Interleukin-5 (IL-5) Increases Spontaneous Apoptosis of B-Cell Chronic Lymphocytic Leukemia Cells In Vitro Independently of bcl-2 Expression, and Is Inhibited by IL-4

By Tryfonia Mainou-Fowler, Virginia A. Craig, J. Adrian Copplestone, Michael D. Hamon, and Archibald G. Prentice

During hematopoiesis, viability factors that suppress apoptosis are required throughout the differentiation process. Some of these factors may also function as growth factors. Interleukin-5 (IL-5) is recognized as a growth factor in hematopoiesis. We examined the involvement of IL-5 as a viability factor of B-CLL in vitro. In 13 B-CLL cases studied, IL-5 at 20 U/mL increased spontaneous apoptosis by a mean percentage of 53% (range, 20% to 129%) (P < .05) after 2 days in culture. On the third day, the mean percentage increase was 37% (range, 18% to 50%). In all cases, IL-4 protected B-CLL cells against IL-5–induced apoptosis by a mean percentage of 47% (range, 18% to 81%) (P < .001). This protection was specific to IL-4 and it was reduced with anti–IL-4 antibody. In addition, expression of bcl-2 protein in untreated cultures was not significantly different from that of the IL-5–treated cells; mean equivalent of soluble fluoro-echrome (MESF) was 5.2 (range, 3.0 to 6.8) and 4.9 (range, 3.0 to 6.3), respectively (P > .2). In freshly isolated B-CLL cells, the MESF was 4.5 (range, 2.4 to 6.6). These results show that IL-5 induced apoptosis in B-CLL cells by a pathway that is independent of bcl-2 expression. IL-4 partially protects against this effect.

MATERIALS AND METHODS

Patients. Thirteen patients with B-CLL who had not received treatment for at least 3 months were studied. They all had high CD5 and low CD2 percentages of positive cells. Monoclonality was achieved by several regulatory processes that include cell proliferation, cell differentiation, and programmed cell death (apoptosis). Apoptosis is an active cell suicidal process that depends upon the transcription and activation of specific endonucleases. This can be influenced or suppressed by intrinsic or extrinsic factors that may be either the appearance or disappearance of a stimulus, or the withdrawal of essential growth factors. Apoptotic death is characterized by specific morphologic and biochemical changes that lead to endonucleolytic degradation of the DNA at nucleosomal intervals. Apoptosis accounts for much of the normal cell death in vivo. Suppression or failure of an essential component of this process could contribute to the development of cancer. Greater understanding of the factors and mechanisms involved in the process of apoptosis could be of value in the development of new therapeutic strategies for cancer.

B-cell chronic lymphocytic leukemia (B-CLL) is a neoplastic disease characterized by sustained lymphocytosis in the peripheral blood (PB) with apparently mature B lymphocytes that have been arrested at an early stage of their differentiation. B-CLL cells have a long life span in vivo. However, in culture, such cells undergo spontaneous apoptosis. The poor survival in vitro may be the result of lack of accessible growth factors because the addition of IL-4 and activation with anti-CD40 monoclonal antibody (MoAb) is required to maintain normal B-cell lines. In addition, IL-4 protects B-CLL cells from spontaneous apoptosis (SA) and dexamethasone (dex)-induced apoptosis in vitro.

Bcl-2 is a proto-oncogene, the product of which blocks cell death after a variety of stimuli. The bcl-2 protein prolongs the survival of hematopoietic cell lines and protects primary neuronal cell cultures from death after withdrawal of growth factors. Bcl-2 protein is detectable in normal fresh lymphoid cells of both B- and T-cell types and in a number of lymphoproliferative disorders such as hairy cell leukemia and B-CLL. The B cells from most follicular lymphomas express high levels of bcl-2 protein, whereas normal germinal centers are negative. Recently, it has been reported that bcl-2 protein expression is downregulated in B-CLL cells entering SA12 and also in hydrocortisone-treated cells, but this has not been confirmed by others.

