



## COMPARATIVE FLOW CYTOMETRIC EVALUATION OF BCL-2 ONCOPROTEIN IN CD5<sup>+</sup> AND CD5<sup>-</sup> B-CELL LYMPHOID CHRONIC LEUKEMIAS

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### ABSTRACT

**Background and Objectives.** Levels of intracellular bcl-2 oncoprotein have been found to be increased in leukemic cells of CD5<sup>+</sup> B-chronic lymphocytic leukemia (CLL) patients. However, it is not clear whether bcl-2 overexpression is a peculiar feature of CD5<sup>+</sup> B-CLL. Based on this background we carried out a quantitative flow cytometric evaluation of intracellular bcl-2 levels on leukemic cells of CD5<sup>+</sup> and CD5<sup>-</sup> B-CLL.

**Methods.** We assessed in flow cytometry levels of bcl-2 protein using a quantitative indirect immunofluorescence assay (QIFI kit) on samples from 46 previously untreated CD5<sup>+</sup> B-CLL patients. Results were compared with those obtained on either normal peripheral blood B-lymphocytes or leukemic cells from 7 CD5<sup>-</sup> B-CLL patients intentionally selected for statistical comparison.

**Results.** A relatively homogeneous amount of bcl-2 protein which did not reflect either clinical-biological features at the time of diagnosis nor *in vivo*

response to therapy was found. Results expressed as antibody binding capacity (ABC) accounted for a mean value of  $12.2 \pm 1.5 \times 10^3$  molecules/cell (range,  $6.4-13 \times 10^3$  molecules/cell). Levels of bcl-2 detected on CD5<sup>+</sup> B-CLL leukemic cells were significantly lower than those of B peripheral blood lymphocytes from healthy donors ( $p = 0.0001$ ). The same applied when comparing CD5<sup>+</sup> and CD5<sup>-</sup> B-CLL patients (bcl-2 ABC,  $8.07 \pm 0.26 \times 10^3$  molecules/cell vs.  $12.2 \pm 1.5 \times 10^3$  molecules/cell;  $p = 0.0001$ ).

**Interpretation and Conclusions.** According to the role of bcl-2 in preventing apoptosis, our results indicate that differences in the pattern of expression of such an oncoprotein, might, at least in part, explain the more aggressive clinical course of CD5<sup>-</sup> B-CLL forms.

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Unlike follicular lymphomas with t(14;18) translocation, there is no rearrangement of bcl-2 in B-cell chronic lymphocytic leukemia (CLL); however, bcl-2 is overexpressed in B-CLL, and thus plays an important role in preventing programmed leukemic cell death.<sup>1-3</sup> Although the mechanisms accounting for bcl-2 overexpression in B-cell CLL are unclear, some preliminary data suggest hypomethylation as a possible cause.<sup>2</sup> Given the pivotal role of bcl-2 in preventing apoptosis, it can be speculated that the increased pattern of expression of this protein might provide an explanation for the accumulation of leukemic CD5<sup>+</sup> B-cells in G<sub>0</sub> phase, thus contributing to the pathogenesis of CLL.<sup>4,5</sup>

Studies dealing with the overexpression of the bcl-2 oncoprotein in CD5<sup>+</sup> B-CLL have previously been reported;<sup>2,3,6-8</sup> however, there is no information concerning the quantitative evaluation of bcl-2 levels in CD5<sup>+</sup> and CD5<sup>-</sup> B-cell leukemias.<sup>9-11</sup> In the present study we comparatively analyzed the levels of bcl-2 oncoprotein on leukemic cells from 46 CD5<sup>+</sup> and 7

CD5<sup>-</sup> previously untreated patients. For this purpose, a routinely quantitative immunofluorescence indirect assay (QIFI kit) was utilized. In conclusion, we attributed our results to either the clinical-biological parameters at the time of diagnosis or the *in vivo* chemotherapeutic sensitivity to a standard regimen consisting of intermittent chlorambucil and prednisone.

