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B Cell Response to Surface IgM Cross-Linking Identifies Different Prognostic Groups of B-Chronic Lymphocytic Leukemia Patients

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On the basis of responses to surface IgM (slgM) cross-linking, B cells from 41 patients with B-chronic lymphocytic leukemia were categorized as 15 nonresponders (group I) and 26 responders (group II). The latter cases were subclassified as those seven where proliferation was induced (subgroup IIa) and the remaining 19 in whom apoptosis occurred (subgroup IIb). Signal disruption in group I was confirmed by the absence of Ca²⁺ mobilization. Activation of PI3K was constitutive in subgroup IIa, but not in subgroup IIb, and that of Akt induced by anti-μ in subgroup IIa, but not in subgroup IIb. Among the MAPK, ERK was more highly activated relative to p38 in subgroup IIa, whereas activation of p38 predominated over that of ERK in subgroup IIb. For subgroup IIb cells, based on tyrosine phosphorylation and translocation into lipid rafts, slgM signaling was shown to be enhanced by Zap70. The different consequences of signaling through slgM were associated with biological prognosis indicators. These included high levels of CD38, lack of mutations in the IgVH chain genes, preferential usage of full-length CD79b, and severe clinical stage. Thus, modification of slgM-induced signaling could be a therapeutic approach. The Journal of Immunology, 2005, 174: 3749–3756.
proapoptotic, and the ERK family, which is antiapoptotic (34, 35). Once activated, phospholipase C γ generates diacylglycerol and inositol-1,4,5-triphosphate from membrane phosphatidylinositol-4,5-biphosphate. These messengers are required for activation of PKC and release of Ca^{2+}, respectively. MAPK are then activated by phosphorylated PKC and intracellular Ca^{2+}, which has just been mobilized. This is reminiscent of defective Ca^{2+} fluxes in some B-CLL (6, 36), based on differences either in the constitutive level (37) or in the phosphorylation status of PTK (34).

The present study showed that cross-linking sIgM did not induce significant changes in spontaneous apoptosis of B cells from 15 of 41 B-CLL patients. Signal transduction occurred in the remaining 26 patients, in whom apoptosis was delayed in 7 patients and accelerated in 19 patients. Reduced apoptosis was assigned to activation of Akt and ERK. In contrast, increased apoptosis was accounted for by activation of p38 and aberrant expression of Zap70, also shown to be functional in B-CLL cells, on the basis of tyrosine phosphorylation and translocation to the LR. These characteristics correlated with clinical stage and biological prognostic features, such as the expression of CD38 (38), the mutational status of IgV_{H} genes, and the CD79b isoform used.

**Materials and Methods**

**Patients and controls**

Forty-one untreated B-CLL patients were enrolled in the study, after they had given informed consent to the institutional review board at the Brest University Medical School. All fulfilled the phenotypic criteria for the diagnosis of B-CLL (39) and were classified as grade A (16 patients), grade B (18 patients), and grade C (7 patients) (40). These 26 males and 15 females ranged from 44 to 86 years, and their blood count varied from 9.6 to 189.2 × 10^{12} lymphocytes/ml. Blood was also taken from 10 healthy volunteers and tonsils from 15 children undergoing tonsillectomy. Daudi B cell line cells were purchased from the American Type Culture Collection.

**Cell preparation**

The Abs used were obtained from Beckman Coulter, unless specified. Ficol-Hypaque-separated mononuclear cells from B-CLL patients, healthy volunteers, and patients’ tonsils were treated with a mixture of unconjugated anti-CD3, anti-CD4, anti-CD8, and anti-CD56 mAbs, and the B cells were purified using goat anti-mouse IgG Ab-coated magnetic beads (Bio-Advance). This yielded cell populations containing >96% B cells, as shown by PE-anti-CD19 and FITC-anti-CD19 staining.