In this study, we examine the involvement of IL-5, a growth factor involved in hematopoiesis, in apoptosis of B-CLL cells in vitro. We also investigate whether there is any relationship between the antiapoptotic effect of IL-4 and any IL-5 effect and whether changes in bcl-2 protein levels are involved.

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CD2-coated magnetic beads (Dynabeads; Dynal, New Ferry, Wirral, UK) and monocytcs, by adherence to plastic for 1 hour at 37°C. All cell preparations were negative for CD15 MoAb. Also they were less than 0.5% positive and less than 1% positive when stained with CD2 and CD3 MoAbs, respectively.

**Immunophenotyping.** Cells (10⁷) were treated with the appropriate dilution of the FITC-labeled MoAb for 30 minutes on ice. They were washed three times in ice-cold phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) before being resuspended in 0.5 mL PBS. Fluorescence analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). A negative control (mouse IgG1) was also included.

**Bcl-2 expression.** Cells (1 to 2 × 10⁶) were made permeable by treatment with 70% ethanol for 10 minutes at 4°C and then washed in cold PBS with 2% BSA. The washed cells were then incubated with unlabeled mouse-antihuman bcl-2 MoAb for 30 minutes at 4°C. After washing (PBS + 2% BSA) the cells were stained with FITC-labeled F(ab')₂ fragments of rabbit-antimouse Ig for further 30 minutes at 4°C, washed as before and the cell associated fluorescence analyzed on a FACScan flow cytometer.

In validating this method, it was established by using an unrelated antibody (mouse IgG1) that permeabilization of the cells did not increase nonspecific absorption of antibody.

To quantify bcl-2 expression, the flow cytometer was calibrated using FITC-labeled microbeads (Quantum 25 series; diameter 9.1 μm) supplied by the Flow Cytometry Standards Corporation for Becton Dickinson. The range of the standard curve was 0 to 1.9 × 10⁴ mean equivalents of soluble fluorochrome (MESF).

Thus, all figures for bcl-2 expression are given as MESF. This method allowed quantification of bcl-2 expression and direct comparison between samples analyzed at different times.

**Cell culture.** B-CLL cells (2 × 10⁶/mL) were cultured with or without IL-5 of varying concentrations (5 to 50 U/mL) initially and then at 20 U/mL, for maximal effect in the presence or absence of IL-4 (100 U/mL) and/or IL-4 (20 μg/mL). Cells were cultured in medium (RPMI 1640; GIBCO, Paisley, UK) supplemented with 5% heat-inactivated (35°C, 30 minutes) fetal calf serum (Flow, Herts, UK), 20 mM HEPES buffer (GIBCO), glutamine (0.3 mM/mL, Sigma, Poole, Dorset, UK), penicillin, and streptomycin (100 μU/mL and 100 μg/mL, respectively). Cell cultures at 4°C and with dex (2 × 10⁻³ mol/L; Sigma Chemical Co, St Louis, MO) were included as negative and positive controls, respectively. At preestablished times, 2 to 3 × 10⁶ cells were washed twice (200g, 10 minutes) with cold PBS (Ca²⁺/Mg²⁺ free; GIBCO) and processed as described below. Another aliquot was centrifuged and the pellet was used for DNA extraction and electrophoresis (see below).

**DNA labeling for flow cytometry.** A modified method of Nicolletti et al. was used. Briefly, the washed cells (4 × 10⁶) were gently resuspended in 2 mL of a hypotonic fluorochrome solution in 12 × 76 polypropylene tubes (Becton Dickinson). The fluorochrome solution consisted of propidium iodide (PI; 50 μg/mL; Sigma) in sterile distilled water also containing 0.1% each of sodium citrate, Triton X-100 (Sigma), and lysophosphatidylcholine (10 μg/mL; Sigma). The tubes were placed in the dark at 4°C for at least 5 hours before flow cytometric analysis. The PI fluorescence of the nuclei was measured using a FACScan flow cytometer (Becton Dickinson) and the data were registered on a logarithmic scale on the FL2 channel. Cell debris was excluded by appropriately raising the forward-scatter threshold. At least 5 × 10⁶ cells of each sample were analyzed. All measurements were done under the same instrument settings.

