Interleukin-8 Induces the Accumulation of B-Cell Chronic Lymphocytic Leukemia Cells by Prolonging Survival in an Autocrine Fashion

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Several cytokines have been suggested to play a regulatory action on the neoplastic clone of patients with B-cell chronic lymphocytic leukemia (B-CLL) by interfering in the differentiation, proliferation, or death/survival pathways. Interleukin-8 (IL-8) is a chemoattractant protein constitutively expressed at the mRNA level and released by B-CLL cells. In view of the presence of the IL-8 receptor mRNA and of specific IL-8 binding, confirmed also by Scatchard analysis using 

125I-IL-8, the study was extended to evaluate the possible regulatory role of this cytokine on B-CLL cells. IL-8 failed to show any in vitro proliferative effect on leukemic B-CLL cells. By contrast, the propidium iodide (PI) staining of the DNA content showed that IL-8 could prolong the survival of resting B-CLL cells in 11 of 16 cases studied. In the remaining 5 cases, 90.6% ± 4.39% SD of the cells after culture remained viable and IL-8 could exert a significant death protection action after pretreatment with 10−4 mol/L hydrocortisone, which reduced the percentage of viable B-CLL cells. The dose range of IL-8 capable of inducing the prolonging survival effect is comparable with the levels of IL-8 released constitutively by B-CLL cells, indicating that the death protection action is exerted at physiologic doses. The in vitro rescue from death induced by IL-8 is reflected by an increased expression of bcl-2 mRNA in B-CLL cases incubated in the presence of IL-8. These findings were further confirmed at the protein level, because in B-CLL cells that displayed a bimodal bcl-2 intracytoplasmatic protein expression IL-8 was capable of upmodulating the bcl-2 peak expression peak. The potential autocrine regulatory action exerted by IL-8 is supported by the evidence that exogenous IL-8 can upregulate IL-8 mRNA in B-CLL cells. These results, together with the demonstration that antibody-mediated neutralization of endogenous IL-8 could induce a significant in vitro reduction in the number of living cells, further support the hypothesis that, in B-CLL, the physiologic doses of IL-8 released constitutively by the leukemic clone may play an autocrine role in the process of cell accumulation characteristic of this disease.

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B-CELL CHRONIC lymphocytic leukemia (B-CLL) is characterized by the accumulation in the peripheral blood of slowly dividing monoclonal CD5+B lymphocytes.

The reasons for this accumulation process, typical of this disorder, are still largely unclear. A better understanding of the underlying mechanisms would open new strategies towards a more physiologic management of the disease. Over the last few years, several cytokines have been shown to interfere in the in vitro process of cell accumulation. At concentrations of 0.5 to 10 ng/mL, interleukin-4 (IL-4) inhibits the spontaneous and induced apoptosis of B-CLL cells; interferon α (IFNα; 1,000 U/mL) increases the survival of B-CLL cells and reduces the spontaneous DNA fragmentation of the leukemic cells; 100 U/mL of IFNγ promotes the survival of B-CLL cells; and IL-10 (100 ng/mL) specifically induces apoptotic cell death of B-CLL cells. The spontaneous death of B-CLL cells observed in vitro in a proportion of cases contrasts with the long survival in vivo of the same cells, further pointing to true autocrine and/or paracrine pathogenetic cytokine loops other than those due only to cellular interactions in the control of the disease in vivo.9

We recently described the constitutive production and release of biologically active IL-8 by B-CLL cells and the increased levels detected in the serum of the patients.10 IL-8 is an inflammatory cytokine with chemotactic properties towards neutrophils, T cells, and eosinophils, but an IL-8–related function on B lymphocytes has never been reported.

In the present study, we further investigated the role of IL-8 in B-CLL. Evidence is provided in support of the possibility that IL-8 may be involved in an autocrine manner in the abnormal accumulation of the neoplastic clone characteristic of this lymphoproliferative disorder.

