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ABSTRACT

Background
Chronic lymphocytic leukemia remains incurable, despite the addition of rituximab to chemotherapy as an available means of treatment. The resistance of certain patients to this monoclonal antibody prompted us to set up *in vitro* studies of another CD20-specific monoclonal antibody, B1 (later termed tositumomab). We hypothesized that the membrane lipid organization of leukemic B cells might be instrumental in the cells' sensitivity to the B1 monoclonal antibody.

Design and Methods
B lymphocytes from 36 patients with chronic lymphocytic leukemia and 13 patients with non-Hodgkin's lymphoma were investigated for B1-triggered cell death. Membrane components, such as sphingomyelin and ganglioside M1, were investigated by flow cytometry, immunofluorescence and co-immunoprecipitation, together with the Csk-binding protein.

Results
Chronic lymphocytic leukemia patients segregated into two groups: B cells from one group were sensitive to B1, whereas those from the second group were not. Further results ascribed the resistance of these latter cases to a defective recruitment of Csk-binding protein, resulting in a lack of sphingomyelin and ganglioside M1 at the outer leaflet of the plasma membrane of their malignant B cells. Sphingolipids were indeed retained in the cytoplasm, because of lowered activity of P-glycoprotein. Supporting this mechanism, rifampicin, an inducer of P-glycoprotein, improved the activity of this transmembrane efflux pump, normalized the quantity of sphingomyelin within the membrane, and thereby restored the efficacy of the B1 monoclonal antibody in the formerly B1-resistant cases of chronic lymphocytic leukemia.

Conclusions
The lipid organization of membranes of B cells from patients with chronic lymphocytic leukemia differs from one patient to another. In practice, given the relevance of the membrane lipid distribution to the efficacy of biotherapies, this observation is of potential importance.

Key words: chronic lymphocytis leukemia, CD20, rituximab, tositumomab, sphingomyelin, P-glycoprotein.

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The online version of this article has a Supplementary Appendix.

Introduction

Awareness that patients with chronic lymphocytic leukemia (CLL) remain at risk of a poor outcome following chemotherapy has sparked a great deal of interest in understanding the pathophsyiology of this unique malignancy. Contrary to the long-held belief that it results from the accumulation of B lymphocytes, the latest data[1] add credence to the view that the high growth rate of tumor cells has been overtaken by defective programmed cell death. Put simply, malignant B lymphocyte production is not offset by a commensurate order of cell eradication.

Improvement of immunotherapy-mediated cellular cytotoxicity would, therefore, be of value in the management of CLL patients. In this respect, a wealth of evidence has made CD20 an ideal target for B-cell-depleting antibodies.[2] Indeed, the first monoclonal antibody approved by the Food and Drug Administration[3] to treat patients with non-Hodgkin's lymphoma (NHL) was specific for CD20. This was termed rituximab, validated as a therapy for NHL, and subsequently tested in CLL with a low response rate.[4] Diverging response rates may reflect low membrane expression of CD20 in CLL, compared with NHL.[5] This lack of efficacy prompted us to investigate *in vitro* further CD20-specific monoclonal antibodies. Among them, B1 (later called tositumomab) appeared to act by lysing a range of rituximab-resistant target B cells, including human CD20-transgenic B lymphocytes in mice.[6]

Theoretically, the antitumor effects of CD20-specific monoclonal antibodies[7] combine antibody-dependent cellular cytotoxicity, complement-mediated lysis, and excessive programmed cell death. For these mechanisms of action to proceed, the CD20 molecules must be cross-linked, and hence translocated into liquid-ordered structures of the membrane.[8] Some of these structures orchestrate B-cell antigen receptor signaling. They have been denominated lipid rafts, which is a strictly operational definition based on insolubility in 1% Triton X-100 and buoyancy on density gradients.[9] These regions are not uniform, consisting of cholesterol and glycosphingolipids, such as ganglioside M1 and sphingomyelin.[10] This does not imply that sphingomyelin is confined to the lipid rafts. Interestingly, sphingomyelin can be hydrolyzed into ceramide by sphingomyelinases. In turn, ceramide is converted into sphingomyelin by sphingomyelin synthases 1 and 2. In practice, the lipid rafts may be detected in the plasma membrane using either cholera toxin B, which recognizes ganglioside M1, or with antibody directed against sphingomyelin-bound lysenin.[11] Aggregation of CD20 activates the phosphoprotein associated with glycosphingolipids which recruits Csk to the lipid rafts to keep the resident *Src*-family kinases inactive.[12] This adaptor has, accordingly, been termed Csk-binding protein (Cbp).

Recent reports have outlined the importance of membrane microdomains in the effect of rituximab, in that CLL cells from patients with high ganglioside M1 expression respond to this monoclonal antibody, whereas the remainder do not.[13] In this respect, following their landmark studies,[14] Cragg *et al.* distinguished the type-I "rituximab-like monoclonal antibodies" which translocate CD20 into lipid rafts and promote complement-mediated lysis, from the type-II "B1-like monoclonal antibodies" which do not translocate CD20 into conventional lipid rafts, but encourage programmed cell death.[6] One step further, according to the same group of investigators, type-II monoclonal antibodies evoke homotypic adhesion of B cells,[6,15] so that membrane exchanges brought about by cell-cell contacts through glycosphingolipid-containing microdomains cause a possibly non-apoptotic death.[16] Anyway, it has never been formally proven what molecular process *in vivo* might mimic the high-affinity cross-linking achieved with monoclonal antibody reagents *in vitro*.[2] Paradoxically, a few CD20-specific monoclonal antibodies are extremely poor at inducing apoptosis, although potent at partitioning CD20 into membrane rafts,[15] and CD20 modulation limits the efficacy of related monoclonal antibodies.[17] Ultimately, so great are disparities in mechanisms induced by CD20-specific monoclonal antibodies to kill B lymphocytes,[18] that their heterogeneity casts doubt on a classification of CD20-specific monoclonal antibodies based solely on their ability to translocate their target antigen into the lipid rafts. In fact, there is no solid evidence that all membrane microdomains fulfill the criteria artificially chosen for the definition of lipid rafts.[19] Such a statement is particularly worrisome because the results of the detergent-based methods used to purify lipid rafts may not reflect the overall membrane organization. Besides, lysis buffers other than Triton X-100 yield new microdomains similarly associated with CD20 molecules,[19] and the B1 monoclonal antibodies carry supplemental CD20 molecules into new insoluble fractions of the plasma membrane.[20] In light of what has recently been learned about the plasma membrane, we set out to dissect the relationships between the amount of glycosphingolipids and the degree of cell destruction caused by B1 monoclonal antibody.