**DNA extraction and electrophoresis.** For DNA extraction, 2 × 10⁶ cells were lysed for 5 hours at 37°C in TRIS-EDTA buffer (50 mMm/L and 10 mmol/L, respectively; pH 8.0) containing 0.5% SDS

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**Table 1. Immunohematologic Data of B-CLL Patients**

<table>
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<tr>
<th>Patient</th>
<th>Sex</th>
<th>Stage</th>
<th>WBC*</th>
<th>CD2</th>
<th>CD5</th>
<th>CD20</th>
<th>FMC7</th>
<th>CD10</th>
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<th>λ</th>
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<td>2</td>
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<td>M</td>
<td>A/O</td>
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<tr>
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<td>83</td>
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<td></td>
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<td>87</td>
<td>7</td>
<td>63</td>
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* Percentage of positive cells determined by immunofluorescence.
† White blood cell (WBC) count × 10⁹/L.
IL-5 INDUCES APOPTOSIS IN B-CLL 2299

Fig 2. Kinetics of the effect of IL-5 on the apoptosis of B-CLL cells in culture. Cells (2 x 10^6/mL) were cultured for the time indicated in the absence or presence of IL-5 (20 U/mL). They were then stained with PI and cell fluorescence was analyzed by flow cytometry. P, patient.

Table 2. Effect of Concentration of IL-5 on the % Increase in Spontaneous Apoptosis in B-CLL Cells

<table>
<thead>
<tr>
<th>IL-5 Concentration (U/mL)</th>
<th>% Increase in Spontaneous Apoptosis*</th>
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</thead>
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<tr>
<td>Patient 4</td>
<td>Patient 10</td>
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<td>10</td>
<td>105</td>
</tr>
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<td>20</td>
<td>137</td>
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</table>

Abbreviation: NI, no increase.

* On day 2 of culture.

RESULTS

Effect of IL-5 on the SA of B-CLL cells in culture. IL-5 at 20 U/mL significantly increased (P < .05) SA of B-CLL cells after 2 days of culture in 13/13 patients studied (Figs 1 and 2, Tables 2 and 3). The mean percentage increase was 53% (range, 20% to 129%). No such effect was seen in 24-hour cultures (Fig 2). The percentage increase of SA with IL-5 was more prominent in 48-hour cultures (Fig 2). Although the percentage apoptosis with IL-5 started to decline, after 48 hours it was still higher than that observed in the absence of IL-5 (Fig 2). The increase of SA with IL-5 depended on the concentration of IL-5 (Table 2) and in 50% of the cases, apoptosis was as high as that induced by dex (Figs 1 and 3A; P < .02). IL-4 significantly reduced both spontaneous (P < .001) and IL-5-induced apoptosis (P < .001) (Figs 1 and 3) and in most cases, the extent of this reduction was very similar (Fig 1, P > .2). This antiapoptotic effect of IL-4 was specific and was significantly reduced or

Table 3. Effect of αIL-4 on the Protection of IL-4 Against IL-5-Induced Apoptosis

<table>
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<tr>
<th>Additions*</th>
<th>IL-5, IL-4, αIL-4</th>
<th>IL-5, IL-4, αIL-4</th>
<th>IL-4</th>
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</thead>
<tbody>
<tr>
<td>Patient No.</td>
<td>SA</td>
<td>IL-5, IL-4</td>
<td>IL-5, IL-4, αIL-4</td>
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<tr>
<td>9</td>
<td>21</td>
<td>31</td>
<td>18</td>
</tr>
</tbody>
</table>

Abbreviation: SA, spontaneous apoptosis.

