Interleukin 10 Induces Apoptotic Cell Death of B-Chronic Lymphocytic Leukemia Cells

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Summary

Recent studies have established that interleukin (IL)-10 induces growth and most notably differentiation of normal human B lymphocytes. We studied here the effects of IL-10 on the proliferation and survival of B-chronic lymphocytic leukemia (B-CLL) cells. IL-10 was found to inhibit 54–96% of the spontaneous tritiated thymidine incorporation observed in 3 of 12 B-CLL samples. Furthermore, IL-10 decreased the viable cell recovery of all five B-CLL samples tested, irrespective of whether cells were spontaneously synthesizing DNA or not. After 1 wk, B-CLL populations cultured with IL-10 were lost while those cultured without IL-10 survived. Flow cytometric analysis, DNA gel electrophoresis, and Giemsa staining all revealed that IL-10 induced B-CLL cells to die from apoptosis. This IL-10-mediated apoptosis was dose dependent and specific as it could be inhibited by a neutralizing anti-IL-10 antibody. B-CLL cells undergoing apoptosis in response to IL-10 showed decreased Bcl-2 protein levels. Addition of IL-2, IL-4, interferon γ, and anti-CD40 monoclonal antibody prevented the IL-10-mediated apoptosis of B-CLL cells. None of the malignant B cell populations obtained from eight non-Hodgkin's lymphomas and three hairy cell leukemias underwent apoptosis after IL-10 treatment, thus suggesting that the apoptotic effect of IL-10 is specific for B-CLL cells. Thus, IL-10 inhibits the DNA synthesis and most notably the survival of B-CLL cells, findings that call for considering IL-10 in the immunotherapy of chemoresistant B-CLL.

Materials and Methods

Antibodies. The monoclonal and polyclonal antibodies used for the phenotyping of the leukemic B cell preparations were purchased from the following manufacturers: FITC-conjugated anti-CD5 (Leu 1), anti-CD19 (Leu 12), anti-CD20 (Leu 16), anti-CD14 (Leu M3), anti-CD10 (Calla), and anti-HLA DR mAbs; all from Becton Dickinson Monoclonal Center (Mountain View, CA); FITC-conjugated anti-CD3 (IOT3) and anti-CD2 (IOT11) mAbs both from Im-
munotech (Marseille, France); FITC-conjugated anti–Bcl-2 mAb and F(ab)'; fragments of goat anti–human IgM, IgD, IgA, and IgG mAbs (from Dako (Glostrup, Denmark); FITC-conjugated F(ab)'; fragments of donkey anti–human κ or λ light chains antibodies from Kallestad Laboratories (Austin, TX). The neutralizing anti-IL-10 rabbit polyclonal purified Ab was produced in the laboratory as described previously (12). 3 μg/ml of anti-IL-10 Ab were determined to totally inhibit the effect of 10 ng/ml of IL-10 in an inhibition assay of IFN-γ production (12). A polyclonal anti-CD4 Ab, produced and purified in the same conditions as the anti-IL-10, was used as unrelated control antibody.

**Flow Cytometric Analysis.** Cell surface staining was performed as described previously (11) and samples were analyzed with a FACScan® (Becton Dickinson and Co., Sunnyvale, CA). Propidium iodide (2 μg/ml) was added in each sample before flow cytometric analysis in order to gate-out dead cells. The negative control was performed with an isotype-matched unrelated mAb. For intracellular detection of Bcl-2 protein, cells were permeabilized by 15-min incubations at 4°C in saponine (0.5 mg/ml) before staining with anti-Bcl-2 mAb or an unrelated mAb as a negative control. Apoptosis was determined by measurement of the incorporation of DNA-binding fluorophores Bisbenzimide Hoechst 33342 (Behring Diagnostics, La Jolla, CA) and propidium iodide as described previously (13). Cells were incubated 5 min with 10 μM Hoechst 33342 and 32 μM propidium iodide immediately before analysis with a double laser equipped FACStar Plus® (Becton Dickinson and Co.). Analysis permitted us to delineate three different populations: (a) permeable cell membranes allow dead cells to incorporate propidium iodide; (b) apoptotic cells incorporate only Hoechst; (c) viable cells incorporate neither propidium iodide nor Hoechst. The validity of this method was confirmed by fluorescent microscopy on the cells sorted from each population.