Design and Methods

Patients, disease and normal controls

Thirty-six untreated patients fulfilling the criteria for the diagnosis of CLL were enrolled in the study, and scored clinically according to Matutes *et al.*[21] Disease stage was distributed as follows: stage A, 13 men plus 12 women (aged 50 to 91 years); stage B, two men plus two women (aged 35 to 77 years); and stage C, four men plus three women (aged 60 to 81 years) (*Online Supplementary Table S1*). An additional 13 NHL patients, eight with marginal zone B cell lymphoma, three with mantle cell lymphoma and two with follicular lymphoma, were included as disease controls for activity of the B1 monoclonal antibody in low-grade NHL.[6] Blood was also taken from three healthy volunteers from the staff, and tonsils from four children undergoing routine tonsillectomy. Informed consent was obtained from all these individuals or their parents, and the protocol was approved by the Institutional Review Board at Brest University under the reference 2008-214.

Reagents

Reagents are described in the *Online Supplementary Design and Methods*.

Cell preparation

The method employed yielded over 97.5±4.50% B lymphocytes in the preparations, as ascertained by phycoerythrin-conjugated anti-CD19, fluorescein isothiocyanate(FITC)-conjugated anti-CD3 and phycocyanin 5-conjugated anti-CD5 monoclonal antibodies using the Epics-XL fluorescence-activated cell sorter (FACS) from Beckman-Coulter. Details on cell preparation are provided in the *Online Supplementary Design and Methods*.

Lymphocyte culture and killing assays

For cultures, 3×10^5 B lymphocytes were seeded into 24-well plates, and incubated at 37°C in RPMI-1640 supplemented with 2 mM *L*-glutamine, antibiotics and 10% fetal calf serum (Gibco). In selected experiments, AB-human serum was used either untreated or heat-inactivated in order to inhibit complement activation. In addition, 10 μg/mL of B1 was either added or not to the cultures, with or without 20 μg/mL rifampicin, an inducer of P-gp.[22] After 24 or 48 h, the lymphocytes were recovered and washed. Cell death was quantified by FITC-annexin-V (AV) with propidium iodide (PI) using the Beckman-Coulter apoptosis kit. After a 15-min incubation, live cells were enumerated. Apoptotic cells were those positive for AV but negative for PI, and necrotic cells those positive for AV and PI. The decrease in the percentage of live cells was calculated according to the formula: (% AV-negative/PI-negative cells without B1) minus (% AV-negative/PI-negative cells with B1).

Fluorescence-activated cell sorter analyses

In these studies, 5×10^5 freshly-isolated B cells were stained by incubation for 30 min at 4°C with saturating concentrations of conjugated anti-CD5, anti-CD19, anti-CD20 monoclonal antibodies or FITC-conjugated cholera toxin B. There was also unconjugated anti-ceramide monoclonal antibody (Alexis) developed by FITC-conjugated goat anti-mouse IgM polyclonal antibody (Beckman-Coulter), and unconjugated rabbit antilysenin polyclonal antibody developed by FITC-conjugated donkey anti-rabbit IgG polyclonal antibody. To render their cytoplasm accessible, the lymphocytes were fixed in 4% paraformaldehyde, and permeabilized by a 20-min incubation at 4°C in phosphate-buffered saline (PBS)/bovine serum albumin (BSA) containing 0.5% saponin (Sigma). This cheap method gave similar results as treatment with a commercial kit. Intra-cytoplasmic lysenin was detected using the two-layer antibody system, as above.

Calculation of mean fluorescence intensities (MFI) of these markers required a minimum of 5,000 events and the results compared with isotype controls. The numbers of CD20 molecules per cell were indirectly quantified by determining the amount of monoclonal antibody binding to the cells at saturating concentrations, using the Quantum kit (Flow Cytometry Standards Corp). Briefly, 50 μL of microbeads were added to 20 μL of monoclonal antibodies, and antibody-binding capacities were derived from the MFI and expressed as arbitrary units.

Confocal microscopy

In all these experiment, 3×10^5 B cells were stained by incubation for 15 min on ice with FITC-conjugated B1 monoclonal antibody (Sigma). In the functional studies, this incubation was followed by a second 10-min incubation at 37°C. The consequential distribution of CD20 into the lipid rafts was studied by re-incubating the cells for 30 min at 4°C with biotinylated-cholera toxin B, or antilysenin polyclonal antibody. After three washes with PBS, the first lipid raft marker was revealed by rhodamine red X (RRX)-conjugated streptavidin, and the second by RRX-conjugated goat anti-rabbit polyclonal antibody. In some experiments, B cells were incubated with the FITC-conjugated B1 monoclonal antibody for 30 min on ice, and subsequently left at 37°C for another 7 min prior to their examination.

