

## DIFFERENTIAL EXPRESSION OF BCL-2 ONCOPROTEIN AND FAS ANTIGEN ON NORMAL PERIPHERAL BLOOD AND LEUKEMIC BONE MARROW CELLS. A FLOW CYTOMETRIC ANALYSIS

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### ABSTRACT

**Background.** Fas antigen (Ag) has recently been identified as the putative surface molecule capable of transducing apoptotic signals into cells. Alterations in the expression of proto-oncogene bcl-2 have been implicated in the regulation of apoptosis.

**Materials and methods.** By employing a monoclonal antibody to bcl-2 protein (124 clone) and to Fas Ag (UB2 clone) the expression of these molecules was analyzed at flow cytometry on bone marrow (BM) and peripheral blood (PB) samples from patients suffering from different lymphoid and myeloid leukemic diseases (27 acute non-lymphocytic leukemia [ANLL]; 14 acute lymphocytic leukemia [ALL]; 19 B-cell chronic lymphocytic leukemia [CLL]; 2 Ph1+ chronic myeloid leukemia [CML]; one CD8+ T-cell chronic lymphoproliferative disorders). Results were compared with those observed on normal PB leukocytes and BM B-cell precursors from patients with non-neoplastic hematological disorders.

**Results.** Fas Ag was constitutively expressed by both monocytes and neutrophils, while lymphocytes expressed bcl-2 with no difference between B and T cell subsets. Interestingly, bcl-2 expression was always absent on neutrophils. When dealing with ANLL patients, a relatively low bcl-2 and high Fas Ag phenotype characterized subtypes with granulocytic (M2) or promyelocytic (M3) differentiation. This observation was confirmed in a small number of patients for whom bcl-2 levels were quantified as antibody binding capacity (ABC) in molecules/cell. Leukemic cells from patients with ALL constitutively expressed bcl-2, the pattern of this expression being quantitatively lower than that of immature B-cell precursors. Finally, high bcl-2 and low Fas Ag expression represented a crucial part of the B-cell CLL immunophenotype.

**Conclusions.** Although based on a small number of patient and control samples, our results suggest that bcl-2 and Fas Ag are coordinately expressed on normal PB leukocytes. Fas Ag is expressed at low levels on B-CLL cells, generally considered long-surviving cells. The relatively lower bcl-2-expression detected in both M2 and M3 subtypes may explain, at least in part, the higher remission rates obtained in these forms of ANLL than in other less differentiated morphological variants.

*Key words:* bcl-2, Fas Ag, peripheral blood leukocytes, bone marrow leukemic cells

Programmed cell death (apoptosis) is a physiological mechanism that plays an important role in cell differentiation and the selection processes necessary for maintaining cellular homeostasis in a variety of tissues.<sup>1</sup> The recently identified Fas antigen (Ag) is a member of the nerve growth factor (NGF)/tumor necro-

sis factor (TNF) receptor superfamily that can mediate apoptosis.<sup>2</sup> Indeed anti-Fas antibodies may effectively accelerate apoptotic cell death in some human cell lines, although the apoptotic effect is in part determined by bcl-2 expression status.<sup>3</sup> Interestingly, a soluble form of APO-1/Fas Ag has been identified in the supernatant

of human B- and T-cell lines and increased serum levels have been detected in B- and T-cell leukemias.<sup>4</sup>

The cytoplasmic product of proto-oncogene bcl-2 has been shown to prolong cellular survival by inhibiting apoptosis.<sup>15</sup> Bcl-2 protein and Fas Ag have opposite effects on apoptosis; therefore, differential expression of these molecules may result in dysregulation of the apoptotic processes.<sup>6,7</sup> Both bcl-2 protein and Fas Ag expression have been analyzed on peripheral blood (PB) leukocytes<sup>3</sup> and on surgical tissues from non-Hodgkin's lymphomas (NHL).<sup>8</sup> Here we show that bcl-2 and Fas Ag are coordinately expressed on normal PB leukocytes. Fas is expressed at low levels on B-CLL cells, generally considered to be long-surviving cells. Acute non lymphocytic leukemia (ANLL) cases belonging to M2 and M3 subtypes displayed a relatively low bcl2/high Fas Ag phenotype, which may explain, at least in part, the higher remission rate obtained in these morphological variants.