**Factors.** Purified recombinant IL-2 (3 × 10^6 U/mg; Amgen Biologicals, Thousand Oaks, CA), IL-4 (1 × 10^7 U/mg; Schering-Plough Research Institute, Kenilworth, NJ), and IFN-γ (1 × 10^7 U/mg; Genzyme Corp., Cambridge, MA) were routinely used at the final concentration of 20 U/ml, 100 U/ml, and 500 U/ml, respectively. Recombinant highly purified IL-10 from Chinese hamster ovary transfected cells, was obtained from Schering-Plough Research Institute and routinely used at a final concentration of 100 ng/ml.

**Patients.** Pathological samples were provided by Dr. A. Bussel (Hôpital Saint-Louis, Paris, France), Dr. J. F. Rossi (Institut du Cancer-Val d'Aurelle, Montpellier, France), Dr. A. Bryon (Hôpital Edouard Herriot, Lyon, France), and Dr. J. J. Sotto (Hôpital A. Michallon, Grenoble, France). This study included eight patients with the diagnosis of B-CLL, eight with the diagnosis of non-Hodgkin's lymphomas of low grade malignancy, according to the Working Formulation (14), and three with hairy cell leukemia. The available specimens included blood, spleen, and lymph nodes. Patient BAR was treated by corticoids and patient FLA was treated by chemotherapy at the time of the study. All other patients had not received any chemotherapy in the 3 mo before this study.

**Purification of B Lymphocytes.** Organs were cut with a scalpel blade and passed through a fine wire mesh to prepare a single cell suspension. Mononuclear cells were separated by standard Ficoll/Hypaque gradient method and were next submitted to E rosetting with sheep red blood cells. Nonrosetting cells (E-fraction) were labeled with anti-T cell (anti-CD2, anti-CD3) and anti–monocyte (anti-CD14) mAbs and subsequently incubated with magnetic beads coated with anti–mouse IgG antibodies (Dynal, Oslo, Norway). Residual non-B cells were removed by applying a magnetic field for 5 min.

IL-10 Inhibits Spontaneous DNA Synthesis and Decreases Viable Cell Recovery of B-CLL Cells. To examine the proliferative response of B-CLL cells to IL-10, 12 B-CLL samples were cultured for 3 d in complete medium with or without IL-10. Four B-CLL samples (Fig. 1 A) spontaneously incorporated significant amounts of [3H]Tdr (2,800-5,700 cpm) after 3 d of culture, and in all four cases, IL-10 significantly reduced this response (54-96% inhibition). As shown in Fig. 1 B...
(representative of three independent experiments), the inhibitory activity of IL-10 was dose dependent. The 50% inhibition was obtained with 1-3 ng/ml (depending on the sample) and maximal inhibition with 10-100 ng/ml of IL-10. The IL-10 dependent inhibition of [3H]TDR uptake was observed from days 2 to 7 (data not shown). The poor [3H]TDR uptake observed in the eight other B-CLL cases (<1,000 cpm) was not significantly affected by IL-10. Trypan blue dye exclusion assays, performed on five B-CLL samples cultured for 4 d with or without IL-10, indicate that IL-10 reduces the viability of B-CLL populations, irrespective of whether cells spontaneously synthesized DNA or not (Fig. 2 A). As illustrated on Fig. 2 B, a significant decrease of viable cell numbers was usually detected after 3 d, and after 7 d, this resulted in the loss of the culture. The effect was specific, since a neutralizing anti-IL-10 antibody was able to prevent the IL-10-induced cell death.