**Measurement of apoptosis**

For each patient, 6 wells of 96-well plates (Nunc) were left uncoated and matched with another 6 wells coated with 50 μg/ml sheep F(ab')_{2}-anti-rabbit F(ab')_{2}, IgG (Jackson ImmunoResearchLaboratories) overnight at 4°C. All cultures were set at 5 × 10^{9} B cells/well in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, and antibiotics. Anti-μ F(ab')_{2}, (Sigma-Aldrich) at 10 μg/ml final concentration was added to coated wells. After 24 h, the cells were stained with FITC-annexin V (AnV) and propidium iodide (PI) and analyzed by FACS. Cells in the early stages of apoptosis were positive for FITC-AnV and negative for PI. For each patient, each of the 6 coated wells (sIgM-triggered apoptosis) was compared with one of the 6 uncoated wells (spontaneous apoptosis). Significances were determined using the Wilcoxon’s rank-sum test for paired data. When p was >0.05, the sample was interpreted as resistant to sIgM cross-linking, and when p was <0.05, it was interpreted as sensitive. For experiments with inhibitors, cells were preincubated with 1 μg/ml wortmannin (Sigma-Aldrich) or 1 μM bisindolylmaleimide (Calbiochem) in RPMI 1640 medium at 37°C for 1 h before stimulation. Anti-μ-induced apoptosis was confirmed using three other assays that we have described previously in great detail (16, 17, 41, 42): examination of nuclear condensation, measurement of hypoploidy, and DNA fragmentation analysis.

**Proliferation assay**

Ki-67 staining was used as a measure of cell proliferation 24 h after ligation with anti-μ. Fixed cells were permeabilized with 1 ml of paraformaldehyde/lysine/periodate solution for 15 min at −10°C. After washes, they were incubated with FITC-anti-Ki-67 mAb (DakoCytomation) and analyzed using an EPICS Elite (Beckman Coulter) FACS. Our negative control was an irrelevant FITC-IgG1.

**Flow cytometry**

PE-anti-CD5, PE-anti-CD79b, or PE-anti-CD38 were combined with FITC-rabbit F(ab')_{2}, anti-μ (DakoCytomation), and 10,000 events measured/sample. The number of positive cells was compared with isotype controls and that of molecules per cell quantified by the amount of Ab binding to the cell at saturating concentrations, using the Quantum Simply Cellular kit (Flow Cytometry Standards). In selected experiments, the cells were permeabilized with 70% methanol for 1 h, washed, and incubated with unconjugated anti-Zap70 mAb or with control IgG_{2a}. Zap70-containing B cells were revealed with FITC-goat-anti-mouse. An irrelevant FITC-conjugated goat Ab was the negative control.

**Ca^{2+} flux measurements**

Cells at a concentration of 3 × 10^{6}/ml were incubated for 20 min at 37°C with 5 μM fluo-4 acetoxymethyl ester (AME; Molecular Probes), 0.02% pluronic acid, and 4 mM probenecid (Sigma-Aldrich). The cells were maintained at 37°C for 30 min to de-esterificate cellular AME. They were then excited at 488 nm and stimulated with 50 μM biotinylated-anti-μ cross-linked with unconjugated streptavidin (Sigma-Aldrich). The mean fluorescence intensity of fluo-4 AME at 525 nm was calculated. Treatment with 2 μg/ml ionomycin (Sigma-Aldrich) was the positive control.

**CD79b transcript analysis**

RT-PCR was necessary to discriminate between the TR and the full-length (FL) variant of CD79b (43). B cells from 21 randomly selected patients were evaluated. For the CD79b gene (21), the primers were 5′-TGTCAC CATGGCCAGGGTGTTTGGT-3′ (exon 1) and 5′-CCATCCTATGTTG GGGGAGGGATC-3′ (exon 6). For the GAPDH gene, they were 5′-CT TAGCACCCTCTGCGCAAGG-3′ and 5′-CTTACATCTGAGGCACTG-3′. DNA fragments of 978 bp for the FL form of CD79b, 602 bp for its TR form, and 542 bp for GAPDH were obtained.

PCR amplification consisted of a 4-min denaturing step at 94°C. Reactions were at 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s, with a final 10-min reaction to complete synthesis. We conducted 35 cycles for CD79b and 30 cycles for GAPDH. PCR band densities were assessed using Molecular Analyst Software (Bio-Rad), and the intensities of the signals of CD79b normalized to that of the GAPDH.

These results were confirmed using real-time PCR conducted in 10-μl mixtures containing 50 ng of template cDNA, 1 × SYBR Green PCR master mix (Applied Biosystems), and 500 nM of each primer. For both isoforms of CD79b, we used 5′-CCCCGAGCGTCATCTGGTTC-3′, which is a 3′-primer located in exon 6, combined with one of the two specific 5′-primers: 5′-CGGTACCGGATTTCCAAGGATCG-3′ encompassing exons 3 and 4 of the FL isoform or 5′-CACAGAGCTGC GAGTCCGGGATCC-3′ encompassing exons 2 and 4 of the TR isoform. Amplification conditions consisted of one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Comparison of cycle thresholds was completed as previously described (44) to evaluate the expression of TR-CD79b, relative to that of FL-CD79b.