### Materials and Methods

#### Patient characteristics

Forty-six patients diagnosed at our Institution as having CLL form the basis of this study. The mean age of the patients was 64.4 years (SD 6.9), and the male to female ratio 31 to 15. B-CLL was diagnosed according to standard criteria that included peripheral blood lymphocytosis greater than  $5 \times 10^9/L$  and BM lymphocytosis greater than 30%.<sup>12</sup> All patients were staged according to the Binet clinical staging system<sup>13</sup> (stage A, 24; stage B, 9; stage C, 13) and the pattern of BM involvement was

evaluated in 39 patients (84.7%). Although four histological patterns were recognized (i.e., nodular, interstitial, mixed and diffuse),<sup>14</sup> for the purpose of the current study, interstitial, nodular and mixed patterns were examined together as a non-diffuse group. Therefore, 28 cases could be classified as having non-diffuse and 11 cases a diffuse histological pattern of BM involvement. According to previously described methods, lymphocyte doubling time (LDT) was assessed during the treatment-free period in 26 patients with an observation time of over one year.<sup>15</sup>

#### Cytomorphologic evaluation

Wright-Giemsa stained peripheral blood films were classified according to the French-American-British (FAB) criteria (16): CLL (>90% small lymphocytes, <10% prolymphocytes, <10% large lymphocytes); CLL/PLL (<90% small lymphocytes, >10% and <55% prolymphocytes, <10% large lymphocytes); CLL/mixed (<90% small lymphocytes, <10% prolymphocytes, >10% large lymphocytes); atypical/mixed CLL (<90% small lymphocytes, <10% prolymphocytes, >5% <30% cleaved lymphocytes). Based on this criteria, 43 out of 46 (93.4%) CD5<sup>+</sup> and 4 out of 7 (57.1%) CD5<sup>-</sup> patients were classified as having typical morphology.

#### Immunophenotype analysis

Fresh peripheral blood samples were used for immunological analyses. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation and cells were stained using both direct and indirect techniques. In the latter cases, a second layer reagent of fluorescence coupled with anti-mouse Ig (Ortho-Pharmaceutic) was used. The different immunomarkers used included CD3, CD5, CD22,  $\kappa$  and  $\lambda$  light chain immunoglobulins (Ortho, Raritan, NY, USA); CD19, CD20, CD23, CD11c (Becton Dickinson, San José, CA, USA); FMC7 (Immunotech, Marseille, France).

In all cases, the predominant leukemic population shared B-cell markers (i.e., CD19, CD20) and CD5 antigen. For the purpose of the present study, leukemic cells were enumerated via the expression of the CD19 antigen, and residual T-cells were identified through the expression of the surface CD3 molecule. The percentage of CD19<sup>+</sup> cells was generally greater than 70% (mean, 86.3±6.7%), and the percentage of residual T-cells accounted for a mean value of 11.8±5.3%. A marker was considered positive when it was expressed in over 30% of the cells analyzed. The stained cells were analyzed on an ABSOLUTE flow cytometer (Ortho Diagnostic System). In order to obtain a higher number of CD19<sup>+</sup> cells for bcl-2 analysis, an acquisition gate was set to the right light scatter and fluorescence intensity so as to collect only cells with CD19 fluorescence signals (Figure 1). Samples from healthy

donors or from CLL patients whose amount of residual T-cell accounted for more than 10% were analyzed in two-color immunofluorescence. A phycoerythrinated (red fluorescence) CD19-PE and a fluorescinated bcl-2 (bcl-2 FITC) monoclonal antibody were used for this purpose.

Lastly, we intentionally selected a disproportionate number of CD5<sup>-</sup> patients (7 cases) with the aim of evaluating the expression of bcl-2 in this relatively less frequent B-CLL variant. Detailed morphological and immunological information which led to a diagnosis of CD5<sup>-</sup> is reported in the *Results* section.

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

In order to detect intracellular bcl-2 in individual cells, the cells were permeabilized by means of a new fixative (OPF, Ortho Permeafix). This is a reliable method of intracellular antigen detection in flow cytometry which makes it possible to obtain a higher number of labelled cells and greater mean fluorescence intensity (MFI) than other methods of fixation. Furthermore, OPF does not affect the binding at saturating concentration of MoAb to the intracellular antigen. However, the presence of intracellular antigens in flow cytometry had already been confirmed by immunocytochemical analyses performed in cytopins by Pizzolo *et al.*<sup>17</sup> who compared both methods. The permeabilized cells were incubated with purified mouse anti-bcl-2 MoAb (124 Clone: IgG<sub>1</sub>,  $\kappa$  isotype, DAKO, Copenhagen, Denmark). 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

