Effect of a Thymic Factor on T Lymphocytes in B Cell Chronic Lymphocytic Leukemia: In Vitro and In Vivo Studies

By F. Lauria, D. Raspadori, and S. Tura

Abnormalities of T lymphocytes in B cell chronic lymphocytic leukemia (B-CLL) have been extensively documented by several immunologic investigations. Following recent studies pointing to the favorable effect of TP-1, a partially purified extract of calf thymus, on the T cell-mediated immunity of several diseases, including Hodgkin's disease, we have used monoclonal antibodies and the enriched T lymphocyte populations of 16 untreated B-CLL patients to evaluate the proportion of T cell subsets before and after the administration of TP-1. In addition, the proliferative response to phytohemagglutinin (PHA) and the helper function in a pokeweed mitogen (PWM) system were assessed. In ten cases, the effect of TP-1 was also studied in vitro by evaluating the same parameters before and after incubation of B-CLL T cells with the drug. The study demonstrated that in vivo administration of TP-1 increases significantly (P < .001) the proportion of the defective helper/inducer T cell population (OKT4-positive cells) in B-CLL, leading to a near-normal OKT4/OKT8 ratio. Furthermore, the improved phenotypic profile was accompanied by an increased proliferative response to PHA and, in particular, by a significant increase (P < .01) of T helper capacity; this increase was, however, insufficient to enable the normalization of the serum immunoglobulin levels. The in vitro incubation of B-CLL T lymphocytes did not succeed in producing significant modifications in distribution and function.

MATERIALS AND METHODS

Heparinized peripheral blood samples were collected from 16 B-CLL patients and 15 normal controls. The patients, ten males and six females, were all untreated; they were aged 50 to 80 years, and according to Rai et al., six were in stage 0, five in stage I, four in stage II, and one in stage III.

TP-1 was prepared as described by Bergesi and Falchetti at the Istituto Farmacologico Serono, Rome. Briefly, calf thymuses were minced and extracted with ammonium acetate. The extract was heated to 70°C, filtered, and precipitated with ammonium sulfate.

Recent studies have pointed to a favorable effect of TP-1 on the T cell-mediated immunity of untreated patients with Hodgkin's disease.17-18 This, as well as the well-known risk of infective complications in B-CLL coupled with the multiple T cell defects, led us to evaluate the proportion of T cell subsets in the enriched T lymphocytes of 16 untreated patients with B-CLL, using monoclonal antibodies (MoAb) before and after administration of TP-1. In addition, we have assessed the proliferative response to PHA and the helper function in a PWM system. In ten cases, the effect of TP-1 was also studied in vitro by evaluating the same parameters before and after incubation of B-CLL T cells with the drug.

This study demonstrates that in vivo administration of TP-1 is capable of increasing the proportion of the defective helper/inducer T cell population (OKT4-positive cells) in B-CLL, leading to a near-normal OKT4/OKT8 ratio. Furthermore, the improved phenotypic profile was accompanied by an increased proliferative response to PHA and also by a significant increase of T helper ability; this was insufficient, however, to enable the normalization of the serum immunoglobulin levels. The in vitro incubation of B-CLL T lymphocytes with TP-1 was incapable of producing significant modifications in distribution and function.
TP-1 was pyrogen free and the endotoxins were always less than 1 ng/mg of the TP-1 in all the samples used for in vitro and in vivo studies.

In Vivo Studies

Immunologic parameters were performed before and after administration of TP-1 by measuring the serum immunoglobulin level and spontaneous rosettes with sheep red blood cells (SRBC).

<table>
<thead>
<tr>
<th>Controls (10 cases)</th>
<th>Patients (10 cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of CyIg-positive cells recovered/well x 10^6</td>
<td>15 ± 17</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.03</td>
</tr>
<tr>
<td>%E Rosette (of total)</td>
<td>26 ± 28</td>
</tr>
<tr>
<td>Percent</td>
<td>0.41 ± 0.42</td>
</tr>
<tr>
<td>OKT3</td>
<td>2.5 ± 18</td>
</tr>
<tr>
<td>OKT4</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>OKT8</td>
<td>0.5 ± 0.5</td>
</tr>
</tbody>
</table>

The rank sum test on log-transformed values was used for statistical analysis.

Cell Subsets Distribution and Functional Studies in 15 Cases of B-CLL Before and After In Vitro Incubation With TP-1

The helper activity of enriched T lymphocytes was assessed by testing the helper T cell function with 5 x 10^4 T lymphocytes in a final volume of 0.2 mL/well.

In ten patients, PBL and enriched T lymphocytes (4 x 10^6/mL) were suspended in RPMI medium containing TP-1 at a final concentration of 50 ng/mL. Previous experiments have shown this to be the ideal concentration in vitro.

The cells were incubated one hour and 24 hours for T surface markers, whereas for the culture period. The tests were then performed as described above.