To access intracellular Cbp, the lymphocytes were permeabilized using the Cytofix/cytoperm Fixation/Permeabilization kit (Becton-Dickinson) which gave much better pictures than those with incubation in saponin-containing PBS/BSA. The cells were then washed in PBS, and stained with mouse anti-Cbp monoclonal antibody plus RRX-conjugated goat anti-mouse IgG polyclonal antibody. The cells were then fixed with 4% paraformaldehyde, placed onto slides (Vector), cytospun at 300 rpm for 1 min, and visualized using a Leica TCS laser scanning confocal microscope equipped with the TCS NT Leica software.

Western blotting

This technique is described in the *Online Supplementary Design and Methods*.

Quantitative reverse transcriptase-polymerase chain reactions

Gene expression levels of sphingomyelin synthases 1 and 2 were determined by quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR), using SYBR-Green PCR Master mix in an ABI Prism 7,000 sequence detection system according to the manufacturer's instructions (Applied Biosystems). Cycle threshold values corresponding to cycle numbers above baseline emission were set. The relative mRNA transcription rate was computed with the $2^{-\Delta\Delta ct}$ method, and normalized to 18S mRNA. Sequences of primers gene were 5'-GACCATCTTGCCAAACAAGTCTCT-3' plus 5'-TCCTGCCTCGGCTGTT-3' for sphingomyelin synthase 1, 5'-GCATGTGTCGTATAGCTCTGRCA TC-3' plus 5'TTCCCCCACCCTT-ATTT-GC-3' for sphingomyelin synthase 2, and 5'-GGCTACCACATCCAAGGAAGF-3' plus 5'CCAATTA-CAGGGCCTCGAAAG-3' for 18S.

The numbers of transcripts for CD20 were determined in ten randomly-selected samples of CLL. Briefly, the RNeasy Mini kit (Qiagen) served to extract mRNA. Quantitative RT-PCR was performed with the ABI PRISM 7,000 Sequence Detector using the 18S rRNA gene to normalize mRNA. Sequence of primers for the *cd20* were 5'CCAATTACAGGGCCTCGAAAG-3' plus 5'-CCAATTAC-AGGGCCTCGAAAG-3.

Calcium flux measurements

We incubated 10^6 lymphocytes/mL for 20 min at 37°C with 5 μM fluo-4 acetoxymethyl ester (AME), 0.02% pluronic acid, and 4 mM probenecid (Sigma). The cells were further maintained at 37°C for 30 min to de-esterify intra-cellular AME. The cell suspension was then excited at 488 nm and stimulated with 25 μg/mL B1, instead of 10 μg/mL B1, as in the other experiments, which did not induce reproducible calcium flux in pilot experiments. The MFI of AME at 525 nm was calculated. Cells treated with 2 μg/mL ionomycin (Sigma) were taken as a positive control for these experiments.

Co-immunoprecipitation experiments

B lymphocytes from two B1-sensitive and two B1-resistant CLL patients were each distributed into three 1×10^7-cell aliquots, and incubated with 2 μg of anti-CD20 B1 monoclonal antibody for 10 min. Importantly, the first aliquot was left at 4°C as a control for non-activation through CD20, the second was incubated at 37°C, and the third was treated with rifampicin for 30 min at 37°C, washed in PBS and incubated for another 10 min at 37°C with B1 similarly to the second cell aliquot. All the resulting pellets were washed again with PBS, and their proteins extracted by a 30-min incubation at 4°C in 1 mL lysis buffer (Miltenyi). The debris was discarded by centrifugation for 15 min at 10,000 rpm and at 4°C, while protein G-coated beads were added to the supernatants. After 30 min at 4°C, they were washed four times with the buffer provided by Miltenyi, and once more with 100 mL Tris HCl 20 μM. The beads were then mixed for 5 min with 20 μL of 95°C pretreated Tris HCl, and applied onto a column. This was washed with 60 mL of Tris HCl and its eluates resolved by electrophoresis on a 10% sodium dodecylsulfate polyacrylamide gel. After transfer of the proteins, the polyvindylidene fluoride membranes were saturated with 5% non-fat milk in 10 mM Tris 0.1% Tween-20

(TBST) for 1 h, and washed three times with PBS. The proteins were revealed with mouse anti-CD20 monoclonal antibodies, mouse anti-Cbp monoclonal antibodies, rabbit anti-*Lyn* polyclonal antibody (Santa-Cruz), rabbit anti-phospho-*Lyn* polyclonal antibody (Abcam), or mouse anti-*Syk* monoclonal antibodies (Santa-Cruz) for 1 h. The membranes were washed with TBST, all bound unconjugated antibodies were incubated with horseradish peroxidase-labeled donkey anti-mouse IgG antibody or horseradish peroxidase-labeled donkey anti-rabbit IgG polyclonal antibody, and binding revealed using enhanced chemiluminescence.

Determination of the P-glycoprotein transport activity

To determine the capability of P-gp to efflux glycosphingolipids, we utilized rho123, as described by Fontaine, Fontaine and Elmquist.[23] Four aliquots of 5×10^5 B lymphocytes from three patients with NHL, four patients with B1-sensitive and four with B1-resistant CLL were incubated with 10 μg/mL of rho123 for 90 min. All aliquots were then washed in PBS before FACS analysis. The first aliquot was studied immediately, the second after 15 min, the third after 30 min, and the fourth after 60 min. In one experiment, B lymphocytes were incubated again with rifampicin for 30 min at 37°C. Then, the amount of sphingomyelin and P-gp, as well as the P-gp transport efficiency were re-evaluated.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Comparisons were made using the Fisher's test, the Mann-Whitney's test for unpaired data and the Wilcoxon's test for paired data. The correlations were established using Spearman's test.