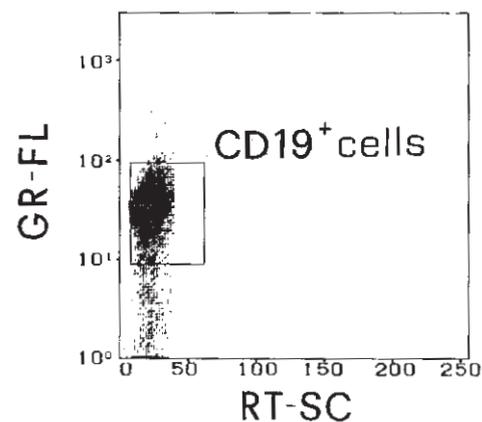


Figure 1. Dot-plot displaying CD19<sup>+</sup> expression versus right scatter to obtain an increased number of CD19<sup>+</sup> events.

dilution for 45 minutes at 4°C). Mouse IgG<sub>1</sub>-FITC was used as an isotype control. For the simultaneous detection of membrane and intracellular molecules before treatment with OPF, cells were incubated with CD19-PE for surface immunostaining.

The Quantitative Immuno-Fluorescence Indirect assay (QIFI kit, DAKO, Copenhagen, Denmark) utilizes an indirect IF analysis in which the fluorescence standards are labelled with the same fluoresced anti-mouse immunoglobulin reagent. It has been shown that these CD5<sup>+</sup> labelled standards can be used to evaluate the number of any IgG Mo Ab molecules/cell that must be tested. Then, 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 between the various groups. The *r* correlation coefficients between parameters were computed in least-squares regression equations.

## Results

#### Detection of bcl-2 in CD5<sup>+</sup> B-cell CLL patients

Of the 46 CD5<sup>+</sup> B-cell CLL cases analyzed, 37 (80.4%) expressed the CD23 antigen on leukemic cells while a positivity for the CD22 and FMC7-antigens was encountered less frequently (28.2% and 15.2%, respectively). When immunological results were translated into the scoring system proposed by Matutes *et al.*<sup>18</sup> it was clear that most patients (80.4%) scored 4-5 while a minority (19.5%) scored 2-3 (Table 1). In almost all patients there were cytomorphological features that suggested a diagnosis of typical CLL. The immunological profile of 3 patients with atypical morphology (2, atypical/mixed; 1, mixed CLL) was consistent with that of common CLL (i.e., CD23<sup>+</sup>, CD22<sup>-</sup>, FMC7<sup>-</sup>), which therefore ruled out the possibility that we were dealing with a CD5<sup>+</sup> lymphoproliferative disorder other than CLL.

A relatively homogenous level of bcl-2 protein was found (ABC, 12.2±1.5×10<sup>3</sup> molecules/cell; range, 6.4-13×10<sup>3</sup> molecules/cell), although did not reflect clinical-biological features at the time of diagnosis. Indeed, the amount of bcl-2 did not change as a function of clinical stages, BM histology and expression of B-lineage immunological markers (Table 1). The same applied when comparing bcl-2 pre-treatment levels of responding and resistant patients whose response to the therapy was evaluated after 4 to 6 courses of intermittent chlorambucil and prednisone.

In order to verify whether bcl-2 protein was over-

expressed on CD5<sup>+</sup> B-CLL cells, results were compared with those obtained in two-color fluorescence (CD19-PE/bcl 2-FITC) on peripheral blood cells of 5 healthy donors. From our experience, it seems that normal B-lymphocytes express significantly lower (*p* = 0.0001) intracellular bcl-2 levels (ABC, 8.07±0.26×10<sup>3</sup> molecules/cell) than CD5<sup>+</sup> B-CLL cells (ABC, 12.2±1.5×10<sup>3</sup> molecules/cell).

#### Detection of bcl-2 in 7 CD5<sup>-</sup> B-cell CLL patients

The clinical-hematological features of 7 CD5<sup>-</sup> B-cell CLL patients intentionally selected for statistical comparison are depicted in Table 2. The leukemic B-cell percentage of this subset of patients was not statistically different from that of patients with CD5<sup>+</sup> B-CLL (75.8±14.8% versus 70.1±14.8%).