In vivo studies are reported in Table 2. The pretreatment period. The tests were then performed as described above.

Spontaneous rosettes with SRBC were performed according to the method of U.S.P. XIX. The endotoxin tested by means of two different functional assays: the enriched T lymphocytes were analyzed with OKT MoAb and rosette-positive cells with sheep red blood cells (SRBC). In addition, by enumerating, on the peripheral blood lymphocytes (PBL), the E rosette-positive lymphoid cells was evaluated by counting at least 200 cells.

Surface Markers

The lymphocyte surface expression of T helper/inducer cells (OKT4), and T suppressor/cytotoxic cells (OKT8) was determined by indirect immunofluorescence microscopy (Zeiss) after labeling with a fluorescein-conjugated goat anti-mouse antisera against all mature peripheral blood T cells (OKT3) (Ortho, Raritan, NJ). T lymphocytes were enumerated and purified by E rosette formation, followed by further separation on Lymphoprep gradient.

Separation and Purification of T Lymphocytes

Peripheral blood lymphocytes were separated on a Lymphoprep gradient. The lymphocyte surface expression of T helper/inducer cells (OKT4), and T suppressor/cytotoxic cells (OKT8) was determined by indirect immunofluorescence microscopy (Zeiss) after labeling with a fluorescein-conjugated goat anti-mouse antisera against all mature peripheral blood T cells (OKT3) (Ortho, Raritan, NJ). T lymphocytes were enumerated and purified by E rosette formation, followed by further separation on Lymphoprep gradient.

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EFFECT OF TP-1 ON B-CLL T LYMPHOCYTES

The response of T lymphocytes to PHA was significantly reduced in all patients studied compared with normal controls ($P < .001$). After TP-1 administration, an increase ($P < .01$) in the response to PHA was observed in 10/14 cases, but only in three patients was a complete normalization reached (Fig 1). Despite treatment with TP-1, the overall mean PHA stimulation index was still significantly depressed compared with normal controls ($P < .001$) (Table 2).

The effect of TP-1 treatment on the helper capacity was assessed on isolated T cells of 16 cases of B-CLL patients and on ten controls cocultured with normal purified B cell in the presence of PWM. As illustrated in Table 2 and Fig 2, B-CLL T lymphocytes showed a constant reduction in helper activity in all cases studied. In 13/16 cases, the administration of TP-1 produced an enhancement of the T helper/inducer capacity. Despite this improvement, however, we were unable to detect any increase in the level of immunoglobulins. In no patient did the administration of TP-1 produce side effects.

### DISCUSSION

Thymic hormones have been shown in both animal and human models to exert an essential role in the differentiation and maturation of thymocytes into functionally mature T lymphocytes, both in periphery and in secondary lymphoid organs. Moreover, an omeostatic activity of thymic hormones has been demonstrated in peripheral T cells, leading to a better in

#### Table 2. Clinical Characteristics, T Cell Distribution, and Functional Studies Before and After In Vivo Treatment With TP-1 in the 16 Patients With B-CLL

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex and Age</th>
<th>Stage</th>
<th>WBC $\times 10^9$/L</th>
<th>E Rosettes*</th>
<th>Percent B</th>
<th>Percent A</th>
<th>Percent T</th>
<th>Percent B</th>
<th>Percent A</th>
<th>Percent T</th>
<th>OKT4/OKT8 Ratio</th>
<th>PHA (cpm)</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.F.</td>
<td>M 58 0</td>
<td></td>
<td>11.7</td>
<td>9.8</td>
<td>23</td>
<td>24</td>
<td>86</td>
<td>91</td>
<td>38</td>
<td>81</td>
<td>26</td>
<td>24</td>
<td>1.46</td>
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<tr>
<td>C.A.</td>
<td>M 64 I</td>
<td></td>
<td>39.1</td>
<td>50.9</td>
<td>11</td>
<td>9</td>
<td>93</td>
<td>89</td>
<td>42</td>
<td>64</td>
<td>47</td>
<td>41</td>
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</tr>
<tr>
<td>Z.A.</td>
<td>F 73</td>
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<td>11.2</td>
<td>13.7</td>
<td>38</td>
<td>32</td>
<td>87</td>
<td>95</td>
<td>29</td>
<td>68</td>
<td>48</td>
<td>45</td>
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<tr>
<td>Z.G.</td>
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<td>48.6</td>
<td>16</td>
<td>15</td>
<td>77</td>
<td>87</td>
<td>28</td>
<td>58</td>
<td>24</td>
<td>30</td>
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<tr>
<td>R.A.</td>
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<td></td>
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<td>83</td>
<td>86</td>
<td>16</td>
<td>50</td>
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<td>31</td>
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<tr>
<td>B.L.</td>
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<td>16.5</td>
<td>11</td>
<td>13</td>
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<td>42</td>
<td>38</td>
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<tr>
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<td>80</td>
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<tr>
<td>N.G.</td>
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<td></td>
<td>19.1</td>
<td>20.7</td>
<td>21</td>
<td>23</td>
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<td>90</td>
<td>50</td>
<td>46</td>
<td>43</td>
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<td>1.16</td>
</tr>
<tr>
<td>M.T.</td>
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<td>10.8</td>
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<td>30</td>
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<td>0.87</td>
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<tr>
<td>N.A.</td>
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<td>98.7</td>
<td>83.8</td>
<td>9</td>
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<td>T.W.</td>
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<td>17</td>
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<td>44.3</td>
<td>21</td>
<td>23</td>
<td>92</td>
<td>88</td>
<td>27</td>
<td>60</td>
<td>32</td>
<td>38</td>
<td>0.84</td>
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<tr>
<td>R.C.</td>
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<td>32.3</td>
<td>30.1</td>
<td>16</td>
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<td>93</td>
<td>95</td>
<td>40</td>
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<td>40</td>
<td>48</td>
<td>1.00</td>
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<tr>
<td>B.F.</td>
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<td></td>
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<td>64.8</td>
<td>12</td>
<td>16</td>
<td>87</td>
<td>92</td>
<td>35</td>
<td>52</td>
<td>41</td>
<td>32</td>
<td>0.85</td>
</tr>
<tr>
<td>M.G.</td>
<td>F 67 II</td>
<td></td>
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<td>51.4</td>
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<td>36</td>
<td>58</td>
<td>36</td>
<td>26</td>
<td>0.95</td>
</tr>
</tbody>
</table>