* Values represent percent apoptosis on day 2. Concentrations of additives were as follows: IL-5, 20 U/mL; IL-4, 100 U/mL; αIL-4, 20 μg/mL.
Fig 3. DNA fluorescence (PI mediated) in B-CLL by flow cytometry profile. (A) Cells from patient 2 (Table I) were cultured at 4°C (control) or 37°C (experimental) with IL-5 (20 U/mL) in the presence or absence of IL-4 (100 U/mL), or with Dex (2 x 10^-6M) or in medium alone (SA). (B) Also, cells (patient 3) were cultured with or without IL-4 (100 U/mL) and all-4 (20 µg/mL). *, the "shoulder" of apoptotic cells.
in some cases abolished with a polyclonal antibody to rhIL-4 (Table 3 and Fig 3B).

Demonstration of oligonucleosomal DNA fragmentation. To confirm apoptosis, DNA was extracted from cells cultured at 37°C or 4°C with or without IL-5 and/or IL-4 and run on agarose gel. DNA from dex-treated cells was also included as a positive control. The results (Figs 4 and 5) illustrate a classical ladder pattern of apoptotic DNA in dex-treated cells and in cells cultured with or without IL-5 and IL-4. The intensity of bands of the fragmented DNA extracted from cells cultured with IL-5 was greater than that obtained in the absence of IL-5 (Fig 4, lanes 1 and 2). However, the intensity of bands was significantly less in the presence of IL-4 compared with SA (Fig 5, lanes 1 and 2). Also, the intensity of bands was significantly less in the presence of IL-4 and IL-5 together compared with IL-5 alone (Fig 4, lanes 3 and 2). As expected, at 4°C there was no DNA fragmentation in the presence of dex or IL-4 (Fig 5, lanes 7 and 4) and in the presence of IL-5 (Fig 3A).

Bcl-2 protein expression. Figure 6 is a typical profile by flow cytometry of bcl-2 expression. This expression was not significantly altered under conditions that induce apoptosis (ie, treatment with dex or IL-5) when compared with bcl-2 expression at 4°C or at 37°C (Fig 6, B and C). Fresh B-CLL cells may express higher levels of bcl-2 than cultured cells (Fig 6, B and C). However, this finding is not consistent and is also unrelated to apoptosis. In addition, in five patients, irrespective of the increase of SA with IL-5, the expression of bcl-2 protein in cells cultured in medium alone was not significantly different from that when cells were cultured in the presence of IL-5 (Table 4; P > 0.2), although one showed a clear reduction (patient 8) and one showed a clear increase (patient 7).

DISCUSSION

In the present study, we report a new function of IL-5: the induction of apoptosis in B-CLL cells. Interleukin-5 significantly increased SA in all B-CLL cases investigated. There was no correlation between phenotype (CD5+ and FMC7+ in particular, either alone or together) or clonality and the percentage of spontaneous or IL-5–induced apoptosis. This effect was specific to B-CLL cells and it was not observed in normal PB B cells (results not shown). However, the apoptotic effect of IL-5 on normal CD5+ B cells has yet to be examined.

The apoptotic activity of IL-5 increased with increasing concentration of IL-5, with half the cases being as great as that induced by dex. Apoptosis in response to IL-5 was not evident until 48 hours of culture. Fresh B-CLL cells may not express receptors for IL-5. However, in the presence of IL-5, receptor expression may be inducible. In all cases, apoptosis induced by IL-5 was significantly reduced in the presence of IL-4. The protective effect of IL-4 was not significantly different from the IL-4 protection against SA. Also, the antiapoptotic effect of IL-4 was specific and it was abolished in the presence of polyclonal anti–IL-4 antibody.
Recently, we and others have shown that IL-4 inhibits spontaneous and dex-induced apoptosis in B-CLL cells. This effect was specific to IL-4 and it was not observed in normal PB cells.

We have also observed that there is interpatient heterogeneity in the protective effect of IL-4 as well as in the apoptotic activity of dex and IL-5. This heterogeneity may be the result of variability of the receptor affinity for the factor in these cells. Recently it has been shown that B-CLL cells express either high- and/or low-affinity receptors for IL-4. The receptors for IL-5 consist of two subunits, and in the murine chronic B-cell leukemia cell line, IL-5 binds with low or high affinity when it binds to one or both subunits, respectively. IL-5 binding to human B-CLL cells has not yet been investigated.