**IL-10 Enhances the Apoptosis of B-CLL Cells.** To further document the deleterious effect of IL-10 on cell viability, we tested whether IL-10-treated B-CLL cells were dying through apoptosis. To this end, after a 24-h incubation with or without IL-10, B-CLL cells were incubated for 5 min with Hoechst 33342 and propidium iodide to discriminate, by flow cytometry, cells undergoing apoptosis from viable cells and from necrotic cells, as described in Materials and Methods. As shown by FACS® analysis (Fig. 3, A and B), IL-10 increased the proportion of FLA B-CLL cells undergoing apoptosis, from 8 to 30%, as shown by a selective uptake of Hoechst 33342 (lower right panel). It also increased the proportion of dead cells from 20 to 30%, as shown by the uptake of propidium iodide (upper panel). To further confirm the relationship between these three populations, double negative cells of the lower left quadrant, and cells incorporating Hoechst 33342 after reculture (Fig. 3 D), thus indicating that the apoptotic process was engaged even though IL-10 was removed. On the 6 B-CLL samples tested, IL-10 enhanced by two- to four-

![Figure 2. IL-10 decreases viable B-CLL cell recovery. B-CLL cells were cultured in complete medium: (A) without or with IL-10 (100 ng/ml) (Trypan blue dye exclusions were performed at day 3); (B) without (□) or with IL-10 (100 ng/ml) (●), anti-IL-10 (3 μg/ml) (▲) or combination of anti-IL-10 and IL-10 (C) (Trypan blue dye exclusions were performed daily from days 0–7). Two experiments performed with other B-CLL samples yielded similar results. The symbol "+" means that this sample spontaneously incorporated [3H]TDR which is inhibited by IL-10 (see Fig. 1 A). Others do not spontaneously incorporate [3H]TDR.

![Figure 3. IL-10 increases the spontaneous apoptosis of B-CLL cells. 5 × 10^6 B-CLL cells were cultured without or with 100 ng/ml of IL-10. After 24 h, cells were incubated during 5 min with Hoechst 33342 (10 μM) and propidium iodide (32 μM). Discrimination of necrotic and apoptotic cells was performed by multiparametric flow cytometry. Ordinates: propidium iodide; abscissa: Hoechst 33342. Numbers indicate the percentage of positive cells in the three populations. The upper quadrant corresponds to dead cells, the lower left to viable cells and the lower right to apoptotic cells. (A) Complete medium; (B) IL-10; (C) apoptotic cells sorted and recultured for 24 h without IL-10; (D) viable cells, sorted and recultured for 24 h without IL-10.](https://example.com/figure3)
Table 1. Effect of IL-10 on the Survival of Malignant B Cells

| Samples | Phenotype | Percent viability | Percent apoptosis |
|---------|-----------|-------------------|-------------------|
|         | CD5       | CD10              | IL-10             | IL-10             |
| FLA     | B-CLL     | +                 | ml                | 58               | 41               | 10    | 26    |
| PAS     | B-CLL     | +                 | Mk                | 67               | 45               | 4     | 14    |
| PIG     | B-CLL     | +                 | mdl               | 81               | 66               | 6     | 15    |
| PON     | B-CLL     | +                 | k                 | 73               | 21               | 8     | 36    |
| VIS     | B-CLL     | +                 | MDk               | 25               | 8                | 26    | 34    |
| GER     | B-CLL     | +                 | MDk               | 36               | 16               | ND    | ND    |
| LES     | B-CLL     | +                 | mk                | 53               | 14               | 6     | 21    |
| BAR     | B-CLL     | +                 | Ml                | 30               | 14               | ND    | ND    |
| GAN     | DSCL      | +                 | Mk                | 7                | 9                | 8     | 8     |
| VER     | DSCL      | +                 | MGl              | 35               | 33               | 16    | 15    |
| MAG     | SLL       | +                 | MDk               | 57               | 56               | 9     | 10    |
| BOU     | FML       | -                 | Gk                | 32               | 36               | 9     | 7     |
| BRE     | FML       | -                 | Ml                | 88               | 87               | 1     | 1     |
| BOR     | FSCL      | -                 | Gk                | 54               | 39               | 15    | 17    |
| MOI     | FLCL      | -                 | MDk               | 37               | 42               | 12    | 14    |
| IEN     | FLCL      | -                 | ND                | 40               | 42               | 5     | 7     |
| MAU     | HCL       | -                 | mdk               | 63               | 59               | 3     | 4     |
| GOD     | HCL       | -                 | mkl               | 71               | 75               | 2     | 3     |
| MAR     | HCL       | -                 | ND                | 15               | 12               | 18    | 27    |

* Lower case letter for low density expression of surface Ig; upper case letters for high density surface Ig.

