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. Harmon, 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 fluorochrome (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.

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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 determined on the basis of membrane monocotypic \( \kappa \) or \( \lambda \) light chain expression (Table 1).

Reagents. Fluorescein-isothiocyanate (FITC)-labeled mouse-antihuman MoAbs to the CD series (CD2, CD5, CD20, CD54, and CD10) and FITC-labeled F(ab)2 fragments of mouse to human \( \kappa \) and \( \lambda \) chains were purchased from DAKO Ltd (High Wycombe, Bucks, UK). The FITC-labeled FMC7 MoAb was obtained from Sera Lab (Crawley Down, Sussex, UK). The mouse MoAb to human bcl-2 protein was purchased from DAKO. Recombinant human IL-5 (rhIL-5) and rhIL-4 were obtained from Advanced Protein Products (Brockmoor, West Midlands, UK). These were more than 97% and 98% pure, respectively, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The bioactivity was \( 5 \times 10^6 \) U/mL for IL-5 (determined by liquid eosinophil differentiation factor assay) and \( 10^7 \) U/mL for IL-4 (determined by PB T-lymphocyte \( \mathrm{H}^2 \)-thyridine incorporation proliferation assay). Polyclonal rabbit-antihuman IL-4 (dIL-4) was purchased from Genzyme (Boston, MA). This was greater than 90% IgG antibody as confirmed by Phast Gel (Pharmacia, St Albans, Herts, UK) analysis.

Cell isolation. Mononuclear cells from PB (EDTA as anticoagulant) were isolated by centrifugation on a Ficoll-Hypaque (Nycomed, Sheldon, Birmingham, UK) gradient. T cells were separated with From The Department of Haematology, Derriford Hospital, Derriford Road, Plymouth, PL6 5DH UK.

Submitted January 5, 1994; accepted June 1, 1994.

Supported by the Plymouth and District Leukaemia Fund. V.A. Craig is a Leukaemia Research Fund Clinical Fellow.

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Blood, Vol 84, No 7 (October 1), 1994: pp 2297-2304
CD2-coated magnetic beads (Dynabeads; Dynal, New Ferry, Wirral, UK) and monocytes, 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 (10^6) 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 x 10^6) were made permeable by treatment with 70% ethanol for 10 minutes at 4°C and then washed in cold PBS + 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 (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 X 10^2 mean equivalents of soluble fluorescent (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 x 10^6/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 (56°C, 30 minutes) fetal calf serum (Flow, Herts, UK), 20 mmol/L HEPES buffer (GIBCO), glutamine (0.3 mg/mL, Sigma, Poole, Dorset, UK), penicillin, and streptomycin (100 U/mL and 100 μg/mL, respectively). Cell cultures at 4°C and with dex(2) (2 x 10^3 mol/L; Sigma Chemical Co, St Louis, MO) were included as negative and positive controls, respectively. At preestablished times, 2 to 3 x 10^6 cells were washed twice (200g, 10 minutes) with cold PBS (Ca^++/Mg^++ 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(20) was used. Briefly, the prewashed cells (4 x 10^6) were gently resuspended in 2 mL of a hypotonic fluorochrome solution in 12 x 76 polystyrene 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 lyophilophosphatidylcholine (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 x 10^6 cells of each sample were analyzed. All measurements were done under the same instrument settings.

DNA extraction and electrophoresis. For DNA extraction, 2 x 10^6 cells were lysed for 5 hours at 37°C in TRIS-EDTA buffer (50 mmol/L and 10 mmol/L, respectively; pH 8.0) containing 0.5% SDS.
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>Patient 4</th>
<th>Patient 10</th>
<th>Patient 9</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>26</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>10</td>
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<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>20</td>
<td>105</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>50</td>
<td>137</td>
<td>24</td>
<td>68</td>
</tr>
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</table>

Abbreviation: NI, no increase.

* On day 2 of culture.

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

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>SA</th>
<th>IL-5</th>
<th>IL-5, IL-4</th>
<th>IL-5, IL-4, αIL-4</th>
<th>IL-4</th>
</tr>
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<tbody>
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<td>5</td>
<td>56</td>
<td>77</td>
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<td>21</td>
<td>31</td>
<td>18</td>
<td>28</td>
<td>15</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 1) 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^-6 M) 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 (Fig 6A) 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 > .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 \( ^{9,11,12} \) and dex \( ^{11,13} \)-induced apoptosis in B-CLL cells. This effect was specific to IL-4 \( ^{9} \) and it was not observed in normal PB cells.\( ^{9} \)

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.\( ^{14} \) 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.\( ^{22} \) 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.\( ^{23} \) Recently, the bcl-2 proto-oncogene has been associated with prolonged survival of hematopoietic cells.\( ^{14} \) 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.\( ^{24} \) Bcl-2 protein is detect-
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 downregulation of the bcl-2 protein expression in B-CLL cells undergoing SA and also in hydrocortisone-treated cells and that IL-4 prevented this downregulation. 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 (e.g., dexamethasone) or negative (e.g., 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 loss 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 rIL-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

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