Results

The B1 monoclonal antibody does not kill malignant cells in all patients with chronic lymphocytic leukemia

We first wanted to ensure that B1 was a type II CD20-specific monoclonal antibody, by assessing its inability to induce complement-mediated lysis.[6,14] This was the case, as its killing activity was not impaired by removal of complement (*data not shown*). Further experiments confirmed that, without any crosslink, like rituximab-like type I CD20 monoclonal antibodies,[24] the B1 monoclonal antibody did not induce calcium flux, although ionomycin mobilized calcium normally in the B lymphocytes studied (Figure 1A).

Based on the behavior of the malignant B lymphocytes to B1-induced programmed cell death (with a cut-off value set at 10% according to spontaneous apoptosis), two groups of CLL patients were identified (Figure 1B). Like B cells from normal blood samples (24.8±5.1%), control tonsils (66.5±7.8%), and all patients with NHL, those from 19 of 36 CLL patients were killed by the B1 monoclonal antibody (this first group was termed B1-sensitive), whereas B cells from the remaining 17 CLL patients were not (the second group was termed B1-resistant).

The known relationship between rituximab activity and expression level of CD20[25] led us to verify whether B1-sensitive B cells were also those bearing high numbers of CD20 molecules. The results (*Online Supplementary Figure S1*) revealed similar antibody-binding capacities for CD20 in the two groups of CLL patients: 74.4±32.3 *versus* 69.3±26.4 $\times10^3$ molecules per cell. Furthermore, as demonstrated by quantitative RT-PCR, reductions in the number of mRNA copies for CD20 were not different in the ten randomly-selected CLL patients, then identified as belonging to one of the two CLL groups described above: 0.20±0.37 in three B1-sensitive *versus* 0.24±0.30 in seven B1-resistant CLL patients. Thus, the expression of CD20 does not influence the B1-induced death of B cells, in contrast to the dependence of rituximab-induced cell death on CD20 density.[25] Since responsiveness to rituximab correlates with CD20 density and with severity markers, dissociation between the responsiveness to B1 and CD20 density is consistent with the absence of associations (*Online Supplementary Table S1*) between, on the one hand, these two characteristics, and on the other hand, the age of the patients (73.8±9.8 years in B1-sensitive patients *versus* 66.2±12.4 years in the remainder), lymphocytosis ($71\pm50\times10^3$/μL in B1-sensitive patients *versus* $65\pm34\times10^3$/μL in the remainder), severity of the disease (13 stage A patients in B1-sensitive patients *versus* 12 stage A in the remainder), and percentage of CD38 expression (8.9±18.1% in B1-sensitive patients *versus* 19.3±25.5% in the remainder).

Relevance of the membrane structure to B1-induced cell death

Besides the division of the CLL patients into responders and non-responders according to their vulnerability to B1, the notion that the extent of B1-induced cell death depends on the capacity of this monoclonal antibody to translocate CD20 into lipid rafts[16,18] raises the possibility that differences between B1-sensitive and B1-resistant CLL patients reflect variations in membrane expression of ganglioside M1 on their B cells.

The results provided evidence for this working hypothesis (Figure 2A). The MFI of lipid raft-bound FITC-conjugated cholera toxin B were much higher in the B lymphocytes from the former group than in those from the latter: 3.1±1.1 *versus* 1.6±0.3 ($P<10^{-4}$). That is, B cells from the B1-sensitive CLL patients went on to have similar amounts of ganglioside M1 as those from marginal zone B-cell lymphoma patients in contrast to those from the B1-resistant CLL patients. Of note, cholera toxin B binding distinguished five cases with the highest levels of ganglioside M1 from 14 cases in the B1-sensitive CLL group (Figure 2A). At the moment, the reason for such dichotomy between patients is unclear, but deserves further experimental evaluation. These FACS analysis results were confirmed by western blotting (close to the two mantle zone B-cell lymphomas, compare the five B1-sensitive to the four B1-resistant CLL patients, *data not shown*), and further documented by confocal microscopy examination of total ganglioside M1. This is evident from comparing the middle to the bottom panel of the second column in Figure 2B for ganglioside M1, and the top to the bottom panel of the second column in Figure 2C for sphingomyelin.

Additionally, confocal microscopy showed that, given their normal membrane expression of ganglioside M1, B cells from patients with mantle zone B-cell lymphoma and B1-sensitive CLL were capable of partitioning the B1-bound CD20 molecules into ganglioside M1-enriched domains, possibly lipid rafts. At first glance, these data are at odds with previous reports, but it has to be kept in mind that lipid raft glycosphingolipids could not be distinguished from non-lipid raft glycosphingolipids in our experiments.[6,16] Figure 2B shows that green-stained CD20 spots and red-stained ganglioside M1 spots merged, so that their overlays turned yellow (third column).