**IgV_{H} gene analysis**

IgV_{H} genes were sequenced as described previously (45). A mixture of oligonucleotide 5′-primers specific for each leader sequence was used to amplify cDNA from the patients. The PCR products were purified with High Pure PCR Columns (Roche Diagnostic Systems) and analyzed with the Big Dye Terminator Sequencing Reaction kit (Applied Biosystems). Nucleotide sequences were aligned to Basic Local Alignment Search Tool and database of human Ig V region genes. The criteria in assigning membership of the D gene families were seven consecutive nucleotides of identity with no more than two differences. The length of the CDR3 was calculated between codon 95 and codon 102, and sequences exhibiting the Big D gene families were seven consecutive nucleotides of identity with no more than two differences. The length of the CDR3 was calculated between codon 95 and codon 102, and sequences exhibiting >2% deviation from a germline IgV_{H} sequence were regarded as mutated.

**Western blot analysis**

Two aliquots of 1.5 × 10^{7} B-CLL cells were left on ice for 30 min. The first was incubated with 15 μg/ml IgG (F(ab')_{2}-anti-IgM (Fab')_{2}) cross-linked with 50 μg/ml F(ab')_{2}-anti-IgG (Fab')_{2}, and the second was with Fab alone. Both were warmed to 37°C for 5 min. The cells were treated for 30 min at 4°C with 1% Triton X-100 in lysis buffer (20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA with 1 mM PMSF, 10 μg/ml aprotonin, and 1 mM sodium orthovanadate).
The protein concentration of cell lysates was determined by the microbicinchoninic acid protein assay (Pierce), and the proteins were resolved by SDS-PAGE and transferred to PVDF membrane sheets (Bio-Rad). The unbound sites were blocked overnight with 1% gelatin in PBS containing 0.05% Tween 20 and probed with phospho-specific anti-Akt S472/S473, anti-ERK1/ERK2 T202/Y204, anti-p38 T180/Y182 mAbs (BD Pharminogen), goat phospho-specific anti-Zap70 Y292, or rabbit phospho-specific anti-Zap70 Y319 Abs (Santa Cruz Biotechnology). These first-layer Abs were developed with HRP-goat anti-mouse IgG Ab, HRP-donkey anti-goat IgG Ab, or HRP-goat anti-rabbit IgG Ab (all from DakoCytomation) and visualized with an ECL detection system.

Two-dimensional electrophoresis

The protein samples were loaded onto nonlinear (pH 3–10) immobilized pH gradient strips (Bio-Rad) and focused for 30,000 V. These were incubated for 15 min with 5 ml of a first equilibration solution containing 0.375 M Tris-HCl (pH 8.8), 6 M urea, 2% SDS, 35% glycerol, and 70 mM DTT. Then they were allowed to activate through the second equilibration solution, which was identical with the first, except that 135 mM iodoacetamide was substituted for 70 mM DTT. The strips were loaded onto the top of 5–15% acrylamide-bis-acrylamide gradient gels, electrophoresed for 4 h at 30 mA/gel, and transferred to PVDF membranes, which were probed with rabbit anti-phospho-tyrosine Ab and HRP-goat anti-rabbit IgG. Following two-dimensional electrophoresis (2DE), some spots were cut out, and the proteins were sequenced by the Edman degradation method (Centre de Séquençage, Institut des Protéines).

Study of the LR

In some experiments, the cells were incubated with 10 nM of the cholesterol-sequestering drug methyl-β-cyclodextrin for 30 min at 37°C before they were cultured. The LR were isolated based on their insolubility in nonionic detergents and buoyant density on a sucrose gradient (45). Following a 5-min incubation at 37°C in medium with or without F(ab′)2, anti-μ Ab, two suspensions of 8 × 10⁷ leukemic B cells were washed with 25 mM ice-cold Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA (TNE). Both suspensions were then lysed for 30 min on ice in 1% Triton X-100 in TNE buffer with protease inhibitors. One milliliter of each supernatant was mixed with 1 ml of 85% sucrose in TNE and transferred to the bottom of a centrifuge tube. This was overlaid with 3 ml of 35% sucrose and 1.5 ml of 5% sucrose in TNE. The samples were centrifuged in a MLA-80 rotor at 180,000 × g for 17 h at 4°C in a Beckman Optima-Max ultracentrifuge (Beckman Coulter). The insoluble fraction at the interface of 35% and 5% sucrose representing the LR membrane fraction were collected. Fractions were resolved by SDS-PAGE and transferred to PVDF membranes, which were probed with rabbit anti-Zap70 Y319 Abs (Santa Cruz Biotechnology), or anti-Zap70 mAbs; the latter two were developed with HRP-goat anti-mouse IgG Ab.