MATERIALS AND METHODS

B-CLL cell purification. Enriched B-CLL cells were obtained after a Lymphoprep (Nycomed AS, Oslo, Norway) gradient separation of mononuclear cells and depletion of monocytes by adherence to plastic for 60 minutes at 37°C. T lymphocytes were removed by rosette formation with sheep red blood cells (E-rosettes) and repeated Lymphoprep fractionations. After purification, the samples to be tested were controlled for viability and analyzed with selected monoclonal antibodies (MoAbs) by flow cytometry using a FACSCAN (Becton Dickinson, Mountain View, CA); they contained greater than 98% CD5/CD19 double-positive leukemic cells, less than 1% CD14+ monocytic cells, and less than 1% CD3+ T cells. As previously described,10 in selected experiments, CD3 and CD14 mRNAs amplifications were also used to analyze the possible contamination of T cells and monocytes, respectively.

Cell cultures. Purified B-CLL cells were cultured for 2 to 14 days at a concentration of 2 × 105/mL in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA). The HL60, U937 (acute myeloid leukemias), and Nalm-6 (B-cell acute lymphoid leukemia) cell lines were cultured under the same conditions and used as controls. Human recombinant monococyte IL-8 (Upstate Biotechnology Inc, Lake Placid, NY) was used at final concentrations of 5 and 50 ng/mL. In a few experiments, higher doses were also used. Hydrocortisone (HC; Sigma Chemical Co, St Louis, MO) was used at 10−4 mol/L. A neutralizing antihuman

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IL-8 rabbit antiserum (Upstate Biotechnology Inc) was used at a dilution of 1:20 and 1:50. An anti–IL-8 MoAb (6E6 clone) kindly provided by Dr J.J. Oppenheim’s laboratory and produced by YSS Biolaboratory LTD (Mississauga, Ontario, Canada) was used at a dilution of 1:100. Recombinant human IL-4 (Upstate Biotechnology Inc) was used at a final concentration of 10 ng/mL. Recombinant human IFNα from Escherichia coli was obtained from Schering Corp (Kenilworth, NJ) and used at 500 U/mL.

Constitutive IL-8 release. IL-8 was measured in the supernatants of purified B-CLL cells incubated for 72 hours with RPMI 1640 medium supplemented with 10% FBS using an enzyme-linked immunosorbent assay (ELISA; generously provided by Medgenix Diagnostics, Fleurus, Belgium) with a detection limit of 0.7 pg/mL, according to the manufacturer’s instructions.

IL-8 binding. The direct binding of phycoerythrin-conjugated IL-8 (IL-8–PE; Fluorokine IL-8 Flow Cytometry Kit; R&D Systems, Minneapolis, MN) to its receptor was analyzed by FACSCAN flow cytometer. B-CLL and control cells (1 × 10⁶) were incubated for 60 minutes at 4°C with 10 μL of IL-8–PE at a concentration of 50 μg/mL or with unconjugated PE. The cells were then washed to remove unconjugated IL-8–PE and resuspended in 0.2 mL phosphate-buffered saline (PBS) for final flow cytometric analysis.²¹ Twenty-eight experiments were performed by using IL-8 (specific activity, 2,000 Ci/mmol) purchased from Amersham (Buckinghamshire, UK). Cells used for binding experiments were resuspended in a buffer (binding medium) consisting of RPMI 1640 medium containing 20 mmol/L HEPES (pH 7.2), 2% bovine serum albumin (BSA), and 0.02% sodium azide to inhibit the internalization of the iodinated ligand by the cells. A total of 1 × 10⁶ cells in 400 μL of binding medium containing various concentrations of [¹²⁵I]–IL-8 was incubated at 23°C for 2 hours (in preliminary experiments, binding at 23°C required 2 hours to reach equilibrium). After incubation, the cells were resuspended and carefully transferred onto 800 μL of precooled FBS and subsequently separated from the medium by centrifugation at 10,000 rpm in a microfuge. Medium and serum were aspirated and the cell sediment was sliced off with a razor blade to measure radioactivity with a gamma counter (Packard, Downers Grove, IL) with a counting efficiency of 70% for [¹²⁵I]. Duplicate determinations were performed for each IL-8 concentration. Specific binding was determined as the amount of binding blocked by competition with a 100-fold excess of unlabeled IL-8. Unspecific binding was defined as the amount of binding not displaced in the presence of excess cold ligand.