The diagnosis of B-cell CLL depended on the morphology in 4 patients whose cytological features matched the criteria of typical CLL. The expression of CD23 prevented us from diagnosing a leukemic phase of lymphoplasmocytic or follicular lymphoma in two patients with morphological features similar to atypical/mixed CLL. The presence of morphological and immunological features belonging to both diseases (i.e., CLL and PLL) led to the diagnosis of prolymphocytoid transformation of CLL (CLL/PLL) in one case. B-cell markers that identify immunologically atypical CLL such as FMC7 and CD22 were expressed in the majority of

**Table 1. Intracellular bcl-2 levels of 46 CD5<sup>+</sup> B-CLL patients stratified on the basis of clinico-hematological features.**

Variable	N. pts	bcl-2 ABC (10 <sup>3</sup> molecules/ cell)	p value
Immunological score			
4-5	37 (80.4%)	12.3±1.5	0.11
2-3	9 (19.5%)	11.4±1.0	
Clinical stage			
A	24 (41.3%)	12.1±1.7	0.91
B	9 (19.5%)	12.1±1.4	
C	13 (28.2%)	12.3±1.0	
BM histology			
Non-diffuse	28 (71.7%)	12.1±1.6	0.85
Diffuse	11 (28.2%)	12.0±1.2	
LDT			
> 12 months	23 (88.4%)	12.0±2.2	0.77
≤ 12 months	3 (11.5%)	11.6±0.4	
Treatment response			
Responsive	8 (66.6%)	10.8±1.6	0.37
Resistant	4 (33.3%)	11.8±2.0	

ABC = Antibody Binding Capacity; BM = Bone Marrow;

LDT = Lymphocyte Doubling Time;

Immunological score according to Matutes *et al.*, *Leukemia* 1994; 8:640-5.

**Table 2. Clinical-biological characteristics of 7 CD5<sup>-</sup> B-cell CLL patients.**

Pts/cytology	Stage/BM	Percent cell positive					bcl-2 (ABC)
		CD20	CD22	CD23	FMC7	CD11c	
1. typical	A/I	55.8	72.6	80.1	65.1	38.7	9.5
2. CLL/PLL	C/ND	97.0	95.8	39.6	80.3	25.3	10.8
3. atypical/mixed	C/N	60.2	78.8	93.0	79.4	37.3	7.7
4. atypical/mixed	C/ND	71.3	93.6	41.8	92.2	87.8	8.8
5. typical	B/I	86.9	76.0	1.5	8.9	25.8	10.2
6. typical	B/I	74.5	76.8	0.1	11.1	0.3	8.9
7. typical	A/I	85.1	56.5	92.8	39.2	38.0	9.4

I: Interstitial; N: Nodular; ND: Not done; bcl-2 ABC (antibody binding capacity) in  $10^3$  molecules/cell.

patients (71.4% and 100%, respectively).

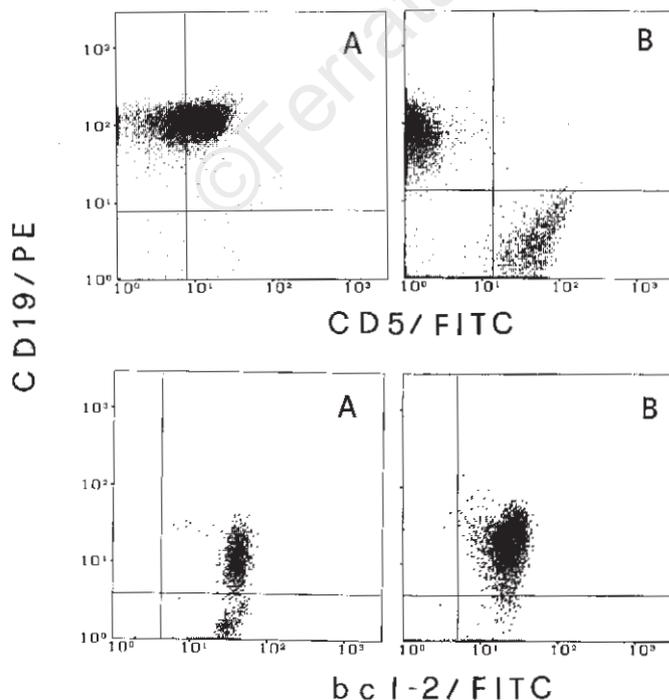
Interestingly, CD23-positivity was observed in 71.4% of cases, a figure not far from that of CD5<sup>+</sup> patients (80.4%). Lastly, when the amount of intracellular bcl-2 of this small subset of CD5<sup>-</sup> patients was compared with that of CD5<sup>+</sup> patients, significantly lower levels were revealed (ABC,  $9.3 \pm 0.9 \times 10^3$  molecules/cell versus  $12.2 \pm 1.5 \times 10^3$  molecules/cell;  $p = 0.0001$ ) (Figure 2).