B cells were incubated for 30 min on ice with Alexa Fluor 594-cholera toxin B (Molecular Probes) to target GM1 to the LR and with FITC-rabbit F(ab′)2, anti-μ Ab. One aliquot of cells was left at 4°C to serve as a control for nonactivation of the BCR, and a second aliquot was warmed to 37°C for 5 min. A third aliquot was kept at 0°C. All three aliquots were analyzed for double-stained cells with or without slgM engagement. They were then fixed in 3% paraformaldehyde and centrifuged at 300 × g onto slides. The cytospins were examined using a confocal microscope (Leica Microsystems).

Statistical analysis

Results were expressed as mean ± SEM, and comparisons were made using the χ² test (with Yates’ correction when required), the paired Wilcoxon’s rank-sum test, and the Mann-Whitney U test for unpaired data.

Results

slgM cross-linking on B-CLL cells leads to no effect, proliferation, or apoptosis

Because we suspected that heterogeneity of the patients might explain the conflicting reports of the B cell fate following slgM cross-linking, responses to anti-μ were measured in 41 patients. They varied from sample to sample but consistently fell into three patterns, referred to as group I and subgroups Ia and Ib (Fig. 1A). In group I (15 patients), anti-μ had no effect on apoptosis because the percentages of AnV-binding cells following slgM cross-linking were 18.4 ± 1.8% compared with spontaneous levels of 18.0 ± 1.8%.

In group II, there were two opposing changes. In subgroup Ila (7 patients), the percentages of AnV-binding cells diminished from 18.7 ± 1.8 to 4.3 ± 0.6% (p < 10⁻⁴), whereas they augmented from 18.0 ± 1.1 to 40.5 ± 1.5% (p < 10⁻⁴) in subgroup IIb (19 patients). Interestingly, the reduction in expression of slgM was more pronounced in group I than in subgroup Ila before (18.6 ± 3.1 vs 36.9 ± 3.1 × 10³ molecules/cell; p < 0.04) but not 24 h after slgM cross-linking (17.9 ± 3.6 vs 17.8 ± 4.4 × 10³ molecules/cell), and than in subgroup IIb before (18.6 ± 3.1 vs 29.4 ± 1.8 × 10³ molecules/cell; p < 0.005) and 24 h after slgM cross-linking (17.9 ± 3.6 vs 42.3 ± 3.5 × 10³ molecules/cell; p < 0.01).

At time 0, the baseline values of AnV binding were <5% in all samples. The variation of its binding to group I cells, following slgM cross-linking, was 5.8 ± 2.7% and not different from that of the spontaneous levels. This absence of effect differs from a 76.9 ± 2.4% decrease in subgroup Ila and a 166.5 ± 15.2% increase in subgroup IIb.

As shown in previous studies (16, 17), the AnV data were confirmed by nuclear condensation, hypoploidy of the lymphoid cells, and DNA fragmentation in 15 randomly selected patients, of whom 4 patients were categorized in group I, 2 patients in subgroup Ila, and 9 patients in subgroup IIb.

Ca²⁺ mobilization following slgM cross-linking

To confirm that the BCR pathway was disrupted in group I B-CLL, the induction of Ca²⁺ flux through slgM cross-linking (5, 36, 37) was measured in 12 of 15 group I, 7 of 7 subgroup Ila, and 16 of 19 group Iib patients (Fig. 1B). There was no Ca²⁺ response in the 12 group I patients, although ionomycin mobilized Ca²⁺ normally. In contrast, an increase in intracellular Ca²⁺ was seen in subgroups Ila and Iib. It was of interest that these subgroups exhibited differences in Ca²⁺ flux curves (Fig. 1B). In the seven subgroup Ila samples tested, Ag treatment was able to induce a sustained Ca²⁺ rise. In the 16 subgroup Iib samples tested, Ca²⁺ was mobilized rapidly but transiently as seen for tonsillar B cells (14 samples tested) and Daudi B cells.

Induction of proliferation following ligation of slgM

As shown by Ki-67 staining, the anti-μ-induced protection against apoptosis was accounted for by an increased proliferation of the cells in all of the seven subgroup Ila patients (Fig. 1C). There was indeed an increase in the percentage of Ki-67-expressing B cells from 9.9 ± 1.1 to 43.5 ± 4.6% (p < 10⁻⁴) on activation through slgM. In addition, DNA flow profiles for subgroup Ila (Fig. 1D) showed that cells from subgroup Ila patients are in S phase. This result correlates to the Ki-67 positivity by 24 h.