Sphingomyelin could be relevant to B1-triggered pro-

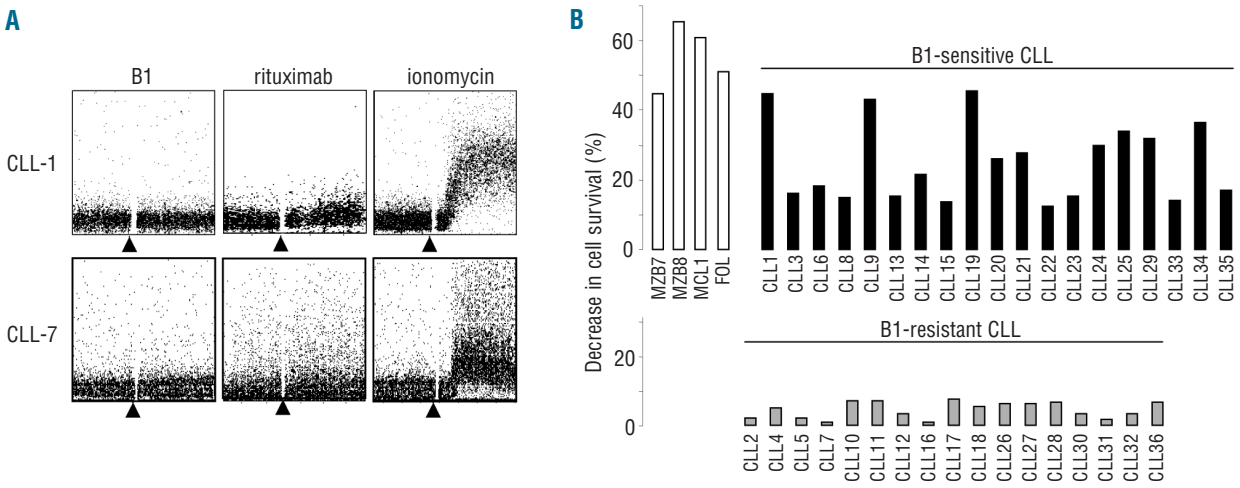

Figure 1. The B1 anti-CD20 monoclonal antibody kills B cells from certain patients with CLL, normal blood and tonsillar B cells, and all those with marginal zone B (MZB), mantle cell (MCL) or follicular lymphoma (FL). (A) Calcium flux was measured in B cells from two CLL patients. The cells were loaded with fluo-4 AME, and rituximab or B1 anti-CD20 monoclonal antibody 3 min later (arrow heads). Calcium mobilization by ionomycin was the positive control for this experiment. (B) Malignant cells from two MZB, one MCL, one FL, and 36 CLL patients were incubated with or without 10 μg/mL of B1. The decrease in the numbers of live cells was analyzed by FACS after FITC-annexin-V plus propidium iodide staining. Two groups of CLL patients were distinguished: 19 patients were B1-sensitive like those with NHL, whereas the remaining 17 patients with CLL were B1-resistant.

grammed cell death. Its involvement was supported using confocal microscopy as B1 and sphingomyelin co-localized in B1-sensitive, but not in B1-resistant CLL patients (Figure 2C). In contrast, the lack of ganglioside M1 in the B lymphocytes from B1-resistant CLL patients was associated with their dysfunction. As can be seen, B1 did not co-localize CD20 with ganglioside M1, since the green CD20 spots remained distinct from the red ganglioside M1 spots (third column).

On the basis that ganglioside M1 is synthesized from ceramide, a decrease in the amount of intracellular ceramide might lead to reduced synthesis of ganglioside M1 in B cells from B1-resistant CLL patients. Unexpectedly, there were no differences in ceramide synthesis between B1-resistant and B1-sensitive CLL patients (Figure 3A). FACS analyses were carried out to determine expression levels of sphingomyelin at the outer leaflet of the cell membrane (Figure 3B). The membranes of B cells from the B1-sensitive CLL patients displayed much more sphingomyelin than the membranes of those from B1-resistant CLL patients: 46.7±18.1 *versus* 16.1.±3.3 ($P<10^{-3}$). To exclude trivial reasons for the lower expression of sphingomyelin at the outer leaflet, e.g. its insufficient synthesis, B cells were permeabilized and total amounts of sphingomyelin inside the B cells were evaluated. The intracytoplasmic quantities of sphingomyelin seemed to be similar (Figure 3C) in B1-sensitive and B1-resistant CLL patients, including a few outliers (10.2±3.4 *versus* 12.3±8.1). Even more convincing, no differential expression of transcripts for sphingomyelin synthases 1 and 2 (Figure 3D) was seen by quantitative RT-PCR in the two groups of CLL patients.

Csk-binding protein expression is down-regulated in B cells from B1-resistant patients

Compared with B1-resistant CLL patients, the abundance of ganglioside M1 and sphingomyelin in B cells from B1-sensitive patients suggests that a specific lipid organization of the outer leaflet of the plasma membrane is mandatory for recruiting proteins to its inner leaflet. Of these organizations, some are implicated in the transduction of CD20-mediated signals for programmed cell death, most notably the transmembrane adaptor Cbp. The blots showed that Cpb was as highly expressed in five B1-sensitive CLL patients as in two patients with NHL, but far less in those from four B1-resistant CLL patients. It is interesting that Cbp co-localized with CD20 in the B lymphocytes from the B1-sensitive, but not in those from the B1-resistant CLL patients (compare the upper to the lower row in Figure 4A).

The consequence was that stimulation of CD20 with B1 encouraged CD20 to interact with Cbp in B1-sensitive, but not in B1-resistant CLL patients, as confirmed by the co-immunoprecipitation experiments (Figure 4B). Once precipitated, B1-bound CD20 carried along with it *Lyn* in the two groups of patients. Importantly, the B1-sensitive, but not the B1-resistant CLL patients, showed *Lyn* partially phosphorylated and associated with *Syk*.

P-glycoprotein is essential for the transportation of sphingolipid to the plasma membrane

Given the role of P-gp in the extrusion of lipids across the plasma membrane,[26] we reasoned that the failure to translocate sphingomyelin from the cytoplasm to the plasma membrane in B lymphocytes from some CLL patients may reflect ineffectiveness of the pump. Due to the retention of sphingomyelin within their cytoplasm, the membrane levels of P-gp were dramatically reduced in the six B1-resistant CLL, compared with the eight B1-sentitive CLL B cell samples (2.4±1.1 *versus* 6.9±2.3, $P<0.005$), and compared with the four patients with NHL (Figure 5A, with representative examples in Figure 5B).