## Materials and Methods

### Patient and control cell samples

Bone marrow (BM) leukemic cells obtained at the time of diagnosis before antileukemic therapy from 27 ANLL and 14 acute lymphocytic leukemia (ALL) patients were utilized for the present analysis. Diagnostic procedures that included cytomorphological, cytochemical and immunological analyses were carried out in all leukemic patients. Morphological examination of the BM smears indicated that over 50% of cells were blasts. According to the FAB classification,<sup>9</sup> ANLL patients were grouped as follows: M1, 11; M2, 6; M3, 3; M4, 5; M5, 2. The ALL classes consisted of 10 common ALL (B-II), 3 pre-B ALL (B-III) and one mature B-ALL (B-IV).<sup>10</sup>

Bcl-2 and Fas Ag expression was also studied on PB lymphocytes from 19 CD5<sup>+</sup> B-cell CLL patients (stage A, 11; stage B, 5; stage C, 3). At the time of the present study three out of the 19 patients were receiving intermittent chlorambucil and prednisone; one patient was on maintenance therapy with interferon- $\alpha$ 2 three times a

week. For immunological analyses B-CLL cells were assessed according to the following formula: B-CD5<sup>+</sup> cells = CD5<sup>+</sup> - CD3<sup>+</sup>. According to this evaluation the mean value of CD5<sup>+</sup> non-T cells accounted for 69.8% $\pm$ 20.

Bcl-2 and Fas Ag expression were evaluated on lymphocytes from a previously untreated patient suffering from a CD8<sup>+</sup> T-chronic lymphoproliferative disorder, and on neutrophils from 2 Ph1<sup>+</sup> chronic myeloid leukemia (CML) patients who were being treated with hydroxyurea. Finally, the expression of either Fas Ag or bcl-2 protein was studied on normal PB leukocytes from 6 healthy donors (HDs) and on BM B-cell precursors from 4 children with idiopathic thrombocytopenic purpura (ITP). The threshold for Fas Ag and bcl-2 positivity was 20%.

### Cell preparation and flow cytometric analysis

Freshly heparinized samples were used for immunological analyses. When dealing with leukemic patients, BM leukemic cells were separated from whole blood by Ficoll-Hypaque density gradient centrifugation. Owing to the different right- and forward angle light-scattering properties of PB cells, the trigger region of the cytogram whose reading area opened on lymphocytes, monocytes and granulocytes were used to separate these cell populations from others. Furthermore, normal PB lymphocytes were enumerated via the expression of the surface CD3-molecule, while monocytes and granulocytes, were recognized via the expression of surface CD14 and CD16 molecules, respectively.

Immunofluorescence analysis of Fas Ag expression on lymphocytes, monocytes and granulocytes as well as on BM leukemic cells was performed as follows: the cells were incubated with anti-Fas monoclonal antibody (Mo Ab) (UB2 Clone, IgG1 isotype, Immunotech, Marseille, France) or irrelevant control antibody, washed and treated with a second layer reagent of fluorescence-coupled anti-mouse Ig (Ortho-Pharmaceutic). For flow cytometric evaluation of intracellular bcl-2 in individual cells, the cells were permeabilized by means of a new fixative (Ortho Permeafix, OPF).<sup>11</sup> The fixed, permeabilized cells were incubated with purified mouse anti-bcl-2 MoAb conjugated

with fluorescein isothiocyanate isomer 1 (124 Clone: IgG1, k isotype, DAKO, Copenhagen, Denmark). Incubation with antibodies lasted for 15 minutes on ice, followed by 2 washings in PBS containing 3% fetal calf serum and 0.1% sodium azide. The stained cells were analyzed on an ABSOLUTE flow cytometer (Ortho Diagnostic System). In selected cases two-color immunofluorescence was utilized. A phycoerythrinated (red fluorescence) MoAb (CD19 for B-lymphoid and CD33 or CD13 for myeloid leukemias) and a fluoresceinated (green fluorescence) MoAb (Fas Ag or bcl-2) were used for this purpose. The mean fluorescence intensity (MFI) was assessed on the basis of the mean channel fluorescence of each positive population sample (linear acquisition, 0 to 250 channels).