*Percent of OKT3-positive cells.
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×10$^9$.
vitro response to subliminal antigenic signals.\textsuperscript{21} These experimental findings were later confirmed by the beneficial effect of thymic hormones, and of TP-1 in particular, in human pathologic conditions, such as immune defects, recurrent viral infections, etc.\textsuperscript{22,23} More recently, TP-1 has been investigated in vitro and in vivo in patients with Hodgkin's disease, and an improvement of the immunologic competence, such as an increase in the proportion of E rosette-forming cells and in the response to PHA, has been achieved.\textsuperscript{17,18} In our study, we did not record any increase in the proportion of E rosette-positive cells, but the administration of TP-1 did increase significantly (\(P < .001\)) the proportion of OKT4-positive cells (helper/inducer) in B-CLL patients, leading to a near-normal OKT4/OKT8 ratio. Furthermore, the improved phenotypic profile was accompanied by an increased proliferative response to PHA (\(P < .01\)) and by an improved helper function in a PWM-stimulated assay (\(P < .01\)). The level of serum immunoglobulins, however, was not affected by the administration of TP-1, and no effect was observed on the T cell distribution and function when B-CLL T lymphocytes were incubated in vitro with TP-1. The fact that a correction of T cell subset imbalance was obtained only when giving TP-1 in vivo (no effect was observed in vitro) suggests that the drug may play an important role in activating or restoring T cell physiologic maturation pathways through the thymic compartment and/or in T-dependent areas of peripheral lymphoid organs. However, it is ineffective on peripheral mature T cells. Interestingly, in six cases, a relatively high proportion of T lymphocytes that were characterized by the absence of both antigens recognized by the OKT4 and OKT8 MoAbs, previously demonstrated mainly in patients with advanced disease,\textsuperscript{4} was recorded; this suggests a maturation impairment that may be restored by the thymic hormone. Thus, it appears that TP-1 does not produce any increase in the total number of T cells, as suggested by the unmodified number of E rosette-forming cells, but does produce a maturation effect on the OKT4 cell compartment.

Although the exact implications of T cell abnormalities in B-CLL are yet to be fully understood, they may, nonetheless, contribute to the gradual accumulation of immature B cells, which occurs as the disease progresses, and especially, to the immunodeficiency that frequently accompanies this disease. The reduced antibody production in B-CLL may in fact relate to the reduced proportion of OKT4-positive cells associated with an increase of OKT8-positive cells, which is complicated also by an intrinsic defect within the T helper cell population.\textsuperscript{9,10} It is intriguing that TP-1 appears to affect not only the percentage of OKT4-positive cells, but also the functional behavior of these cells. In this respect, although the exact mechanism of the action of TP-1 on the T-cell compartment of B-CLL needs to be clarified, the possibility that it may act by promoting an increase of T-soluble factors (eg, interleukin 2) should be taken into consideration. More studies are warranted in order to assess whether the positive effect on the T cell compartment may be prolonged by a “maintenance” regimen, as preliminary data suggest that the effect of TP-1 may decline rapidly within 1 month after ceasing treatment. This study will clarify whether “continuous” treatment is capable of influencing the level of serum immunoglobulins in B-CLL and whether it may tangibly affect the course of the disease, particularly in patients with more advanced and complicated disease.

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