This quantitative difference prompted us to assess whether P-gp was less efficient in B cells from B1-resistant CLL patients than in B cells from B1-sensitive CLL patients. To this end, we measured the shedding of rho123 15, 30

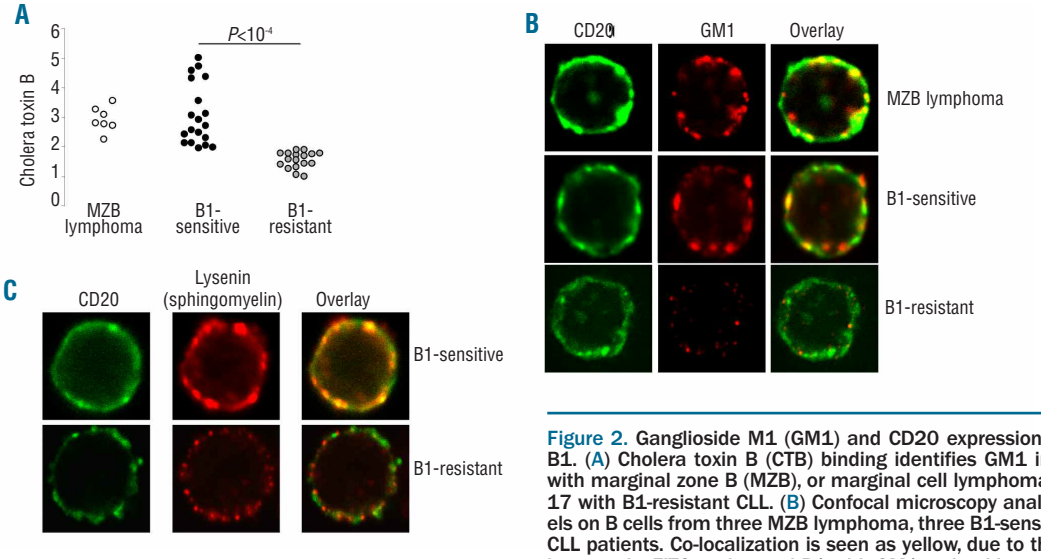

Figure 2. Ganglioside M1 (GM1) and CD20 expression on B cells incubated with B1. (A) Cholera toxin B (CTB) binding identifies GM1 in B cells from six patients with marginal zone B (MZB), or marginal cell lymphoma, 19 with B1-sensitive and 17 with B1-resistant CLL. (B) Confocal microscopy analysis of CD20 and GM1 levels on B cells from three MZB lymphoma, three B1-sensitive and three B1-resistant CLL patients. Co-localization is seen as yellow, due to the overlay of CD20 stained in green by FITC-conjugated B1 with GM1 stained in red by rhodamine-conjugated CTB. (C) Co-localization of green-stained CD20 with sphingomyelin stained in red with rhodamine-conjugated anti-lysenin antibody (magnification x100).

and 60 min after its engulfment. The amount of rho123 released was inversely proportional to that left inside the cells. Thus, the reduction in the intensity of rho123 accounted for the activity of P-gp. The quantity of rho123 (Figure 5C) still in the B cells from B1-sensitive CLL patients was diminished at 15, 30 and 60 min ($P<0.05$ for the three comparisons), but virtually not in those from B1-resistant CLL patients (non-significant at 15, 30 and 60 min). As documented by normalization of rho123 efflux in these B lymphocytes (Figure 5D), rifampicin activated P-gp at 15 and 30 min ($P<0.05$ for the two comparisons).

Remarkably, following P-gp induction with rifampicin, the membrane expression levels of sphingomyelin were augmented ($P<0.01$) to such an extent that there were no longer any differences in the quantity of sphingomyelin between untreated or rifampicin-treated cells from B1-sensitive CLL patients on the one hand, and rifampicin-treated cells from B1-resistant CLL patients on the other (Figure 6A). Accordingly (please, look back at the right lanes in Figure 4B), B1-stimulated CD20 co-immunoprecipitated Cbp, while *Lyn* was phosphorylated and accompanied by *Syk*. Thus, increasing amounts of sphingomyelin conferred CD20 the capacity to co-localize with sphingomyelin (*Online Supplementary Figure S2A*), as well as with Cbp (*Online Supplementary Figure S2B*). As a result (Figure 6B), B1-induced programmed cell death was restored in these B lymphocytes, while cells originally resistant to the monoclonal antibody turned out to be sensitive and thereby induced into apoptosis ($P<0.01$). These results suggest that the lack of sphingomyelin is sufficient to keep apoptosis at bay.

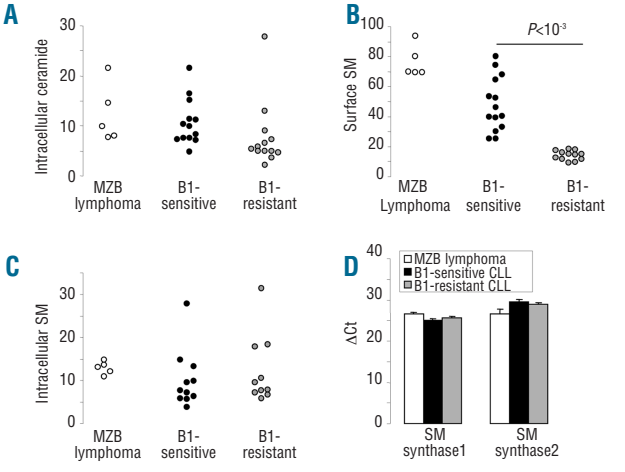

Figure 3. Expression of ceramide and sphingomyelin (SM) in B cells from patients with B1-resistant and B1-sensitive CLL. (A) Intracellular content of ceramide in permeabilized B cells from five patients with marginal zone B cell (MZB) lymphoma, 13 with B1-sensitive and 13 with B1-resistant CLL. (B) The MFI for SM-bound lysenin on the membrane were measured in B cells from five patients with MZB cell lymphoma, 14 with B1-sensitive and 12 with B1-resistant CLL. (C) SM, i.e. lysenin, was quantitated in permeabilized B cells from five patients with MZB, 11 with B1-sensitive and 10 with B1-resistant CLL. (D) Transcripts for SM synthases 1 and 2 were evidenced by quantitative RT-PCR in B cells from three patients with MZB, four with B1-sensitive, and three with B1-resistant CLL.