#### *Indirect IF assay and assessment of bcl-2 antigen density by the QIFI technique*

Leukemic cells from 9 ANLL patients (6 M1; 2 M2; 1 M3), whose BM samples were cryopreserved were utilized for the assessment of bcl-2 antigen density by the Quantitative Immunofluorescence Indirect assay (QIFI Kit, DAKO, Copenhagen, Denmark). Cell viability was determined by trypan blue staining, which was more than 80% in all samples. Indirect staining for bcl-2 was performed by incubating permeabilized cells with anti-bcl-2 MoAb (1:20 dilution for 30 min at 20°C), followed by washing and further incubation with affinity purified goat anti-mouse Ig FITC (1:100 dilution for 45 minutes at 4°C). This test was analyzed with the QIFI kit assay. Briefly, the method utilizes an indirect immunofluorescence analysis in which the fluorescence standards included were labeled with the same fluoresceinated anti-mouse immunoglobulin reagent. It has been shown that these CD5-labeled standards could be used to evaluate the number of any IgG MoAb molecules/cell to be tested.<sup>12</sup> Fluorescence intensity measured on the different standards was used to calculate the standard regression line between fluorescence intensity and antigen density expressed as antibody binding capacity (ABC) in molecules/cell.

#### *Statistical analyses*

The Student's t-test and analysis of variance

were used to evaluate differences in the mean among various disease groups. The "r" correlation coefficients between parameters were computed in least-squares regression equations.

## **Results**

### *bcl-2 and Fas Ag expression by normal and leukemic PB leukocytes*

Fas Ag, bimodally expressed on the PB T-lymphocytes of HDs (Figure 1), was virtually absent on normal B-lymphocytes. Similar trends were observed in chronic leukemic lymphoproliferative disorders of both B- and T-lineage. Indeed the low Fas Ag expression encountered on B-CLL cells ( $m=13.2\pm 14.1\%$ ) contrasted with the strong pattern of positivity observed in a patients suffering from a CD8<sup>+</sup> T-chronic lymphoproliferative disorder (Figure 2). Monocytes and neutrophils of HDs constitutively expressed Fas Ag (Figure 1). Interestingly, a similar pattern of Fas Ag expression was displayed by neutrophils from 2 patients with Ph1<sup>+</sup> CML (Table 1).

When dealing with cytoplasmic bcl-2 expression, significant differences among PB leukocyte populations were detected. Normal PB lymphocytes expressed bcl-2 constitutively with no differences between B- and T-cell subsets (Table 1).

Table 1. Expression of Fas Ag and bcl-2 oncoprotein on peripheral blood cells from normal controls and patients with chronic leukemias.

<i>Cell samples</i>	<i>Cell type</i>	<i>Fas Ag</i>	<i>bcl-2</i>
Normal controls (n = 6)	B-lymphocytes	-	+++
	T-lymphocytes	++	+++
	Monocytes	++	++
	Neutrophils	+++	-
B-CLL (n = 19)	B-lymphocytes	+	+++
CD8 <sup>+</sup> -T-CLL (n = 1)	T-lymphocytes	++	+++
CML (n = 2)	Neutrophils	+++	-

*Abbreviations: CLL (chronic lymphocytic leukemia); CML (chronic myeloid leukemia). +++ , more than 50% stained cells; ++, up to 30% stained cells; +, up to 10% stained cells; -, less than 10% stained cells;*