BCR signaling directs the fate of B-CLL cells: blocking of transduction factors

The reduction in the rate of apoptosis has been ascribed to several effector molecules. To dissect the mechanisms that result in inhibition of apoptosis, we used reagents to block specific molecules (Fig. 2). Blockade of PI3K with wortmannin in subgroup Ila and subgroup Iib cells prevented activation of Akt and thereby obstructed anti-μ-induced proliferation of subgroup Ila cells, whereas it did not have any apparent influence on antiapoptotic mechanisms of subgroups Ila and Iib cells. This finding is consistent with the results of 2DE where, following BCR engagement, tyrosine-phosphorylated proteins in subgroup Ila (Fig. 3B), but not in subgroup Iib cells (Fig. 3D), were PI3K and Akt. Some spots showed stereotypical localizations, whereas others were identified with certainty by their sequences.
The role of MAPK

Whereas activation of p38 encourages apoptosis, ERK has been shown to be another survival factor (34) regulated in two ways, of which one is dependent on PKC. Inhibition experiments showed that, not only the blockade of PKC with bisindolylmaleimide, which is a potent and selective inhibitor of PKC (46), prevents proliferation (Fig. 2), but this treatment also resulted in sIgM-induced apoptosis in subgroup IIa cells. In contrast, apoptosis was enhanced in subgroup IIb cells. This indicates that ERK is activated in a PKC-dependent manner and that PKC spontaneously exerts its antiapoptotic activity. Changes in different patterns of MAPK activation direct different B cell fates. Again, the results of the inhibition experiments were confirmed by the 2DE profiles. Whereas ERK was tyrosine phosphorylated in subgroup IIa, p38 was not (Fig. 3B). An opposite pattern was obtained in subgroup IIb (Fig. 3D) where p38, but not ERK, was tyrosine phosphorylated.

The differing roles of ERK and p38 were confirmed by specific mAbs (Fig. 4B). Tyrosine phosphorylation of ERK was constitutive and additionally increased by sIgM cross-linking in subgroup IIa. In contrast, it was hardly detectable at rest and not increased by sIgM cross-linking in subgroup IIb. On the contrary, p38 became tyrosine phosphorylated in subgroup IIb but not in subgroup IIa.

Phosphorylation of Zap70

The BCR threshold is higher for death than for survival (35). Thus, for apoptosis to occur, the signal must be sustained. Given aberrancies in the BCR, Zap70 could be involved in lifting the threshold in B-CLL. Consistent with this notion (Fig. 4A), there were...

FIGURE 1. Influence of sIgM cross-linking on spontaneous apoptosis and proliferation of B-CLL cells. B cells from 41 B-CLL patients were cultured for 24 h in the absence or in the presence of 10 μg/ml F(ab')2 mouse anti-μ cross-linked with sheep F(ab')2-anti-mouse IgG F(ab')2. A. Apoptosis was quantified by binding of FITC-conjugated AnV to PI-negative cells. Two groups of patients were identified: in group I, percentages of AnV-binding cells did not change (15 patients), whereas in group II, sIgM engagement resulted in changes (26 patients). This second group was subdivided into two subgroups: in 7 patients, percentages of AnV-binding cells decreased after sIgM cross-linking (subgroup IIa), whereas cells from 19 patients were sensitive to anti-μ-mediated apoptosis (subgroup IIb). B, Ca2+ mobilization was measured in the leukemic cells of seven patients from each subgroup. B-CLL B cells were loaded with fluorescent indicator fluo-4 AME and analyzed using the FACS, and cross-linked anti-μ was added 150 s after starting the analysis (arrows). Anti-μ Ab induced Ca2+ responses in subgroups IIa and IIb but did not mobilize Ca2+ in group I. That the cells were capable of showing a Ca2+ response was confirmed using ionomycin. Data are presented as the mean of the shift in the fluo-4 AME fluorescence over time. A representative example of group I and subgroups IIa and IIb is shown. C, sIgM engagement induced proliferation of group IIa B CLL cells. This response was measured as the percentage of Ki-67-positive cells by FACS. D, The DNA flow profiles show that B cells are hypoploid in subgroup IIb, but not in group I, and in S phase in subgroup IIa.

