Defective Interleukin-2 Production and Responsiveness by T Cells in Patients With Chronic Lymphocytic Leukemia of B Cell Variety

By Olcay Ayanlar-Batuman, Ellen Ebert, and Stephen P. Hauptman

The present studies were designed to investigate the mechanism(s) of the defective T cell proliferative response to various stimuli in patients with B cell chronic lymphocytic leukemia B-CLL. In 14 patients with advanced B-CLL (stage III or IV) we found the T cell response in the autologous (auto) and allogeneic (allo) mixed lymphocyte reaction (MLR) to be 35.7% and 30% of the controls, respectively. Proliferation in the MLR depends upon the production of and response to interleukin 2 (IL 2). A T cell growth factor. IL 2 production in eight B-CLL patients was 22% of the control. The response to IL 2 was measured by the increase in the T cell proliferation in the MLR with the addition of IL 2. T cell proliferation in both the auto and allo MLR of CLL patients was significantly lower than in the controls after the addition of IL 2. The proliferative response of normal T cells to stimulation by CLL B cells was 50% of the control. This latter response was increased to control levels when cultures were supplemented with exogenous IL 2, suggesting that CLL B cells could stimulate IL 2 receptor generation in normal T cells in an allo MLR, but not IL 2 production. The presence of IL 2 receptors on activated T cells was directly determined using anti-Tac, a monoclonal antibody with specificity for the IL 2 receptor. Of the mitogen- or MLR-activated T cells in CLL patients, 6% and 10%, respectively, expressed Tac antigen, whereas identically stimulated control T cells were 80% and 47% Tac+, respectively. Our findings suggest that T cells in B-CLL are defective in their recognition of self or foreign major histocompatibility antigens as demonstrated by their impaired responsiveness in the MLR. Thus, these cells are unable to produce IL 2 or generate IL 2 receptors.

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B-cell chronic lymphocytic leukemia (B-CLL) is a malignancy in which immature, immunoinefficient B cells progressively accumulate in the bone marrow, peripheral blood, and other organs of the reticuloendothelial system.1,2 Recently, T cells in B-CLL were shown to be abnormal, demonstrating a diminished proliferative response to T cell mitogens, as well as to autologous (auto) or allogeneic (allo) cells in the mixed lymphocyte reaction (MLR).3,4 Effector and regulatory T cells, ie, cytotoxic, helper, inducer, or suppressor cells, are generated during the MLR.5,6 Aberrant T cell responses in the auto and allo MLR could reflect a defect in the immunoregulatory T cell generation in B-CLL patients.

Mitogen- or MLR-activated T cells produce a T cell growth factor, IL 2, and generate its receptors on the cell surface.6,7 After IL 2 binds to its receptor, the T cells proliferate and differentiate into regulatory or effector cells with helper, suppressor, or cytotoxic functions. Abnormalities in IL 2 production and responsiveness by T cells have been implicated in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus, the acquired immunodeficiency syndrome (AIDS), and in acute lymphocytic leukemia.12,13 In the present study, T lymphocytes from 14 patients with advanced B-CLL were found to be defective in IL 2 production and receptor generation upon stimulation with mitogen or antigen. We suggest that these defects could account for the T cell abnormalities noted in B-CLL.

MATERIAL AND METHODS

Patients. A total of 14 untreated patients with B-CLL were studied (Table 1). The diagnosis was made by physical examination and by observation of patients' Wright-Giemsa-stained peripheral blood and bone marrow smears. All patients studied had B-CLL as determined by surface immunoglobulin (SIg) evaluation of their peripheral blood lymphocytes (PBL).14 All patients were staged according to the Rai classification and had stage III or IV disease.15 Controls were healthy age-matched volunteers.