### Discussion

B-cell CLL, a lymphoproliferative disorder characterized by the accumulation of long-lived monoclonal B-cells with low proliferative capacity, is the most frequent leukemia in Western countries. The relatively indolent clinical behavior of CLL is due to the fact that neoplastic cells are optimally organized to escape apoptosis.<sup>5</sup> Thus, overexpression of bcl-2 might contribute to pathogenesis of B-CLL by preventing apoptosis. In addition, B-CLL cells do not express Apo-1 Fas, a surface antigen involved in the transduction of the apoptotic signals.<sup>8,19,20</sup> The result of these features is an accumulation of B-CLL cells in G<sub>0</sub> phase.<sup>21</sup>

Although CD5<sup>+</sup> is by far the most common B-cell chronic leukemia, less than 10% are CD5<sup>-</sup>. In clinical practice, it is important to recognize this small subset of B-cell leukemias; in fact, the CD5<sup>-</sup> phenotype is associated with more advanced disease and shorter survival.<sup>9-11</sup> The biological bases of the diverse clinical results of CD5<sup>+</sup> and CD5<sup>-</sup> B-cell chronic leukemias are not clear; however, the higher amount of bcl-2 levels of CD5<sup>+</sup> B-cell leukemias might contribute to the expansion of the neoplastic cell population by prolonging cell survival; in contrast, the relatively low bcl-2 expression observed in CD5<sup>-</sup> B-cells might increase tumor mass by accelerating the rate of cell division.

The results of the present study support those recently reported by Gottardi *et al.*<sup>6</sup> who analyzed expression of the bcl-2 gene family in two subgroups of CD5<sup>+</sup> B-cell lymphoproliferative disorders



**Figure 2. Intracellular levels of bcl-2 protein on leukemic cells of a CD5<sup>+</sup> (A) and CD5<sup>-</sup> (B) B-CLL patient. The two-color immunofluorescence was carried out using FITC- and PE-conjugated antibodies.**

characterized by different clinical outcome such as B-CLL and mantle cell lymphoma (MCL). Although the impossibility of using a reproducible quantitative approach in the assessment of bcl-2 amount in the Gottardi *et al.*<sup>6</sup> study does not allow a comparison with our results, it is evident that bcl-2 overexpression is a unique feature belonging to CD5<sup>+</sup> cells; the more aggressive clinical course of MCL in comparison to B-CLL is due to bcl-1 overexpression, an additional genetic abnormality which characterizes MCL.

Increased levels of bcl-2 protein were detected by Pepper *et al.*<sup>7</sup> in a series of 22 B-CLL patients in whom the expression of *bax*, a homologue of bcl-2 which promotes cell death, was also analyzed. Quantitative cytometric evaluation expressed as molecules of equivalent soluble fluorochrome (MESF) made it possible to detect increased levels of bcl-2 in B-CLL cells in comparison to normal controls. Unfortunately, information concerning the pattern of CD5-expression is not clearly explained.<sup>7</sup>

Our study suggests that the amount of bcl-2 expression is not correlated with clinical stages, BM histology or LDT. Such a finding confirms results of Robertson *et al.*<sup>3</sup> who demonstrated a longer survival rate of patients displaying a lower bcl-2 expression. The relatively small number of patients analyzed and the events recorded make the results by Robertson *et al.*<sup>3</sup> difficult to interpret. On the other hand, it seems that a higher bcl-2 amount is associated with a better life-expectancy in both acute lymphoblastic leukemia (ALL) and acute non-lymphocytic leukemia (ANLL).<sup>22,23</sup>

The cell-killing effect of most chemotherapeutic agents relies on the induction of apoptosis.<sup>24</sup> Indeed, increased levels of bcl-2 and reduced levels of *bax* could be responsible, at least in part, for the resistance to therapy recorded in some CLL patients.<sup>7,25</sup> According to our results, it seems that the intracellular bcl-2 amount does not affect *in vivo* response to therapy, thus suggesting that drug-induced apoptosis is mediated *via* a p53-independent pathway.<sup>26</sup>

In conclusion, the new information that emerges from the present study concerns the different level of bcl-2 oncoprotein of CD5<sup>+</sup> and CD5<sup>-</sup> B-cell chronic lymphoid leukemias. Whether this different pattern of expression might provide the biological basis for the different clinical outcome of these lymphoproliferative disorders is not clear. Several genes including bcl-2, Apo-1 Fas, *c-myc* and p53 are involved in programmed cell death.<sup>27</sup> Abnormalities of these oncogenes might also explain the in Go block of B-CLL cells.<sup>28</sup>

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