1 SLL, small lymphocytic lymphoma; DSCL, diffuse small cleaved lymphoma; FML, follicular mixed lymphoma; FSCL, follicular small cleaved lymphoma; FLCL, follicular large cell lymphoma; HCL, hairy cell leukemia.

Figure 4. IL-10 induces apoptosis of B-CLL cells. (A and B) Giemsa staining of B-CLL cells cultured for 48 h in medium alone (A) or with IL-10 (B); V, viable cells; A, cells undergoing apoptosis, showing chromatin condensation and nuclear fragmentation indicate apoptosis; G, ghost cells. (C) DNA gel electrophoresis from B-CLL cells cultured in medium alone (lane 2) or with 10 ng/ml of IL-10 (lane 3); lane 1, marker sizes (marker VIII, 1114-19 bp). Giemsa staining and DNA gel electrophoresis were performed with the same B-CLL sample, after a 2-d culture. Figures are representative of experiments performed on five different B-CLL samples.
fold the percentage of cells undergoing apoptosis measured at day 3 (Table 1).

The IL-10–induced apoptosis was further confirmed by microscopic examination of Giemsa-stained smears. As shown in Fig. 4 A, B-CLL cells cultured without IL-10 for 3 d displayed an homogeneous morphology. In contrast, cells cultured with IL-10 displayed a considerable heterogeneity after staining, with three principal types: "V" for viable cells, "A" for apoptotic cells, and "G" for ghost cells. In fact, sorted viable cells (lower left quadrant of Fig. 3 B) yielded V cells of Fig. 4 B, while sorted cells incorporating Hoechst 33342 undergoing apoptosis (lower right quadrant of Fig. 3 B) yielded A cells. Finally, cells incorporating propidium iodide of the upper quadrant yielded G cells.

Typical “ladder” pattern of internucleosomal DNA cleavage was observed on gel electrophoresis from DNA of B-CLL cells cultured for 3 d with IL-10 (Fig. 4 C, lane 3). A slight DNA fragmentation was obtained with B-CLL cells cultured without IL-10, reflecting spontaneous apoptosis (Fig. 4 C, lane 2).

IL-10 promoted the B-CLL apoptosis in a dose-dependent manner, 50% of the maximal effect was obtained with 0.6 ng/ml of IL-10 and optimal effect was obtained with 3 ng/ml of IL-10 (Fig. 5 A). The induction of B-CLL cell apoptosis was specific to IL-10, as a neutralizing anti-IL-10 purified antibody inhibited it (Fig. 5 B).

The in vitro survival of B-CLL cells has been shown earlier to be enhanced by addition of IL-4 (reference 16; Fig. 6 A), IFN-γ (reference 17; Fig. 6 A) and anti-CD40 mAb (references 18, 19; Fig. 6 A). As shown on Fig. 6 B, these agents also prevented the B-CLL apoptosis induced by IL-10. Interestingly, IL-2, which per se had only marginal effects on the survival of B-CLL cells, was able to counteract IL-10–induced cell death.

IL-10 decreases the levels of Bcl-2 protein in B-CLL cells. Studies on mechanisms controlling apoptosis have indicated a key role for protooncogene bcl-2 in prevention of apoptosis of lymphocytes (20), neuronal cells (21), and fibroblasts (22). Thus, Bcl-2 protein levels were determined in B-CLL cells by flow cytometry after permeabilization of cells with saponin and staining with a FITC-labeled anti-Bcl-2 monoclonal antibody. Freshly isolated B-CLL cells expressed Bcl-2 protein in a unimodal fashion, at high level (Bcl-2 high) (Fig. 7 A). After a 4-d culture in medium alone, 74% of the B-CLL cells expressed as much Bcl-2 as freshly isolated B-CLL cells (Bcl-2 high), whereas 26% of the cells expressed low levels of Bcl-2 (Bcl-2 low) (Fig. 7 B). Addition of IL-10 to cultures resulted in a decrease of Bcl-2 expression, with 83% being Bcl-2 low and 17% being Bcl-2 high. The mean percentage of Bcl-2 low cells of seven samples cultured for 3 d with IL-10, was 58% ± 26 (mean ± SD), contrasting with 38% ± 25 in cells cultured without IL-10 (p = 0.018, according to a Wilcoxon rank test). FACS® analysis performed on these samples cultured with IL-10 showed a mean of 50% ± 29 of nonviable cells, including those incorporating either Hoechst 33342 or propidium iodide or both dyes. The percentage of Bcl-2 low cells was correlated with the per-
As shown in Fig. 8 B, representing the results pooled from three independent experiments, the IL-10–induced decrease of Bcl-2 expression, observed at day 3, was prevented by addition of IL-2, IL-4, IFN-γ, and anti–CD40 antibody presented on CDw32 L cells (15). These results are in line with the suppression of IL-10–mediated apoptosis of B-CLL cells, observed with those agents (Fig. 6). Note that anti–CD40 activation was able to prevent the spontaneous decrease of the proportion of Bcl-2 high cells in cultures of B-CLL cells without IL-10, while IL-2, IL-4, and IFN-γ were unable to do so (Fig. 8 A).