Isolation of human lymphocytes and subpopulations. PBL from patients and controls were separated from heparinized whole blood (50 mL) by Ficoll-Hypaque centrifugation (FHC) (lymphocyte separation medium, Bioinetics laboratory products, Litton Bioinetics, Kensington Md).15 Unfractionated mononuclear cells were depleted of macrophages by adherence to plastic and adherent and nonadherent cells collected. Macrophage-depleted mononuclear cells were then separated into E rosette-positive (E+) and E rosette-negative (E-) populations with neuraminidase (Sigma Chemical Co, St Louis) treated 5% sheep red blood cells (SRBC) (GIBCO Laboratories, Madison, Wis). E+ cells were enumerated and recovered as described.17 Briefly, macrophage-depleted PBL (4 x 106/mL) in 2 mL of RPMI 1640 (GIBCO Laboratories, Chagrin Falls, Ohio) were mixed with 2 mL of 1% neuraminidase (25 U/mL of 5% SRBC) treated SRBC and 0.5 mL of fetal calf serum (GIBCO Laboratories, Chagrin Falls, Ohio) (inactivated at 56 °C for one hour). The mixture was incubated at 37 °C for five minutes, centrifuged at 200 g for five minutes, and incubated on ice (4 °C) for four hours. The rosettes were carefully resuspended by rotation of the tubes, incubated for an additional 15 minutes on ice, and subjected to FHC. E+ cells were recovered from the pellet after lysis of attached SRBC by treatment with tris(hydroxymethyl)amino-methane-buffered 0.83% ammonium chloride (pH 7.2) for five minutes at 37 °C, and the T cells were washed three times with RPMI 1640 and rerosetted with SRBC. The E+ cells separated after double rosetting of PBL contained <1% SIg+, <3% alpha-naphthyl-acetate-esterase-positive cells, and ~97% formed rosettes with SRBC. Of the E+ cells, 95% ± 1% reacted with a monoclonal antibody, anti-T11 (Ortho Diagnostics, Westwood, Mass), that defines the SRBC receptor present on all mature peripheral lymphocytes.18 Unrosetted cells at the Ficoll-medium interface are referred to as E rosette-negative cells, anti-T11 negative, SIg+ cells (E- SIg+). Proliferation in the MIR depends upon the presence of IL 2 and requires the presence of accessory cells (ie, monocytes or macrophages) that provide the necessary growth factor required for the T cells to proliferate.
cells were typed for IgM, IgD, IgG, \( \text{ic} \) alpha-naphtyl-acetate-esterase-positive cells. In the case of CLL, B and >95% reacted with a monoclonal antibody, MS (Coulter Immunometric System). These cells were 98% alpha-naphtyl-esterase-positive expression.

a or A light-chain or A light-chain and 98% of these cells were positive for either \( \text{ic} \) or A light-chain expression.

cells alone. The lymphocytes from each patient were compared to cultures containing responder cells alone plus the cpm of stimulator cultures containing stimulator and responder cells and cpm of stimulator cells alone. The lymphocytes from each patient were compared to two controls simultaneously isolated and cultured.

**IL 2 production and assay.** Monocyte-depleted T cells (5 \( \times \) 10^6/mL) were incubated with 1 \( \mu \)g/mL of phytohemagglutinin (PHA-P) (Burroughs Wellcome Co, Greeneville, NC) and 4 ng/mL of 4B phorbol 12-myristate 13-acetate (PMA) (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 48 hours in complete medium supplemented with 2% human AB serum in 12 x 75-mm culture tubes (Fisher Scientific Co, Pittsburgh, Pa). Cell viability after culture was greater than 95%. The supernatant was collected and filtered through a 0.45-μ filter (Nalgene Labware, Nalco Co, Rochester, NY) before storage at −70 °C. Preliminary experiments demonstrated this cell concentration and dosage of mitogen to be optimal for IL 2 production. The IL 2 concentration in the supernatants was measured using a cytotoxic T lymphocyte (CTL) assay as previously described. The units of IL 2 were determined by probit analysis. The IL 2 standard, a 1:16 dilution of rat IL 2 that was the supernatant obtained from spleen cells (10 \( \times \) 10^6/mL) incubated with 5 \( \mu \)g/mL of Concanavalin A for 48 hours, was denoted as 1 U/mL.

**Response to IL 2.** The auto MLR was cultured in medium alone or medium supplemented 1:4 (vol:vol) with various concentrations of IL 2 (Electro Nucleotics, Inc, Silver Spring, Md). This IL 2 preparation contained less than 20 pg/mL PHA as determined by an indirect, enzyme-linked, immunoabsorbent assay. This amount of PHA did not cause an increase in T cell proliferation when added to the auto MLR. To some cultures purified IL 1 (Genzyme, Boston, 100 U/mL) was added (1:80, vol:vol).