The modes of action of IL-5 in inducing apoptosis in B-CLL cells and IL-4 in protecting such cells from dying are unknown. Generally, very little is clear about the regulation, biochemical mechanism, and identity of genes or their products that regulate apoptosis. Recently, the bcl-2 proto-oncogene has been associated with prolonged survival of hematopoietic cells. The bcl-2 oncogene is reported to be involved in the selection of T and B lymphocytes and is not detected in areas of high apoptotic cell death. Bcl-2 protein is detect-

### Table 4. Bcl-2 Expression of B-CLL Cells Cultured in the Presence or Absence of IL-5 (20 U/mL)

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Without IL-5</th>
<th>With IL-5</th>
<th>% Increase</th>
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<td>Bcl-2 Expression*</td>
<td>Apoptosis</td>
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</tr>
<tr>
<td>6</td>
<td>6.1</td>
<td>6.0</td>
<td>(41)</td>
</tr>
<tr>
<td>7</td>
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<td>(46)</td>
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<td>5.7</td>
<td>3.5</td>
<td>(24)</td>
</tr>
<tr>
<td>9</td>
<td>3.0</td>
<td>3.0</td>
<td>(41)</td>
</tr>
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</table>

* Mean equivalent of soluble fluorochrome; P > .2 (paired t-test).

![Diagram](image_url)
able in the neoplastic B-cells of some follicular lymphomas (normal germinal centers are negative) and in some lymphoproliferative disorders such as hairy cell leukemia and B-CLL. Recently, Dancescu et al using Western blot analysis, have shown in one patient by SDS-PAGE only that there is down-regulation of the bcl-2 protein expression in B-CLL cells undergoing SA and also in hydrocortisone-treated cells and that IL-4 prevented this down-regulation. These authors also showed that in two other patients studied, the expression of bcl-2 after 1 day of culture with IL-4 increased when compared with that of the fresh cells. Similarly, Panayiotidis et al reported that IL-4 inhibits loss of bcl-2 protein in cultured B-CLL cells. These experiments were done without positive (re, dex-treated) or negative (re, 4°C) controls. Some loss of protein may occur during Western blot analysis and this does not occur with the flow cytometry technique. Therefore, by contrast, in the present study using flow cytometry we observed no significant difference of bcl-2 protein in cells cultured with IL-5 when compared with that in the absence of IL-5. We did not observe any correlation between bcl-2 protein expression and degree of apoptosis. We have previously reported no significant difference in bcl-2 protein expression in dex- or IL-4-treated B-CLL cells when compared with untreated cultured or freshly isolated cells. Recently, Boise et al and Oltvai et al have reported the existence of bcl-2-related genes that produce a range of heterodimers that function as dominant regulators of apoptosis. These findings may explain the conflicting reports on the involvement of bcl-2 proto-oncogene in apoptosis in B-CLL.

Human IL-5 stimulates growth and differentiation of human eosinophils. Recently, Hayes et al reported abnormal IgM production by mitogen-activated B-CLL cells in response to rhIL-5, when compared with normal PB B cells. Also, murine IL-5 has been described as a growth factor for murine CD5+ B-cell lymphoma and also for normal CD5+ cells. This study reports that human recombinant IL-5 induces apoptosis in B-CLL cells.

In addition, IL-5 and also IL-4 are produced by T cells (T-helper). The present study was performed using a purified B-CLL cell population that contained less than 0.5% and less than 1% CD2 and CD3 cells, respectively, indicating that the observed effects of IL-5 and IL-4 were probably not the result of a paracrine factor. Generally, little is known about how T cells interact with malignant B cells in B-CLL. A clearer understanding of these interactions and identification of the biochemical mechanisms controlling apoptosis in B-CLL could be of value in developing new therapeutic strategies.

REFERENCES

Interleukin-5 (IL-5) increases spontaneous apoptosis of B-cell chronic lymphocytic leukemia cells in vitro independently of bcl-2 expression and is inhibited by IL-4

T Mainou-Fowler, VA Craig, JA Copplestone, MD Hamon and AG Prentice