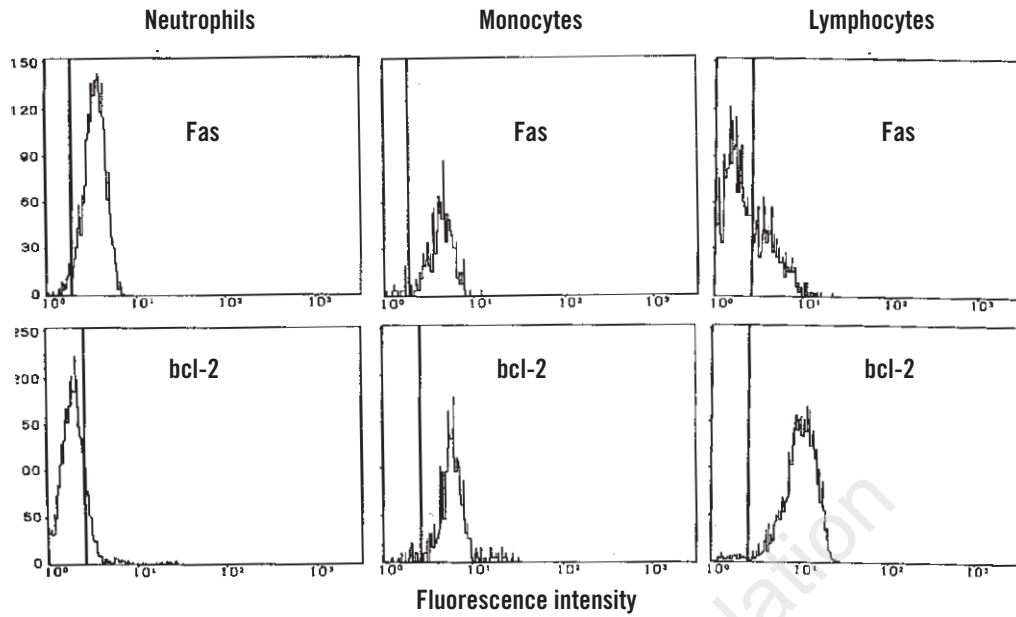


Figure 1. Differential expression of Fas Ag and bcl-2 protein on peripheral blood lymphocytes, monocytes, and neutrophils. Immunofluorescence profiles were displayed as single-parameter histograms.

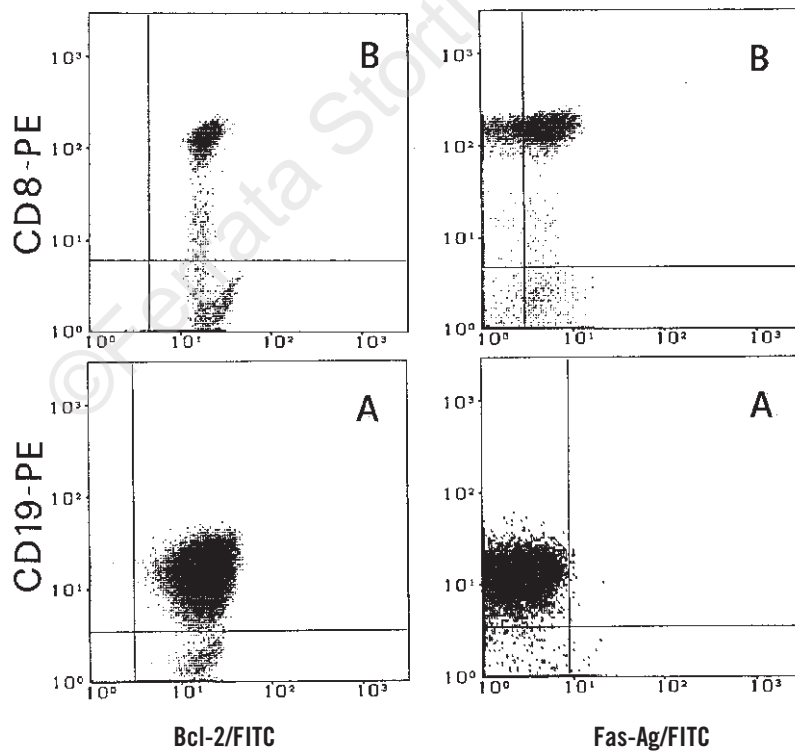


Figure 2. Two color Fas Ag and bcl-2 analysis on peripheral blood lymphocytes from a B-cell CLL (A) and CD8<sup>+</sup> lymphoproliferative disorder (B) patient sample, respectively.

Table 2. bcl-2 protein and Fas Ag expression in B-cell CLL.

	Clinical stages			p*
	A = 11	B = 5	C = 3	
<i>bcl-2</i>				
% positive cells (SE)	92.1 (4.4)	95.5 (2.9)	93.9 (3.9)	0.878
MFI (SE) <sup>o</sup>	84.5 (3.1)	83.8 (5.5)	76.2 (2.4)	0.475
<i>Fas Ag</i>				
% positive cells (SE)	21.3 (2.2)	21.3 (11)	12.2 (4.5)	0.33

\*p value refers to trend from stage A to C. <sup>o</sup>MFI = Mean fluorescence intensity of positive cells (linear acquisition, 0 to 250 channels).

reflected in the lower MFI they displayed (MFI 62.2±6) with respect to lymphocytes (MFI 78.5±6). Finally, bcl-2 expression was always absent on neutrophils (Figure 1).