**Immunofluorescence with anti-Tac.** Patient or control T cells (2 \( \times \) 10^6/mL) activated for two days with PHA-P (1 \( \mu \)g/mL) and PMA (4 ng/mL) or for six days with auto or allo stimulator cells (2 \( \times \) 10^6/mL) were tested for reactivity with anti-Tac (a generous gift from Dr TA. Waldman) antibody (1:500) by indirect immunofluorescence using FITC-labeled goat antimouse IgG antibody (GAMlgG). Immunofluorescent cells were examined using a fluorescent microscope (Ortholux II, Leitz, Wetzlau, Germany) as described.

**Statistical evaluation.** Student’s t test was used to determine the significance of the difference between experimental results obtained from patients and controls.

**RESULTS**

To determine the nature of T cell defects in patients with B-CLL we first investigated the proliferative responses of their T cells to auto and allo B cells in a MLR. In the patient population the mean T cell proliferation in an auto MLR was significantly lower than control values, 1,800 ± 582 cpm v

<table>
<thead>
<tr>
<th>Patients</th>
<th>Hemoglobin (mg/100 mL)</th>
<th>Lymphocytes 10^9/mL</th>
<th>E+ (%)</th>
<th>Slg+ E- (%)</th>
<th>Slg+ Cells Light-Chain Isotype</th>
<th>Platelets/mm^3</th>
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cells in CLL are monoclonal, displaying either κ or λ light chains, it is possible that they have a limited display of class II major histocompatibility locus (MHC) antigens and thus would be defective as stimulator cells in the autologous MLR. Therefore, normal B cells were used to stimulate CLL T cells to determine whether these cells responded appropriately in an autologous MLR. T cells from CLL patients had a defective response to allo control B cells compared to control values: 6,000 ± 750 cpm vs. 22,582 ± 2,200 cpm respectively (P < .001) (Fig 2). These findings suggested that T cells were defective as responders in both autologous and allogeneic MLR in B-CLL patients.

To determine whether there was, in addition to a responder T cell defect, an impairment in the B cells to act as stimulators, an autologous MLR was performed using normal T cells as responders and CLL B cells as stimulators. In these allo MLR, normal T cells were stimulated by either CLL B cells or allo B cells from healthy controls. As seen in Fig 2, the CLL B cell induced a T cell proliferation that was 50% of that induced by control B cells, ie, 10,231 ± 2,310 cpm vs. 22,582 ± 2,200 cpm respectively (P < .05). These results indicate that defects in both stimulatory B cells as well as responder T cells are responsible for the low autologous and allo MLR in patients with B-CLL.

During autologous and allogeneic MLR, T cells proliferate in response to class II MHC antigens present on the surface of non-T lymphocytes and as a consequence of this activation T cells produce IL 2 and generate IL 2 receptors. Since the binding of IL 2 to its receptor induces the proliferation and clonal expansion of the activated T cells, we measured the IL 2 production and receptor generation in T cells from B-CLL patients. In eight of our patients we determined the capacity of their T cells to produce IL 2 upon activation with PHA-P and PMA rather than auto MLR activation since the amount of IL 2 generated in control and patient auto MLR was below the sensitivity of the CTLL assay. After lectin stimulation, patients' T cells produced 22% of that of normal allo MLR cultured with IL 2, 49,000 ± 27,000 cpm vs. 4,900 ± 3,200 cpm respectively (P < .001) (Fig 3). The proliferation increased from 6,000 cpm to 17,000 cpm with the addition of IL 2, whereas there was a significant rise from 5,033 ± 1,200 cpm to 27,000 cpm in the control auto MLR (P < .001) (Fig 1). In the allo MLR with CLL T cells as responders and normal B cells as stimulators, the proliferation increased from 6,000 cpm to 17,000 cpm with the addition of IL 2 (Fig 2). This increase was substantially less than the proliferative increase one sees with control T and B cells in allo MLR upon the addition of IL 2, ie, 49,000 ± 2,990 cpm (P < .05). These results, presented in Table 2, are consistent with the results of immunofluorescence experiments using anti-Tac antibody and demonstrate that not only is Tac expansion significantly diminished in T cells activated in an allo MLR but there are no other functional IL 2 receptors on these cells in B-CLL.