FIGURE 2. Effects of kinase inhibitors on anti-μ-induced apoptosis or proliferation in subgroup IIa and IIb B-CLL cells. Leukemia B cells were incubated with drugs for 1 h before stimulation with F(ab')2 anti-μ. Apoptosis was quantified by the binding of FITC-AnV to PI-negative cells. Proliferation was measured as the binding of the percentage of Ki-67-positive cells. BisI, Bisindolylmaleimide.
more Zap70+ B cells in 18 subgroup IIb compared with 7 subgroup IIa and 15 group I patients: 66.2 ± 6.8 vs 16.8 ± 8.6%, \( p < 10^{-3} \), and vs 9.2 ± 1.9%, \( p < 10^{-4} \).

To determine whether Zap70 was functional, lysates of B-CLL cells from seven subgroup IIb cases that contained Zap70 and six subgroup IIa cases that did not were probed with phospho-specific Abs. Representative phosphotyrosine patterns of each subgroup without stimulation (A and C) and after 5-min stimulation with cross-linked anti-\( \mu \) Ab (B and D) are shown. pI, Isoelectric point.

The role of LR in signaling mediated by sIgM cross-linking

Depletion of cholesterol from the LR with methyl-\( \beta \)-cyclodextrin (Fig. 5A) reduced the percentage of Ki-67-stained cells from 52.4 ± 137 to 18.4 ± 0.7% in six subgroup IIa samples (\( p < 0.05 \)) and that of AnV-binding cells from 38.2 ± 1.1 to 21.2 ± 1.3% in six subgroup IIb samples tested (\( p < 0.05 \)). This pilot experiment indicated that the LR were critical for anti-\( \mu \)-induced proliferation and apoptosis. Whereas BCR did not aggregate into LR (Fig. 5B, left panel), it was seen that sIgM was located in the LR following cross-linking: overlay of green-stained sIgM with red-stained LR was seen yellow in subgroup IIa (Fig. 5B, middle panel), as well as subgroup IIb cells (Fig. 5B, right panel).

To confirm that Zap70 was involved in BCR signal transduction, we showed that it moved into LR in response to sIgM engagement. LR were isolated in B cells from three subgroup IIa and three subgroup IIb patients, and their position in the sucrose gradient

FIGURE 3. 2DE separation and phosphotyrosine pattern of cellular proteins from subgroup IIa and IIb B-CLL patients. Protein lysates were analyzed with a 3- to 10-linear strip in the first dimension and a 5–15% PAGE in the second. The proteins were then blotted and probed with anti-phosphotyrosine Abs. Representative phosphotyrosine patterns of each subgroup without stimulation (A and C) and after 5-min stimulation with cross-linked anti-\( \mu \) Ab (B and D) are shown. pI, Isoelectric point.

FIGURE 4. Specific phosphorylation patterns and Zap70 expression triggered by sIgM cross-linking in B-CLL cells from subgroup IIa and IIb patients. A, Cells were permeabilized with 70% methanol for 1 h and incubated with anti-Zap70 mAb (solid histograms) or isotype control (dotted histogram) for 30 min at 4°C. The binding was detected with FITC-goat anti-mouse by FACS. B, Cells were treated with Alexa Fluor (AF) 594-conjugated cholera toxin B, unconjugated F(ab')2 anti-IgM, and FITC-F(ab')2 anti-F(ab')2. The localization of red (AF-594) and green (FITC) staining is shown as yellow. C, LR of B cells from 7 subgroup IIa and 15 subgroup IIb patients were isolated, blotted, and probed with anti-GM1, anti-IgM, anti-Syk, and anti-Zap70 mAbs. Representative examples are shown.

FIGURE 5. Involvement of the LR in signaling mediated through sIgM cross-linking of B cells from group I and subgroups IIa and IIb B-CLL patients. A, Subgroup IIa and IIb cells were treated with 19 mM methyl-\( \beta \)-cyclodextrin (M\( \beta \)CD) for 30 min at 37°C before they were cultured for 24 h with 10 \( \mu \)g/ml F(ab')2 anti-IgM cross-linked with F(ab')2 anti-F(ab')2. AnV and Ki-67 staining were measured. B, B cells were incubated with Alexa Fluor (AF) 594-conjugated cholera toxin B, unconjugated F(ab')2 anti-IgM, and Ki-67 staining was measured. C, LR of B cells from 7 subgroup IIa and 15 subgroup IIb patients were isolated, blotted, and probed with anti-GM1, anti-IgM, anti-Syk, and anti-Zap70 mAbs. Representative examples are shown.
was determined by the presence of GM1 (Fig. 5C). This did not reside in the LR or the non-LR membrane of resting cells but remained in the cytoplasm, as shown later, before BCR cross-linking. Whereas Syk moved into the LR following cross-linking of sIgM in cells from subgroups IIa and IIb, Zap70 was associated with the LR marker in subgroup IIb but not in subgroup IIa.