#### *Bcl-2 and Fas Ag expression by BM leukemic cells of the myeloid lineage*

Bcl-2 was expressed constitutively by BM leukemic cells of ANLL patients. Despite the lack of correlation with CD34 positivity ( $r = 0.228$ ;  $p = 0.372$ ), due at least in part to the small number of patients analyzed, bcl-2 expression decreased as a function of granulocytic or promyelocytic differentiation. Indeed ANLL cases belonging to FAB subtype M2 or M3 showed a lower percentage of bcl-2-positive cells than leukemias with less differentiated (M1) or monocytic (M4-M5) features ( $P = 0.031$ ) (Table 3). Furthermore, in 9 ANLL (6 M1; 2 M2; 1 M3) patients changes in bcl-2 protein were quantified as ABC in molecules/cell. The mean bcl-2 antigen density was

$12.3 \times 10^3$  molecules/cell (range 7.8- $14.2 \times 10^3$  molecules/cell). Interestingly, FAB M1 cases showed a significantly higher mean bcl-2 ABC than cases with M2-M3 subtype ( $13.3 \pm 0.7 \times 10^3$  molecules/cell versus  $10.2 \pm 2.1$  molecules/cell;  $p = 0.012$ ).

When dealing with Fas Ag expression we found a heterogeneous pattern of expression; Fas Ag positivity increased as a function of granulocytic or monocytic differentiation (Table 2). This is reflected in the lower mean percent value of Fas positive cells among M1 ANLL cases ( $p = 0.008$ ). Moreover, a weak inverse correlation was detected between Fas Ag expression and the MFI of bcl-2 ( $r = -0.372$ ;  $p = 0.052$ ).

#### *Bcl-2 and Fas Ag expression by B-ALL leukemias and B-cell precursors*

Bcl-2 was constitutively expressed by BM cells of all 14 B-lineage ALLs analyzed (mean 97.7%; SD 1.0) (Table 4). These results were compared with those obtained on immature BM B-cell precursors. For this reason we studied bcl-2 expression on BM samples from 4 children with ITP who displayed an excess of CD19<sup>+</sup>/CD10<sup>+</sup> BM cells ( $55.8\% \pm 9.3\%$ ).<sup>13</sup> Immature BM B-cell precursors were found to be bcl-2 positive (range 20% to 95.6%), although the pattern of this expression was quantitatively lower than that observed in B-ALLs (MFI  $61.2 \pm 13.4$  versus  $85.5 \pm 4.3$ ;  $p < 0.001$ ) (Table 4). Finally, low Fas Ag positivity was encountered in common ALL (B-II) cases, in contrast with a relative increase of Fas Ag positive cells observed in both pre-B-ALL (B-III) and mature B-ALL (B-IV) cells

	ANLL FAB subtypes					p
	M1	M2	M3	M4	M5	
<i>bcl-2</i>						
positive cases	11/11	6/6	3/3	5/5	2/2	NS
% positive cells (SE)	96.7 (1.4)	86.4 (7.9)	72.4 (12.5)	96.9 (1.4)	97 (1.8)	0.031
MFI (SE)	98.8 (4)	83.5 (7.4)	88.9 (15.7)	87.2 (3.8)	97.2 (9.7)	0.475
<i>Fas Ag</i>						
positive cases	6/11	6/6	3/3	4/5	1/2	0.197
% positive cells (SE)	32.2 (1.5)	56.9 (2.0)	57.5 (2.3)	54.8 (3)	48.9 (44.7)	0.008
MFI (SE)	47.9 (3.6)	50.1 (5.9)	56.1 (1.9)	53.7 (9.6)	58.8 (12.9)	0.801

Table 3. bcl-2 protein and Fas Ag expression in ANLL.

Abbreviations: P value refers to trend among different groups (analysis of variance); MFI = Mean Fluorescence Intensity of positive cells (linear acquisition, 0 to 250 channels).

The threshold for Fas Ag and bcl-2 positivity was 20%

Table 4. bcl-2 and Fas antigen expression on ALL and B-cell precursor cells.

Cell type	bcl-2	
	% positive cells/MFI	Fas Ag (% positive cells)
Common ALL (B-II) (n = 10)	97.5±4.2/88.0±22.1	13.9±19.6
Pre-B-ALL (B-III) (n = 2)	97.7±3.8/88.2±21.9	37±44.7
Mature B-ALL (B IV) (n = 1)	98.8/80.5	40.8
Normal B-cell precursors (n = 4)	86.0±25.8/61.2±13.4	6.7±4.3

Abbreviations : MFI = Mean Fluorescence Intensity of positive cells (linear acquisition, 0 to 250 channels).

(Table 4).

### Discussion

In the present study we confirm that bcl-2 and Fas Ag expression may be associated with survival of hematopoietic cells. In fact, mature polymorphonuclear cells that have the shortest half-life among PB leukocytes and easily undergo apoptosis *in vitro*<sup>3</sup> were characterized by a high Fas high absent bcl-2 phenotype. In contrast, relatively longer-lived cells such as monocytes constitutively expressed Fas Ag, while bcl-2 expression was lower than that of PB lymphocytes.