Receptor numbers and binding affinities were estimated by Scatchard analysis²² using the Enzfitter computer program (Elsevier Biosoft, Cambridge, UK). Reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted according to the method of Chomczynski and Sacchi²³ using the RNAzol solution (Cinna/Biotech, Houston, TX). RNAs were then treated for 30 minutes at 37°C with 1 U/μg of RNase-free DNase (Promega, Madison, WI) in 40 mmol/L Tris-HCl, pH 7.9, 10 mmol/L NaCl, 6 mmol/L MgCl₂, and 0.1 mmol/L CaCl₂. cDNAs were prepared by reverse transcription at 42°C for 30 minutes in a 50-μL reaction mixture containing 2 μg of total RNA, 0.5 μg oligo-dT, 1 mmol/L dNTPs (2′-deoxynucleotide-5′-triphosphate), 5 μL 10× RT-buffer (100 mmol/L Tris-HCl [pH 8.8], 500 mmol/L KCl, and 1% Triton X-100), MgCl₂ 5 mmol/L, 40 U RNAse, and 25 U avian Moloney reverse transcriptase (AMV)-RT. Five microliters of cDNA (corresponding to 0.2 μg of total RNA) was amplified in the presence of 40 μL of each primer, 10× buffer (0.5 mmol/L KCl, 0.4 mmol/L Tris-HCl, pH 8.8, 0.025 mmol/L MgCl₂, 0.002 mmol/L each dNTPs, and 2 mg/mL BSA), and 0.5 U of Taq (Thermus aquaticus) polymerase.¹⁴ The mixture was overlaid with mineral oil and then amplified in a Thermal Cycler (Cetus Corp, Emeryville, CA) with PCR cycle conditions individual for the different primers tested. Fifteen microliters of PCR product was electrophoresed in a 2% agarose gel in Tris/boric acid/EDTA buffer. Gels were stained with ethidium bromide and photographed. Specific primers for high-affinity IL-8 receptor mRNA (5′-TCACGTGGC-CCCATGCCAAACCTGC; 3′-CAGCGGCGACGGTACGACG) were designed on the basis of published sequences.²⁵ Bcl-2 mRNA primer sequences (5′-TTCCGGAGATGTCCAGCC; 3′-TCACGT- GTGGCCCGAGATGTCCAGCC; 3′-TCACGTGGCCCGAGATGTCCAGCC; 3′-GCTACATGCTCGATCCCACTTAA) specific mRNA primers were purchased from Clontech (Palo Alto, CA). All reagents for cDNA synthesis and PCR are from Promega.

Cytofluorimetric detection of bcl-2 protein. For intracellular detection of bcl-2 protein, 1 × 10⁶ cells were fixed in 2% paraformaldehyde and permeabilized after 10 minutes of incubation at 4°C in saponine (1 ng/mL in PBS) before staining with an FITC-conjugated anti-human bcl-2 IgG, MoAb (DAKO, Glostrup, Denmark) or with a mouse IgG, FITC-conjugated MoAb.

3H-thymidine ([H-TdR]) uptake. A total of 2 × 10⁵ cells were cultured at 37°C in flat-bottom microplate wells in 0.2 mL of RPMI 1640 medium containing 10% FBS and supplemented with nothing or with 5 or 50 ng/mL of IL-8. After 2 days of incubation at 37°C, cultures were pulsed for 18 hours with 1 μCi/well of [H-TdR] (specific activity, 2 Ci/mmol; New England Nuclear, Dreieich, Germany) and the incorporated radioactivity was determined using a liquid scintillation counter.

Propidium iodide (PI) staining. A total of 1 × 10⁶ cultured cells were centrifuged and cell pellets were gently resuspended in 1.5 mL hypotonic hypotonic solution (PI) at 50 μg/mL in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma Chemical Co). The tubes were placed at 4°C in the dark overnight. The PI fluorescence of individual nuclei was measured using a FACSCAN flow cytometer.

Statistical analysis. The paired t-test was used to analyze the statistical significance of the experimental results.

RESULTS

IL-8 mRNA expression and release. In agreement with our previous report,⁹ all 9 B-CLL samples tested constitutively expressed the mRNA for IL-8 and released between 0.3 and 6.5 ng/mL (3.4% ± 2.3% SD) of IL-8.