**Correlation of different patterns of responsiveness induced by sIgM cross-linking with biological prognosis features and clinical stage**

Studies on stimulation through sIgM suggest that the ability or not to signal may parallel prognosis features (13). Hence, we attempted to correlate the differential responses of B cells to anti-μ with four of these features (Table I). CD38 expression is associated with good responses to sIgM cross-linking (38). Therefore, it is not surprising that there were more subgroup IIb than subgroup IIa and group I patients in whom at least 30% of the B cells expressed CD38 (10 of 18 vs 0 of 7, p < 0.04, and vs 2 of 15, p < 0.02). There were also fewer TR-CD79b in subgroup IIa and subgroup IIb than in group I leukemia cells (TR/FL: 0.73 ± 0.04 and 0.77 ± 0.08 vs 1.14 ± 0.12, p < 0.02 and p < 0.02, respectively). These results were confirmed by real-time PCR, which showed more TR-CD79b, relative to FL-CD79b products (1.07 ± 1.55) in group I, and fewer TR-CD79b, relative to FL-CD79b products in subgroup IIa (0.10 ± 0.07) and subgroup IIb (0.03 ± 0.09). The expression of Zap70 was restricted to B cells from subgroup IIb patients. As previously reported (25–28), this expression paralleled sequencing data from IgVH genes because five of five subgroup IIa, compared with only one of seven group I samples tested, belonged to the unmutated B-CLL group. Finally, these biological characteristics correlated with clinical stages. Indeed, group I consisted of 11 grade A and 4 grade B patients, consisted of subgroup IIa of 2 grade A, 4 grade B, and 1 grade C patients, and subgroup IIb consisted of 3 grade A, 10 grade B, and 6 grade C patients (p < 0.05).

**Comparison of responses to sIgM or CD5 cross-linking**

Thirty-eight of the 41 patients tested in this study had been investigated previously for CD5-induced apoptosis (16, 17). Therefore, we questioned whether a similar pattern of response was observed with both stimuli. All of the 14 anti-μ group I samples, also tested for anti-CD5, failed to induce apoptosis in response to both anti-μ and anti-CD5 (Table II). The cells from seven subgroup IIa patients proliferated following the engagement of sIgM, although they were unaffected by that of CD5. Fifteen of the 17 subgroup IIb tested underwent apoptosis in response to CD5 or sIgM cross-linking. Thus, cells classified into the different groups in this study showed quite the same responses to two different ligands. This applied only to the apoptotic response because we failed to induce proliferation by cross-linking CD5.

**Discussion**

These studies were aimed at clarifying the role of sIgM in modulating spontaneous apoptosis in B-CLL cells and correlating differential responses to anti-μ with known prognostic features. The patient population was divided into two broad groups based on resistance (group I) or sensitivity (group II) to anti-μ. Group I comprised 40% of the patients, whereas the remaining 60% consisted of those where proliferation was triggered (subgroup IIa) and those where it was apoptosis (subgroup IIb). It is important to note that the levels of spontaneous apoptosis were similar in group I and subgroups IIa and IIb. That is, the cases with the highest levels of background AnV staining in control medium did not tend to have the highest levels of anti-μ-induced AnV staining. The disruption of signal transduction was confirmed by the absence of BCR-triggered mobilization of Ca²⁺ in group I samples. One intriguing question is how the same ligand-receptor interaction with sIgM favors proliferation in subgroup IIa, as opposed to apoptosis in subgroup IIb. This helps explain the conflict between reports of the responses to anti-μ of B cells from different B-CLL patients where it was claimed to prolong their survival (5, 6, 13) or to accelerate their apoptosis (8–10). It is likely that the former population corresponds to our subgroup IIa and the latter to our subgroup IIb. The functional dichotomy seen in group II patients is also consistent with the Ca²⁺ flux data in that the response was moderate in proliferation and vigorous in apoptosis. The plateau was due to a prolonged release of intracellular Ca²⁺, rather than a subsequent Ca²⁺ influx, because the cells were cultured in Ca²⁺-free medium. Although Ca²⁺ mobilization profiles similar to ours in B-CLL cells that did or did not proliferate to anti-μ treatment have been described previously (5, 36), these profiles have not been associated previously with proliferation or apoptosis.