**IL-10 Does Not Induce Apoptosis of Non-Hodgkin's Lymphomas and Hairy Cell Leukemias B Cells.** We then wondered whether other malignant B cells would also undergo apoptosis in response to IL-10. Thus, malignant B cell samples from eight non-Hodgkin's lymphomas and three hairy cell leukemias were cultured in complete medium with or without IL-10. After 3 d, cell viability was determined by Trypan blue exclusion and apoptosis was determined by FACS® analysis after incubation with propidium iodide and Hoechst 33342. Before culture, expression of CD5, CD10, and surface Ig were determined on all samples tested. As shown in Table 1, results were compared with those obtained on a panel of eight B-CLL samples. In all cases, IL-10 decreased the viability and increased the apoptosis of the eight B-CLL samples (p = 0.01). In contrast, IL-10 failed to alter cell viability or to induce apoptosis of all the eight lymphoma samples (p = 0.67), whatever their expression of CD5 and CD10 antigens. Similarly, the three hairy cell leukemia samples tested were not significantly induced to undergo apoptosis in response to IL-10.

**Discussion**

Previous studies have demonstrated that B-CLL cells spontaneously die from apoptosis during in vitro culture (23), although it remains low compared to the important cell death observed with normal B cells (10% vs. 35%, respectively, after 24 h; mean of five experiments; our unpublished data). The spontaneous apoptosis of B-CLL cells was earlier found to be enhanced by glucocorticoids (16), Ca2+ ionophore and anti-IgM antibodies (24). In the present report, we demonstrate that IL-10 is also able to enhance apoptosis of B-CLL cells. This is indicated by: (a) flow cytometric analysis showing Hoechst 33342 incorporation; (b) Giemsa staining, showing cells with condensed chromatin; and (c) gel electrophoresis, showing internucleosomal DNA fragmentation, all characteristic of apoptosis. Low concentrations of IL-10 (1–10 ng/ml) induced B-CLL cells into successive steps toward death and 10-fold this optimal concentration (100 ng/ml) did not result in the recruitment of a larger proportion of apoptotic B-CLL cells. However, cells progressively entered into apoptosis with time, ultimately resulting in the loss of cultures. It is not clear: (a) why some cells of a given leukemic clone enter into apoptosis early in the culture while others do it later, and (b) why some B-CLL samples are more susceptible to the apoptotic effect of IL-10 than others. It could possibly be related to the age of the B-CLL cells at the time of their isolation from the patient, or to their cell cycle. The level and promptness of sensitivity to IL-10 could not be related to a particular phenotype such as expression of slgD, CD23, CD39, CD38, and CD25. Nevertheless, all B-CLL cells tested were found to enter apoptosis in response to IL-10, thus indicating an homogeneous response of this leukemic cell type. This property seems to be restricted to B-CLL cells, as malignant B cells from non-Hodgkin's lymphomas, expressing or not the CD5 antigen, and hairy cell leukemias did not show increased cell death when cultured with IL-10. In this context, increased circulating IL-10 levels have been detected in sera of patients suffering from non-Hodgkin's lymphomas and the high levels of circulating IL-10 have been correlated to poor prognosis (25–27). In HIV-associated lymphomas, this IL-10 production was ascribed to the neoplastic clone, thus suggesting a possible autocrine role in the proliferation of these cells (26), as demonstrated in EBV-transformed cell lines (12, 28). In contrast, we did not detect any IL-10 in the sera of 20 untreated B-CLL patients, whatever their clini-
Bcl-2 expression by B-CLL cells, a result contrasting with B-CLL cells, to an extent that correlated with the level of which may explain the lack of IL-10 induced apoptosis in those samples (Table 1). In fact, in the present study, IL-10 was found to downregulate the levels of Bcl-2 protein on B-CLL cells, to an extent that correlated with the level of induced apoptosis. The pooled results of three different B-CLL samples demonstrated a nonsignificant effect of IL-4 on the Bcl-2 expression by B-CLL cells, a result contrasting with a previous study (16). In line with our results, the lack of effect of IL-4, Bcl-2 protein expression has been recently reported in five B-CLL samples (30). However, Bcl-2 expression in one of the three samples tested herein was significantly increased in response to IL-4 while two others remained unaffected. Such an heterogeneous regulation of Bcl-2 expression in B-CLL cells in response to IL-4 remains to be explained. Nevertheless, IL-4 was found, in all cases, to counteract the IL-10 induced decrease of Bcl-2 expression.