IL-8 receptor mRNA expression in B-CLL cells. The presence of the IL-8 receptor mRNA was investigated in 9 unstimulated B-CLL samples after T and monocyte depletion. Using RT-PCR analysis, the 439-bp IL-8 receptor-specific fragment was detected in 7 cases (5 representative samples are shown in Fig 1). The 2 negative results were derived from stock RNA samples; this prevented further IL-8 binding studies to be performed on these cases (see below). B-CLL cells maintained a stable mRNA expression of the IL-8 receptor after 2 to 3 days of culture.

IL-8 binding to B-CLL cells. To sustain the mRNA RT-PCR data, we searched for the presence of the surface IL-8 receptor on B-CLL cells. The cytofluorimetric detection of IL-8–PE bound to B-CLL cases was positive in all 10 enriched B-CLL samples tested (a representative case is shown in Fig 2) and the presence of the IL-8 receptor remained unchanged over a 6-day culture period. IL-8–PE binding to B-CLL membranes was inhibited after coinoculation of the cells with 1 μg of recombinant unlabeled IL-8 (Fig 2). Bind-
ing and competition studies with $^{125}$I–IL-8 showed specific IL-8 receptors on B-CLL cells (Fig 3). The Scatchard analysis is compatible with a single binding affinity model. The data are consistent with an average number of approximately 902 receptors/cell with a $K_d$ of 0.4 nmol/L.

**IL-8–induced B-CLL cell survival in culture.** The detection of the IL-8 receptor on B-CLL cells prompted us to investigate a possible functional role for IL-8 in this form of leukemia. No $^3$H-TdR proliferative signal was induced on 5 B-CLL samples by 5 to 50 ng/mL of IL-8. A possible involvement of IL-8 in controlling the survival in culture of B-CLL cells was then assessed. The PI staining of permeabilized B-CLL cells was performed after incubation with and without the addition of IL-8 at 5 and 50 ng/mL to quantify the percentage of surviving cells. The addition of IL-8 never induced B-CLL proliferation or division, and living B-CLL cells formed a well-defined diploid peak, whereas dying and/or apoptotic cells formed different populations with lower amounts of DNA. In 11 of the 16 (69%) cases studied, an increase in the percentage of living cells after IL-8 treatment was observed (Fig 4A). This effect became apparent at the 5 ng/mL IL-8 dose ($P = .001$) and increased further in the presence of 50 ng/mL ($P = .0001$). In all but 2 cases, higher concentrations of IL-8 (100 to 500 ng/mL) induced a greater proportion of dying cells than in the control. In the majority of cases ($n = 6$), the effect was evident after 2 days of

![Fig 1. IL-8 receptor mRNA RT-PCR analysis. The specific 439-bp PCR fragment is visible in 5 representative B-CLL cases (lanes 1 through 5) and in the HL60 cell line (lane 7). The Nalm-6 cell line (lane 6) represents the negative mRNA control. Lane 8 contains a control PCR reaction performed with all reagents, but without the cDNA. M is the molecular weight marker. At the bottom of the figure, the $\beta_2$-M mRNA amplifications represent the cDNAs control reactions.](image1)

![Fig 2. Cytofluorimetric profile of IL-8–PE specific binding and IL-8–PE binding inhibition after incubation with 1 $\mu$g/mL of unlabeled recombinant IL-8 in a representative B-CLL case. The dotted lines represent the background of unconjugated-PE, the solid lines represent the IL-8–PE staining, and the grey line represents the IL-8–PE staining after coincubation with IL-8.](image2)

![Fig 3. Saturation curve and Scatchard analysis (inset) for binding of $^{125}$I–IL-8 to B-CLL cells. Cells (1 x $10^6$/tube) were incubated with increasing concentrations of $^{125}$I–IL-8 at 23°C for 2 hours and cell bound radioactivity was separated from unbound radioactivity by centrifugation and cold FBS. Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled IL-8. Each point is the mean of at least two replicates. Scatchard data were analyzed by an Enzfitter computer program.](image3)
culture; in the other cases, the best response was observed at 7 days (n = 5). The simultaneous presence of a neutralizing anti–IL-8 MoAb abolished and reversed the IL-8–induced prolonging survival effect (see below).