![Fig 1](image1.png)

**Fig 1.** Autologous MLR was performed as described by incubating 2 × 10⁶ responder T cells (a) with 2 × 10⁶ mitomycin C-treated auto B cells (b) for six days at 37°C and 5% CO₂/95% air in a humidified atmosphere. To some of these cultures purified human IL 2 was added (1:4 vol:vol) (c) The number of individuals studied is (d), and the cpm of responder T cells is (e).

![Fig 2](image2.png)

**Fig 2.** Allogeneic MLR was performed as described by incubating 2 × 10⁶ responder T cells (a) with 2 × 10⁶ mitomycin C-treated allo control B cells (c) or leukemic B cells (p) for six days at 37°C and 5% CO₂/95% air in a humidified atmosphere. To some of these cultures purified human IL 2 was added (1:4 vol:vol) (c) The number of individuals studied is (d), and the cpm of responder T cells is (e).
C-treated B cells by indirect immunofluorescence. Cells (1 x 10^6/mL) for six days. B cells were stimulated with mitomycin C-treated autologous cells (5 x 10^5/mL) and PMA (4 ng/mL) for 48 hours at 37°C and 5% CO2/95% air in a humidified atmosphere. The IL 2 concentration in the supernatants was measured using a CTLL assay. The number of patients and controls who were studied for IL 2 production is 12.

DISCUSSION

These results demonstrate that T cells as well as B cells of patients with B-CLL have intrinsic defects that result in ineffective cellular interactions required for certain immune responses. The T cells of patients with advanced CLL are unable to produce IL 2 or generate IL 2 receptors following activation with mitogen, auto cells, or allo cells. The B cells of these patients are unable to induce normal T cells to produce IL 2, although they are capable of inducing IL 2 receptors on normal T lymphocytes. These experiments do not allow us to determine which step of T cell activation prior to IL 2 receptor generation is defective in B-CLL. The release of IL 2 by T cells occurs in response to two signals provided by antigen-stimulated macrophages, ie, presentation of antigen and production of interleukin 1 (IL 1), a mediator that binds to activated T cells and stimulates the production of IL 2. By performing our experiments using adherent cell-depleted T cells, we were able to provide similar numbers of macrophages, <3%, for patients and controls. In experiments measuring IL 2 synthesis, we added PMA to cultures since it enables lymphocytes to produce IL 2 in response to mitogens in the absence of adherent cells. The quantity of IL 2 in the supernatant of PHA-P-activated T cells was similar regardless of the presence or absence of PMA, suggesting that defective IL 1 production alone could not explain the inability of T cells to produce IL 2 in B-CLL. Furthermore, the addition of purified IL 1 to MLR cultures did not change the proliferation of CLL T cells (not shown).

In all patients, there was a significantly diminished T cell proliferation in response to auto and allo cells compared to control T cells. In the auto MLR, auto T cells recognize the class II MHC antigens (DR antigens) on the surface of non-T cells and respond by proliferating. Lack of DR recognition could result in an inability to induce Tac antigen expression, an IL 2 receptor, following T cell activation with auto stimulated cells. The diminished proliferation and expression of Tac antigen following auto stimulation could be due to a defect in the responder cell, the stimulator cell, or both. Therefore, we measured the response of T cells from B-CLL patients to normal B cells in an allo MLR. T cells from B-CLL patients had a substantially decreased proliferative response in the allo MLR. Proliferation in the allo MLR is also dependent upon IL 2 production and receptor generation. Exogenous IL 2 partially corrected the defective allo MLR, although it was still significantly less than controls. Although a suppressive effect of leukemic B cells on T cell functions cannot be ruled out, our results suggest that T cells have an intrinsic defect in B-CLL since they did not respond to either auto or allo cellular antigens by IL 2 production and receptor generation as normal T cells did. An alternative explanation of our findings could be the presence of a low helper (T4+)/suppressor (T8+) cell ratio in the peripheral blood in these patients. This is unlikely since in four of the presented patients isolated T4+ as well as T8+ cells had a diminished proliferative response to T cell mitogens and to allo cells. In addition, production of IL 2 was significantly decreased in both of the stimulated T cell subsets (not shown).