As an approach to defining differences in responses to BCR engagement in our subgroups, we have focused on some molecules for which the presence of GM1 was positively correlated with a good response to sIgM cross-linking.
recognized to be key in signal transduction. Differences in the patterns of activated kinases offer an explanation for the described heterogeneity. In this study, investigation with a restricted number of selected kinases distinguished subgroups within the responding B-CLL group II. Constitutive activation of PI3K (6, 29) was confirmed by our finding that its inhibition enabled apoptosis to occur. However, aside from its role in B cell survival, this kinase is involved in their growth, as documented by wortmannin prevention of their anti-μ-induced proliferation.

Several molecules, such as Akt, normally prevent apoptosis of B cells. However, Akt, which is an immediate downstream target of PI3K (47), although not phosphorylated in resting B-CLL cells (31, 32), becomes serine phosphorylated following ligation of slgM and, at least in part, regulates BCR-mediated proliferation. In addition, PI3K can activate novel PKC (33), including PKC δ (32), but this was not addressed in the present work. ERK, which is also activated in the presence of bisindolylmaleimide, may contribute to preventing apoptosis in B-CLL (31). Indeed, its activation, together with inactivation of p38, seems to be critical for survival (5). Thus, the proliferative response of B cells to slgM cross-linking in subgroup IIa patients is likely to be due to the higher activity of ERK, relative to p38.

The opposite situation was seen in subgroup IIb B cells. Engagement of slgM was a prerequisite for the phosphorylation of p38, relative to ERK, encouraged apoptotic responses. Such disturbed regulation may result from a long-lasting activation, as reflected by the extension of Ca2+ fluxes. The outcome of signal transduction through the BCR is determined by its strength and duration. Zap70 might enhance the capacity of the BCR to signal, based on its association with elevated BCR signal transduction in B-CLL (26). We provide additional evidence that Zap70 protein, which undergoes tyrosine phosphorylation and translocates to the LR (48) following slgM ligation, is functional in subgroup IIb B cells.

At this moment, the major challenge is to define reliable markers identifying B-CLL patients at risk of an aggressive outcome. The integrity of the different signalizing pathways might help in predicting this outcome. In a separate study, a CD5-induced signal was shown to proceed through CD79B, such as slgM (17). This could explain, at least in part, the different consequences of incubating B-CLL cells with TGF (49) and anti-Fas-mAb (50). As a corollary, the cells from some B-CLL patients resisted apoptosis, irrespective of the stimulus. This intransparency correlated with their inability to mobilize Ca2+. Impaired signaling through the BCR has been associated with low densities of CD38 (8–13, 38), mutations in the IL-1R signaling pathway (8), and preferential usage of TR-CD79b (16, 17, 21, 43). Indeed, its activation, together with inactivation of p38, seems to be critical for survival (5). Thus, the proliferative response of B cells to slgM cross-linking in subgroup IIa patients is likely to be due to the higher activity of ERK, relative to p38.

Other B-CLL cases with more intact BCR-signalizing pathways are better able to convey signals for either apoptosis or proliferation. Subgroup IIb cells that apoptosis carry high densities of CD38, lack mutations in IgVH genes, express Zap70, and use FL-/CD79b preferentially. Interestingly, those patients with poor prognostic features are not present in subgroup IIa, although the signal transduction pathway is functional. Cells from this subgroup do not apoptose but proliferate in response to slgM ligation. Thus, the same ligand-receptor interaction can promote opposing outcomes of B cell activity, not only on costimulatory signals, such as CD40L and the excess of survivin (51), but also on the differentiation stage the cells have reached. Presumably, the cells from subgroup IIa patients that proliferate are more differentiated than those from the subgroup IIb that apoptose. This is consistent with the fact that the majority of cases of mutated B-CLL fail to signal via slgM, although their prognosis is better than that of unmutated B-CLL (Ref. 13; for review see Ref. 52), and with the recent finding (53) that Zap70, which is associated with worse prognosis, enhances slgM signaling in B-CLL. Therefore, it is not surprising that the prognosis was better in the signal-resisting than in the signal-sensitive group of patients. However, it would be important to evaluate the survival, viz, the response to therapy in the three groups of patients. This study has now been initiated.

In conclusion, unresponsiveness mediated by BCR cross-linking defines those patients with indolent B-CLL, whereas the propensity to apoptose (rather than to proliferate) characterizes those with severe disease. Such insights into the BCR transduction pathways may help develop therapeutic agents aimed at interfering with signaling.

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References


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