In the remaining 5 of 16 cases, very high spontaneous survival percentages (90.60% ± 4.39% SD) were recorded after 2 days of incubation and this prevented the addition of exogenous IL-8 to induce a further death protection effect (Fig 4B). However, treatment for 2 days with 10−4 mol/L of HC reduced in these 5 cases the percentage of residual living cells to 55% ± 13.8% SD, and again, under these conditions, the addition of 5 ng/mL of IL-8 induced a significant rescue of dying cells (P = .05; Fig 4C). Figure 5 illustrates the cytotofluorimetric profile of the IL-8–mediated rescue from cell death in 2 representative untreated (Fig 5A) and HC-treated (Fig 5B) B-CLL cases.

A complete or partial B-CLL spontaneous death abrogation was observed after culture of B-CLL cells in the presence of IL-4 (10 ng/mL) or IFNα (500 U), respectively (data not shown). However, the IL-4– and IFNα–induced B-CLL survival did not synergize with IL-8 death abrogation.

IL-8–mediated regulation of bcl-2 mRNA expression in B-CLL cells. Constitutive mRNA expression of bcl-2 was detected by RT-PCR in the 7 B-CLL samples examined. When the cells were incubated with 5 ng/mL of IL-8 for 2 days, in 5 of the 7 samples analyzed an increase of the

![Diagram](image-url)
specific bcl-2 signal was recorded compared with untreated cells, whereas the control β2-M amplifications of the same cDNAs showed a constant signal (3 representative cases are shown in Fig 6). In 5 cases, the cells were incubated with HC. This produced a downmodulation in the expression of bcl-2. Again, and to a further extent compared with untreated cells, bcl-2 mRNA was recovered after IL-8 treatment (Fig 6). In all but one case, the best effect was observed with 5 ng/mL of IL-8, and higher concentrations of IL-8 (50 and 500 ng/mL) did not increase the bcl-2 mRNA expression compared with the basal level.

Regulation of bcl-2 protein by IL-8 in B-CLL cells. bcl-2 protein levels were determined in permeabilized B-CLL cells by flow cytometry in 10 cases. All cases constitutively expressed bcl-2 protein at a high level (bcl-2-high) and in a unimodal fashion. After 12 days of culture, 5 cases maintained the starting expression of bcl-2, and, under these conditions, IL-8 could induce no regulatory effect. In the other cases, a bimodal expression of bcl-2 protein was observed during the time course due to the proportion of cells (44.4% ± 17.4% SD) that expressed low levels of bcl-2 (bcl-2-low). In these latter cases, the addition of 5 ng/mL of IL-8 to the cultures resulted in a statistically significant (P = .01) augmentation of bcl-2-high expression, with a concomitant decrease of bcl-2-low expression. In these 5 cases, the overall mean percentage of bcl-2-high expression increased to 60.4% ± 16.6% SD compared with 53% ± 17.2% SD in cells cultured without IL-8. A profile of bcl-2 protein upmodulation induced by IL-8 in a representative B-CLL case is shown in Fig 7.

IL-8–mediated upregulation of IL-8 mRNA in B-CLL cells. The possibility that IL-8 could modulate the mRNA expression of IL-8 in B-CLL cells was investigated. The RT-PCR analysis performed showed that, in 10 of the 12 B-CLL cases incubated for 2 days with 5 ng/mL of IL-8, an increase of the 289-bp IL-8 specific signal was recorded (6 representative cases are reported in Fig 8), suggesting a potential autocrine regulation of IL-8 mRNA expression in B-CLL cells. In 2 cases, the RT-PCR data were confirmed by detecting increased IL-8 levels in the supernatants of B-CLL cells cultured in the presence of IL-8.