The capacity of B cells from CLL patients to stimulate a proliferative response in normal T cells (allo MLR) was noted to be significantly diminished. This stimulatory defect

![Fig 3. IL 2 production was quantitated in supernatants obtained from monocyte-depleted T cells (5 x 10^5/mL) stimulated with PHA-P (1 µg/mL) and PMA (4 ng/mL) for 48 hours at 37°C and 5% CO2/95% air in a humidified atmosphere. The IL 2 concentration in the supernatants was measured using a CTLL assay.](www.bloodjournal.org)

Table 2. Defective T Cell Activation in B-CLL Patients.

<table>
<thead>
<tr>
<th>Source of T Cells</th>
<th>Mitogen*</th>
<th>Auto B Cells†</th>
<th>Allo B Cells‡</th>
<th>CLL B Cells§</th>
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</thead>
<tbody>
<tr>
<td>Controls (6)</td>
<td>60 ± 10</td>
<td>25 ± 5</td>
<td>47 ± 5</td>
<td>47 ± 10</td>
</tr>
<tr>
<td>B-CLL (6)</td>
<td>6 ± 2</td>
<td>7 ± 2</td>
<td>10 ± 5</td>
<td>ND</td>
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Abbreviation: ND, not determined.

*T cells (1 x 10^5/mL) were stimulated with PHA-P (1 µg/mL) and PMA (4 ng/mL) for three days.

†T cells (1 x 10^5/mL) were stimulated with mitomycin C-treated auto B cells (1 x 10^5/mL) for six days.

‡T cells (1 x 10^5/mL) were stimulated with mitomycin C-treated allo B cells (1 x 10^5/mL) for six days.

§Control T cells (1 x 10^5/mL) were stimulated with mitomycin C-treated CLL B cells for six days.

1Number of persons studied.

Tac+ cells were determined using monoclonal anti-Tac antibody and indirect immunofluorescence.
was corrected by exogenous IL 2. Further studies demonstrated that the CLL B cells were able to induce IL 2 receptors on normal T cells, although they lacked the capacity to induce IL 2 synthesis in normal T cells. Thus the addition of IL 2 would be expected to normalize the allo MLR using CLL B cells to stimulate normal T cells. Although all of the patients studied expressed DR antigen on their leukemic B cells (not shown), as determined with immunofluorescence using a monoclonal anti-Ia antibody (antinonpolymorphic HLA-DR), it is possible that the leukemic B cells lack the MHC class II determinant that is required to induce IL 2 production by T cells although they express another determinant required for IL 2 receptor generation. It has been shown that leukemic B cells in certain patients with B-CLL and hairy cell leukemia do not express a DR-linked determinant, DQ, although they react with MAb against monomorphic DR determinants. The relationship between DQ and/or DR expression by B cells and their capacity to induce IL 2 production and/or receptor generation among T cells is currently being studied.

These results raise two important questions: why are T cells intrinsically defective in B-CLL and what role do the T cell defects play in the pathogenesis of this disease? Initial studies using G6PD expression or cyogenic analysis as clonal markers of hematopoietic cells have shown that T cells and leukemic B cells arise from different clones of hematopoietic stem cells in a few patients with B-CLL. A larger population of patients will have to be analyzed using chromosome banding to determine whether leukemic B cells and T cells do indeed arise from the same clone of a leukemic lymphoid stem cell. It is also possible that T cells are transformed by a virus that changes their immunoregulatory effect on B cells or that both T and B cells are transformed. Human T cell leukemia virus, HTLV-1, a retrovirus, has recently been found to be responsible for inducing a T cell leukemia/lymphoma in certain areas of the world. Proviral sequences of this retrovirus were shown to be present in T cells from 35 B-CLL cases diagnosed in Jamaica. Effector and regulatory T cell defects in B-CLL could be of pathogenic significance.

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Defective interleukin-2 production and responsiveness by T cells in patients with chronic lymphocytic leukemia of B cell variety

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