IFN-γ, IL-2, and anti-CD40, which blocked IL-10 induced apoptosis, also prevented the downregulation of Bcl-2 protein induced by IL-10. Interestingly, IL-2 was able to totally overcome the apoptotic effects of IL-10. This was observed not only in cells that spontaneously synthesized DNA in response to IL-2, but also in samples that failed to do so. This is better explained by the capacity of IL-10 to upregulate the expression of high affinity IL-2 receptors on B-CLL cells (11). These IL-2 receptors will then permit to transmit a survival signal to the B-CLL cells as was shown earlier with ionomycin-treated normal B lymphocytes (31). This further indicates that the survival signal provided by IL-2 prevails over the apoptotic signal of IL-10. While a positive correlation between the level of Bcl-2 protein expression and B-CLL cell apoptosis was observed, a more detailed analysis will be required to determine the pathways of IL-10-induced B-CLL apoptosis. In particular, it will be worthy to determine how IL-10 affects the expression of bcl-x and bax genes, which were recently reported to play a key role in the regulation of apoptotic cell death (32, 33).

IFN-γ was also found to inhibit the IL-10–induced apoptosis of B-CLL cells. Previous studies have demonstrated that IFN-γ sustained B-CLL cell survival (17) and promoted their proliferation (34). Furthermore, IFN-γ has been detected in sera of patients with B-CLL (17), thus suggesting a direct role in the in vivo expansion of the leukemic clone. As B-CLL cells have been shown to produce IFN-γ (16) and as IL-10 has been shown to inhibit IFN-γ production (35–36), it will be worth studying whether the effects of IL-10 on B-CLL cells are due to an alteration of the secretion of IFN-γ or any other autocrine cytokines such as TNF-α (37). In vivo, the direct effect of IL-10 on B-CLL cells may be further strengthened by its inhibitory effect on the production of IFN-γ (35, 36).

The IL-10–induced inhibition of viability observed with B-CLL cells contrasts with the stimulation of viability observed in normal resting murine B cells (38). Several reasons may account for this discrepancy, the most likely being the neoplastic status of the B-CLL cells. Accordingly, our preliminary studies failed to detect increased apoptosis of normal tonsillar CD5+ B cells in response to IL-10 but further studies are required to determine whether other normal B cell subpopulations may undergo increased apoptosis after exposure to IL-10.

The finding that IL-10 induces apoptosis of B-CLL cells, increases the array of IL-10 activities and constitutes another example of a proapoptotic effect of cytokines, as recently demonstrated for the cytotoxic effect of TNF-α (39–41). This contrasts with other cytokines, such as GM-CSF or IL-3, that act as growth factors and whose removal results in cell apoptosis (42).

In summary, we have shown that IL-10 induces apoptotic cell death of B-CLL cells, a finding that calls for considering IL-10 in the immunotherapy of chemoresistant B-CLL.

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