IL-8 neutralization in B-CLL culture. The possibility that endogenous IL-8 may mediate a survival signal in B-
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CLL was investigated. Leukemic cells from 10 B-CLL cases were cultured in the presence of a neutralizing antihuman IL-8 polyclonal rabbit antiserum. Under these culture conditions, the PI evaluation of the number of living cells showed an overall 30% reduction in the number of living cells (Table 1, \( P = .003 \)). By contrast, the preimmune rabbit serum used at the same dilutions used for the anti–IL-8 antiserum maintained or increased the number of untreated living cells. B-CLL cells (6 cases) were also cultured in the presence of an antihuman IL-8 MoAb; again, a statistically significant (\( P = .01 \)) decrease in the percentage of living cells (25% of inhibition) was observed, whereas the control MoAb exerted no effect (Table 1). The anti–IL-8 antiserum displayed its neutralizing action within 7 days of culture, whereas the anti–IL-8 MoAb required a more prolonged culture time (up to 14 days) to exert its inhibitory effect. Coincubation of 7 B-CLL samples with either the polyclonal antiserum or the monoclonal anti–IL-8 antibody and 5 ng/mL of IL-8 contrasted, although not completely, the death induction effect mediated by IL-8 neutralization (62.9% ± 23% SD of living cells in the control cultures vs 44.4% ± 22.3% SD in the presence of the anti–IL-8 antibody vs 53.2% ± 16% SD in the presence of the anti–IL-8 antibody plus IL-8). The addition of 50 ng/mL did not increase further B-CLL survival.

A possible nonspecific effect of the antibodies was ruled out by incubating the Nalm-6 pre-B-cell line with the same reagents; under these conditions, no modification in cell survival was observed. It should be noted that this cell line does not express the IL-8 mRNA and does not release IL-8. We also excluded an antibody-mediated lysis directed against cell surface receptor bound IL-8, because HL60 cells, which bind IL-8, did not modify their living features after culture for up to 14 days in the presence of anti–IL-8.

**DISCUSSION**

Several cytokines have been suggested to play a role in the leukemic growth or in prolonging cell survival of B-CLL cells in vitro. The cytokines may be produced by the leukemic cells themselves or by cells of the immunocompetent system. Thus, it is still uncertain whether this potential cytokine-mediated regulatory network involving the neoplastic clone establishes itself in an autocrine or paracrine fashion. The evidence that IL-8 is the only cytokine always produced constitutively by B-CLL cells suggests that this molecule may exert an autocrine regulatory function on the leukemic population. The results of this study help to shed light on the role of IL-8 in B-CLL.

The demonstration that leukemic B-CLL cells constitutively express the mRNA for the IL-8 receptor and that a specific IL-8 binding occurs on B-CLL cells clearly indicates that B-CLL cells can represent a target of IL-8 function. The IL-8 receptor is expressed on a wide range of cells, including phytohemagglutinin-activated T cells, CD4+ T cells, and...
monocytic cells and cell lines, synovial fibroblasts, neutrophils, the HL60 and THP-1 myeloid cell lines, and primary acute myeloid leukemia cells. Thus, the findings hereby reported represent the first evidence of the constitutive presence of the IL-8 receptor mRNA on human B cells. The functional competence of such receptor in B-CLL was confirmed by the demonstration that the neoplastic cells can effectively bind IL-8. The results of Scatchard analysis are indicative of a single binding affinity model; further studies will be necessary to clarify the presence and the features of IL-8 receptors on normal and leukemic B-cell subsets.

Because a proliferative effect of IL-8 on B-CLL cells was ruled out, the possibility that this cytokine could interfere in the death/survival pathways of the neoplastic clone was investigated. In most B-CLL samples, a prolonged survival effect on the leukemic cells could be documented after incubation with IL-8. In the cases that after culture maintained a high percentage of living cells, the protective effect induced by IL-8 became apparent after pretreatment of B-CLL with HC. These findings are indicative of a heterogeneous behavior of B-CLL samples with respect to the spontaneous and induced death in vitro and to the response to IL-8. The heterogeneity accounts also for the levels of IL-8 necessary to induce the prolonging survival effect. Responses to IL-8 observed by B-CLL cells seem to reflect an IL-8 threshold dose that ranges from 5 ng/mL to a maximum of 50 ng/mL for the protective effect; at doses greater than 50 ng/mL, IL-8 exerts an opposite action. This may be due to a saturation of the receptors and is in agreement with previous studies with other chemotactic agents, including IL-8. Overall, the 5 ng/mL dose of IL-8 appears to be the most effective. This is also confirmed by the mRNA induction experiments and by the functional studies on B-CLL cells. In this respect, it is worth noting that the 5 ng/mL dose is in the range of IL-8 levels constitutively released by B-CLL cells (present study). It appears, therefore, that the levels of IL-8 released physiologically by the leukemic cells are potentially sufficient to trigger in vivo the survival protective effect hereby documented in vitro.

