2 25.0 (4.0/21.0) 1.0 |
ERIC LB-3 No binding
BL-1 No binding

A, adenopathy; S, splenomegaly; ERA, estrogen receptor assay; C, cytoxan; O, oncovin; P, prednisone; CH, chlorambucil; A, adriamycin; RAD, radiation.

*Very low protein is responsible for the disproportionately high value.

to insure that a greater than 95% lymphoid population was being studied.

Immunoglobulin Studies

The Ficoll-Hypaque separated peripheral blood CLL cells were phenotyped using monoclonal antibodies (Bethesda Research Laboratories, Bethesda, MD) directed against human heavy and light chain immunoglobulins. The CLL cells were air dried onto multwell slides (Cel-Line Associates, Newfield, NJ) and immediately fixed in 2% formaldehyde (Polysciences, Warrington, PA) and diluted in PBS for 10 min at room temperature. The slides were washed in PBS and then treated with acetone for 5 min at −20°C, rinsed, and stored at 4°C in PBS until use. Indirect immunofluorescence studies were performed with an FITC-conjugated goat anti-mouse [F(ab')2-specific] (Cappel Laboratories, Cochranville, PA) diluted 1:40 in PBS containing 1 mg/ml bovine serum albumin.

RESULTS

Estrogen Receptor Binding Activity

An outline of the ER determinations and patient characteristics is listed in Table 1. Eight of 11 patients (73%) showed ER binding activity that ranged from 431 fmole/mg cytosol protein to 4.3 fmole/mg cytosol protein. ER binding activity in both the 8S and 4S sedimentation peaks was noted in each instance. No correlation was noted among lymphocyte count, sex, race, time from diagnosis, clinical status, and ER binding activity. Of interest, the three oldest patients in our series (ages 69, 73, 74) were those without evidence of ER binding activity. One of the three
EBV-transformed B-lymphoblastoid lines also showed ER binding activity, with elevations in both the 8S and 4S sedimentation peaks. Two graphs of estrogen binding in lymphocyte cell suspensions are shown in Figs. 1A and 1B.

**Immunoglobulin Studies**

As shown in Table 2, all of the CLL cases expressed cytoplasmic IgM heavy chains, with the exception of patient D.C., whose cells contained IgG heavy chains. The monoclonal derivation of these tumors was demonstrated by their lambda/kappa exclusivity, and in all cases, greater than 95% of the cells were positive for cytoplasmic immunoglobulin by these indirect immunofluorescence techniques.

**Case Report**

Patient R.L., a 65-yr-old black male who was refractory to standard chemotherapy (chlorambucil, cytoxan, oncovin, and prednisone) and radiation treatment (total body irradiation, splenic irradiation) and had progressive disease, was treated with Tamoxifen 20 mg (10 mg/sq m) twice daily following the determination of his ER binding activity of 16.4 fmole/mg cytosol protein (14.4 fmole/mg cytosol protein at the 8S peak and 2.0 fmole/mg cytosol protein at the 4S peak). When therapy was initiated, the patient had extensive lymphadenopathy and hepatosplenomegaly, a peripheral lymphocyte count of 175,000/cu mm, a platelet count of 22,000/cu mm, and a hemoglobin of 7.5 g/dl.

The patient showed a subjective response, gained 5 kg, and had stabilization of his hematologic parameters. He no longer required biweekly blood transfusions (possibly an anabolic rather than a cytotoxic effect of Tamoxifen). The patient's lymphadenopathy and splenomegaly showed less than a 50% regression consistent with a minor response. Following Tamoxifen therapy for 4 mo, the patient's ER status was reassessed after discontinuing the drug. ER binding activity was no longer detectable at 2 wk and at 3 mo post Tamoxifen. Progressive disease was documented after the fourth month of hormone therapy, and therefore the Tamoxifen was discontinued.

**DISCUSSION**

The present report suggests that ER binding activity is present in the malignant lymphocytes of patients with CLL. The biological significance of the presence of receptors in this malignant population remains to be determined.

Estradiol receptors were found both in the 8S and the 4S sucrose density gradient regions. It is known that, in humans, the specific binding protein for estrogens migrates with the 8S fraction, while the 4S fraction usually contains serum estrogen binding proteins, as well as other sex hormone binding proteins. In 7 of the 8 patients where there was binding, the values of 8S fractions were above 3 fmole/mg cytosol protein, which is the cut-off point suggested by McGuire and other investigators for a definitely measurable amount of ER.

ER have been found in malignancies other than breast cancer, particularly in tumors of the colon, pancreas, chromaffin cells, central nervous system, head and neck region, and in melanomas. The values of estrogen binding proteins within the 8S and 4S sucrose density regions had been reported for tumors of the colon and the head and neck region. ER were found in the 8S region that are considered specific by Jensen and De Sombre for "true" receptors in the series of carcinomas of the colon reported by McClendon et al. Molteni et al. found that, in a series of head and neck neoplasms, the majority of tumors with ER had a preponderance of binding in the 8S fraction, although some of them also had high levels in the 4S fraction. Conclusions can be drawn from extensive study relating levels of ER to patient survival rate in both breast and gynecologic cancer, but only preliminary and contradictory results have been reported for most of the other neoplasms studied. In fact there has been

<table>
<thead>
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<th>Table 2. Expression of Cytoplasmic Immunoglobulin in Chronic Lymphocytic Leukemia Peripheral Blood Cells</th>
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<tbody>
<tr>
<td>Patient</td>
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<tr>
<td>--------</td>
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<td>H.B.</td>
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<td>R.W.</td>
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</tbody>
</table>
considerable debate whether specific estradiol binding activity is being measured in these tumors. The use of different assay techniques makes comparisons even more difficult. Whether the survival rate of patients with neoplasms other than in breast or female genital malignancies differs when the estrogen binding protein is found elevated in the 8S fraction rather than in the 45 fraction requires further investigation.

Seventy-three percent of the CLL patients studied displayed a measurable ER level. Reports in the literature and our limited experience suggest that the cell population with ER binding activity may be sensitive to therapy with steroid hormones. The assay of Jensen et al., utilized in this study, provides information about the total cell population. Antibodies directed against ER may help to assess cell-to-cell variation in ER binding activity. At present, the quantity of ER has no predictive value for determining response to hormone therapy in CLL.

The presence of ER binding activity in an EBV-transformed B-lymphoblastoid cell line suggests that EBV receptor-bearing populations of B lymphocytes may also express ER. Further study of established EBV-transformed B-lymphoblastoid cell lines and study of peripheral lymphocytes from healthy donors must be performed before the significance of an elevated ER can be assessed. We are intrigued by the possibility that hormone therapy may provide a means of manipulating subpopulations of lymphocytes in both benign and malignant conditions.

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REFERENCES
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ST Rosen, Z Maciorowski, F Wittlin, AL Epstein, LI Gordon, MS Kies, O Kucuk, HC Kwaan, H Vriesendorp and JN Winter