Given the critical role in the functioning of the immune system played by Fas Ag and bcl-2, observations dealing with the baseline condition of different lymphocyte subsets are of interest. Fas Ag, expressed by a minority of T-cells, was virtually absent on B-lymphocytes. In contrast, both B and T lymphocyte subsets constitutively expressed bcl-2. The activation state occurring in patients suffering from neoplastic diseases such as multiple myeloma may induce T-lymphocytes to assume a Fas-high/bcl-2-low phenotype as a consequence of long-term exposure to tumor cells.<sup>14</sup> Under these circumstances T-cell ability to exert antitumor activity *in vivo* is reduced.<sup>14</sup>

Increased bcl-2 expression has been found on blasts from patients with myelodysplastic syndromes (MDS), ANLL and ALL,<sup>15-19</sup> although the

impact on either response to chemotherapy or prognosis is controversial.<sup>20-22</sup> In comparison to results of the present study, a relatively low number of bcl-2<sup>+</sup> ANLL blasts has been reported in a larger series.<sup>15</sup> This could be due to different permeabilization methods used. Our results are in keeping with those recently reported by Porwit-Mac Donald;<sup>18</sup> in both these studies experiments were carried out by using a new fixative (Ortho-Permeafix) that permeabilizes the cells without altering scatter properties or membrane or cytoplasmic staining.<sup>11</sup>

Although not correlated with CD34 expression, the percentage of bcl-2-positive blasts was, in our experience, significantly lower in M2/M3 FAB subtypes, two morphological variants characterized by a high remission rate after induction chemotherapy. Similar results were obtained in a smaller number of patients from our series in whom bcl-2 levels were assessed by using a quantitative method which makes it possible to evaluate antigen density as antibody binding sites per cell, thus removing any variability due to equipment or reagents.

Fas Ag may be expressed as part of the differentiation program of hematopoietic cells in the presence or absence of interferon- $\gamma$  (IFN- $\gamma$ ) and/or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>23,24</sup> This is reflected in the lower percent value of Fas-positive cells encountered in FAB M1 ANLL cases. Interestingly, when considering the whole series of ANLL patients, an inverse correlation was detected between the mean percentage of Fas Ag positivity and the MFI of bcl-2.

In agreement with the experience of Coustan-Smith *et al.*,<sup>25</sup> we show that bcl-2 expression in B-lineage ALL is markedly higher than that detected in CD10<sup>+</sup> B-lymphoid progenitors from BM of patients suffering from non neoplastic hematological diseases. As recently reported, high levels of bcl-2 are not associated with biological or clinical characteristics in ALL. Nonetheless, strong bcl-2 expression does not confer a poor prognosis. On the contrary, lower bcl-2 expression was associated with poorer outcome.<sup>25</sup>

In B-CLL, deregulation of bcl-2 expression might contribute to disease pathogenesis by preventing the induction of apoptosis.<sup>26</sup> The mech-

anisms responsible for high expression of bcl-2 protein in B-CLL cells are not clear, although this overexpression may result from hypomethylation of this gene in CLL cells.<sup>27</sup> As far as Fas Ag expression is concerned, leukemic cells from B-CLL patients have been reported to be only weakly positive, although *in vitro* activation by *Staphylococcus aureus* Cowan I (SAC), interleukin 2 (IL-2) or  $\alpha$ -interferon may increase its expression.<sup>28,29</sup> Our results show that fresh leukemic cells from 9 out of 19 (47.3%) B-cell CLL patients weakly expressed Fas Ag (more than 10% Fas positive cells). It should be pointed out that the patient whose leukemic cells expressed Fas Ag at a high level (64.5%) was receiving  $\alpha$ -interferon therapy, thus suggesting an *in vivo* upregulation of this molecule.

Although based on a small number of patient and control samples, our results reveal that bcl-2 and Fas Ag are coordinately expressed on normal PB leukocytes. The increased bcl-2 protein levels seen in B-CLL cells may represent a crucial part of their malignant phenotype as it has been shown for other molecules.<sup>30</sup> Fas Ag is expressed at low levels in B-cell neoplasms such as B-CLL with low proliferative capacity and long-lived cell survival.<sup>31</sup> The relatively lower bcl-2 expression detected in both M2 and M3 ANLL subtypes may explain, at least in part, the higher remission rate obtained in these forms of ANLLs than in other less differentiated morphological variants.<sup>32</sup>

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