Discussion

The present study is the first to confirm and extend the fact that B cells diverge from one CLL patient to another with respect to their membrane lipid organization, as originally described by Meyer zum Büshenfelde *et al.*[13] This observation must be of practical importance, inasmuch as the efficiency of biotherapies depends on lipid distribution in the plasma membrane. Its relevance is further exemplified by a recent study showing that edelfosine triggers apoptosis of malignant B cells by clustering Fas within the lipid rafts,[27] and that the deficiency of sphingomyelin in lymphoma patients prevents stabilization of Fas into the lipid rafts, thereby hindering the efficacy of the drug.[28]

In the current work, CLL B cells characterized by high ganglioside M1 and sphingomyelin levels in their plasma membrane possessed active P-gp pumps, recruited Cbp, and ultimately were sensitive to B1-induced programmed cell death. This holds true for half of the CLL patients and for all those with NHL. In contrast, B cells from certain CLL patients exhibited a down-regulated membrane expression of ganglioside M1 and sphingomyelin. The local defect was assigned to intracytoplasmic accumulation of glycosphingolipids, rather than its insufficient synthesis. The cause was a lowered transport by the efflux pumps from the cytoplasm to the plasma membrane. Confirming our proposition, rifampicin-induced activation of P-gp increased sphingomyelin at the outer leaflet of the membrane and, by doing so, facilitated recruitment of Cbp. In other words, it is the distribution of sphingomyelin that governs the attraction of Cbp to the membrane.

Likewise, rituximab has been claimed to activate the sphingomyelin cycle in lymphoma B cells with intracellular ceramide production, mainly due to sphingomyelin hydrolysis by sphingomyelinase stimulation.[29] Interestingly, B1-mediated activation of lymphocytes does not influence the synthesis of ceramide, which suggests that variations in its killing efficacy are likely to be due to differences in the lipid organization at the outer leaflet of the plasma membrane. The implication of this influence is that several types of microdomains and/or lipid rafts must exist. As a matter of fact, they would display distinct properties, and react differently in the presence of 1% Triton X-100. Moreover, the interrelationships between these distinct structures may modulate response to CD20-specific monoclonal antibodies. Similar characteristics have been ascribed to Fas-induced apoptosis which can be regulated at the membrane level by lipid rafts in T lymphocytes.[30] Alternatively, the glycosphingolipids identified above might belong to the non-raft, rather than to the raft areas. Thus, in CD5-expressing CLL B lymphocytes differences have also been described in the pro-apoptotic activity of anti-CD5 monoclonal antibody,[31] and also attributed to the composition of membrane microdomains and their subsequent association with specific proteins.

We believe that other glycosphingolipids or precursors of ganglioside M1 are accumulating upstream. Some of these structures are present with high metastatic potential in cancer.[32] This raises the issue that certain B1-resistant CLL may consist of tumor cells that warrant further characterization. At the moment, our results show that the total amount of sphingomyelin is the same in the two groups of CLL patients. Consequently, should sphingomyelin lack at the external membrane, the missing sphingomyelin is sequestered within intracellular compartments. In agree-

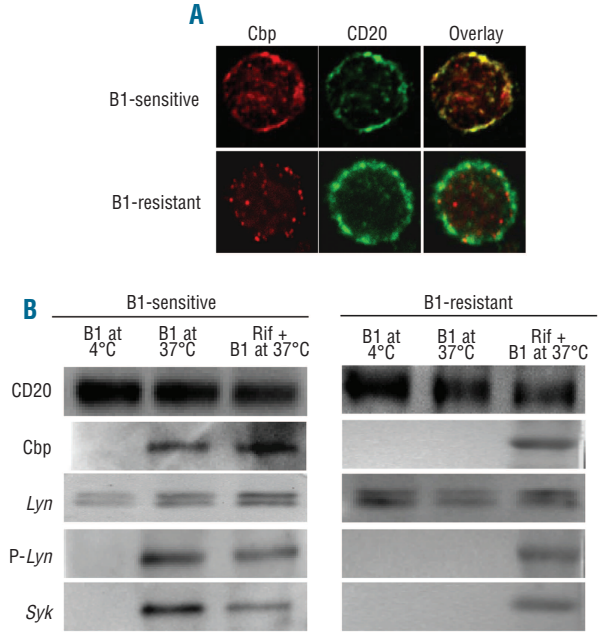

Figure 4. Csk-binding protein (Cbp) expression in CLL B cells. (A) Confocal microscopy of B1 colocalization with Cbp and CD20 in B cells from patients with B1-sensitive CLL, but not in those from patients with B1-resistant CLL (magnification x100). (B) CD20 was immunoprecipitated by B1 plus protein G-coated beads. The CD20 precipitates carried along Cbp and *Lyn*. Importantly, this kinase was phosphorylated and associated with *Syk* in B1-sensitive, but not in B1-resistant CLL B cells. Treatment of B cells from the second group of CLL patients with the P-gp induced by rifampicin (rif) normalized co-immunoprecipitations.