Previous reports have suggested that the in vitro cytokine-mediated prolonging survival effect was due to a rescue from spontaneous apoptotic death of B-CLL cells. In our experiments, we could not reach the same interpretation because neither a consistent spontaneous DNA ladder (indicative of DNA fragmentation) nor a clear morphology with apoptotic bodies could be documented on primary untreated B-CLL—cultured cells. Also, the PI profile rarely showed a hypodiploid peak of DNA, indicative of a homogeneous apoptotic population, but rather a profile representative of asynchronous populations at different levels of death. Only when the cells were treated with HC was a simultaneous programmed cell death induced in a high proportion of cells, and, under this condition, a PI-stained apoptotic peak was observed (Fig 5B).

After treatment with IL-8, an upregulation in the mRNA expression of bcl-2 by B-CLL cells was documented. The IL-8—mediated bcl-2 upregulation was even greater on B-CLL cells pretreated with HC and in which the cytolytic action of HC had induced a downmodulation of bcl-2. These data have been corroborated by the observation of an upregulation of intracellular bcl-2 protein mediated by IL-8; this effect consists of an upmodulation of the bcl-2 mRNA expression when B-CLL cells express bcl-2 protein in a bimodal fashion. It has, in fact, been reported that B-CLL cells constitutively express high levels of bcl-2 and that, during spontaneous or induced (by IL-10 or HC) apoptosis, a downmodulation of bcl-2 expression occurs that is shown by the progressive appearance of an expanded peak of bcl-2 mRNA expression documented by a cytofluorimetric intermediate bimodal profile (personal unpublished data). Overexpression of the proto-oncogene bcl-2 has been associated with the survival and with the prevention of the physiologic program of cell death by B cells. Thus, the evidence of an upregulation of bcl-2 induced in B-CLL cells by physiologic doses of IL-8 further points to the role played by this cytokine in the accumulation process characteristic of this disorder.

The final purpose of this study was to verify a possible autocrine regulation of the neoplastic clone through IL-8 production. The first observation in favor of this possibility is the demonstration of the IL-8 mRNA upmodulation after exposure to IL-8. The second and more suggestive finding is the death-induced effect exerted on B-CLL cells following endogenous IL-8 neutralization following incubation in the presence of both polyclonal and monoclonal anti–IL-8 antibodies. IL-8 neutralization on cells that produce and bind IL-8 (HL60) or, conversely, that neither release, nor express, nor bind IL-8 (Nalm-6) exerted no effect. These findings support the hypothesis that, in B-CLL, the neoplastic clone contributes to its maintenance by using self-produced IL-8.

In conclusion, our analysis points to a functional role of IL-8 in B-CLL. This consists of a prolonging survival effect on spontaneous or induced dying cells mediated by physiologic doses of IL-8. This protective effect is exerted through a bcl-2—dependent pathway and appears to occur via an autocrine loop. A reduction in the number of living B-CLL cells is necessary to induce and recognize the IL-8 promoting survival effect, both in terms of the percentage of living cells and of bcl-2 upregulation. This offers an explanation for the individual behavior heterogeneity observed and suggests that mediators other than IL-8 are likely to be involved in regulating the survival mechanisms in B-CLL.

This study adds to the recent observation of a cell death protection induced in B-CLL cells by IL-4, IFNα, and IFNγ. However, these cytokines are usually not produced constitutively and are not expressed (except for IFNγ, which has been detected at the mRNA level after in situ hybridization) by the neoplastic clone, and their most likely paracrine action occurs at nonphysiologic doses. IL-8, which does not synergize with these molecules, is at the moment the best candidate for an autocrine cytokine prolonging survival effect on B-CLL cells. These findings extend our knowledge on the biologic properties of the leukemic growth in B-CLL and open the prospect of new therapeutic strategies aimed at interfering in the survival control of the neoplastic clone in vivo. Finally, the evidence of a functional role of IL-8 different from the known chemotactic activity and the characterization of a new potential cell target for IL-8 indi-
cate that further studies directed at unraveling the possible effects of this chemokine family on normal and neoplastic B-cell populations are necessary.

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