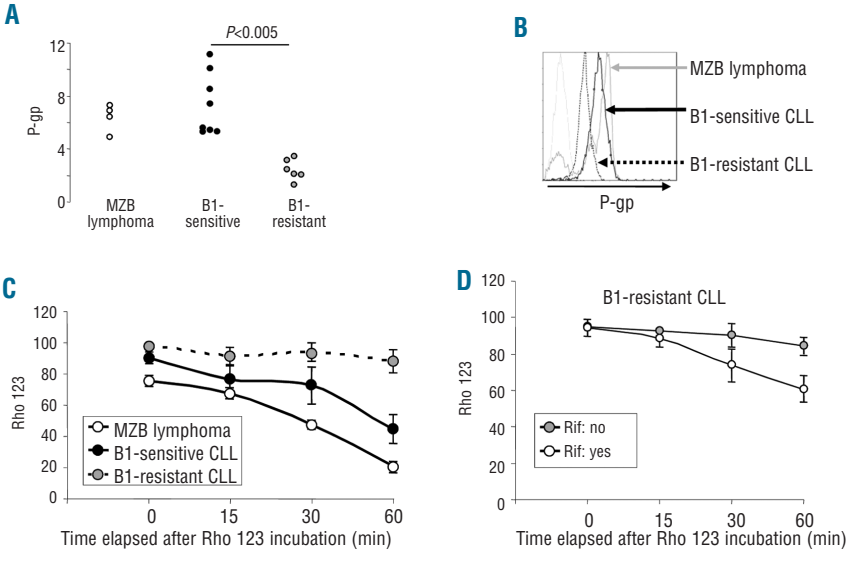

Figure 5. Membranes of B cells from patients with B-1 resistant CLL exhibit reduced level and efficacy of P-glycoprotein (P-gp). (A) MFI for surface P-gp was determined in B cells from four patients with marginal zone B cell (MZB) lymphoma, eight with B1-sensitive and six with B1-resistant CLL. (B) Examples of P-gp expression in B cells from patients with B1-sensitive and B1-resistant CLL. (C) Examination of the efflux of P-gp. Four aliquots of $5x10^5$ CLL B cells were incubated with rhodamine (rho) 123 for 90 min. The first aliquot was FACS analyzed immediately, and 15, 30 and 60 min later, and P-gp activity determined in four patients from each group of CLL patients by the reduction in intracytoplasmic rho123. (D) Rifampicin (rif) improved the P-gp activity in B cells from three cases of B1-resistant CLL patients.

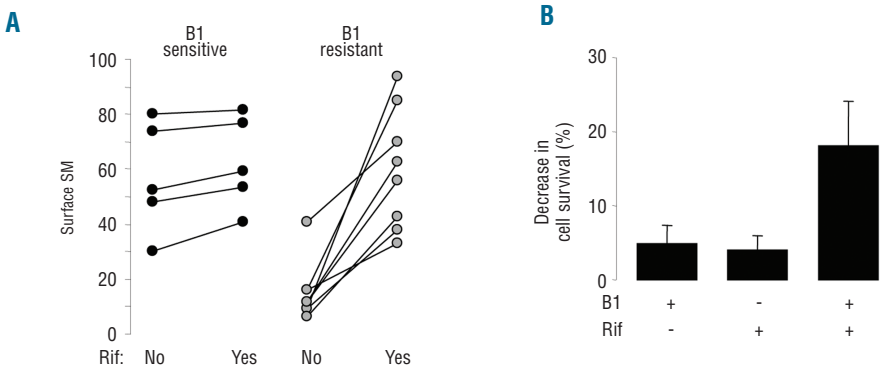

Figure 6. Rifampicin (rif) increases sphing (SM) at the membrane external leaflet and thereby restores sensitivity of B1-resistant CLL B cells to B1. (A) B cells from five B1-sensitive and eight B1-resistant CLL patients were stimulated for 30 min at 37°C with B1. (B) The pro-apoptotic effects of B1 were assessed by annexin-V plus propidium iodide staining in resistant lymphocytes before and after treatment with rif.

ment with this view, P-gp has the capacity to induce translocation of lipids, such as sphingomyelin and glucosylceramide, across the plasma membrane. One step further, differences in P-gp activity distinguish B cells of a given patient with CLL from another,[33] and the present study associates these cells with the susceptibility to B1-induced programmed cell death.

Simply, on the basis that B1 does not proceed through conventional lipid rafts, this monoclonal antibody is distinguished from rituximab, and grouped as a type II anti-CD20 monoclonal antibody. Yet, a major concern exists regarding the definition of lipid rafts, which relies historically on insolubility in 1% Triton X-100.[9,34] As clearly highlighted by Pike,[35] a number of other detergents have since been used to isolate membrane fractions, and the protein and lipid content of lipid rafts prepared by these various methods display significant differences, suggesting that they do not yield identical membrane fractions. Non-detergent methods have also been developed for isolating lipid rafts.[36] According to some investigators, detergent resistance is a highly subjective approach.[37] This claim is based on differences in the results of numerous studies strongly supporting the fact that lipid rafts are not uniform.[38] Such heterogeneity might thus account for disparities in the definition of lipid rafts, and differences in their requirements for anti-CD20 on antibody-induced apoptosis. In line with this cautious interpretation, our fluorescence-based experiments revealed that, in B cells from patients with B1-sensitive CLL and NHL, B1 co-localizes CD20 with sphingomyelin, ganglioside M1 and Cbp. Moreover, in B cells from B1-resistant CLL patients, in which sphingomyelin was reduced at the cell surface, rifampicin promoted P-gp, and improved co-localization. So, this antibiotic increases the level of sphingomyelin and thereby enables its association with CD20 and Cbp in B cells from B1-resistant CLL patients. Finally, programmed cell death reappeared after rifampicin treatment in these CLL B cells after incubation with B1. Very interestingly, to explain their seminal finding that B1 does not activate caspases, Ivanov *et al.* speculated that it may operate in a non-apoptotic way,[15,16] which is difficult to reconcile with the findings of others.[39] In fact, these differences may simply be accounted for by specificities of the various techniques used by these investigators.[40] Nonetheless, the present results confirm that the B1 monoclonal antibody produces direct cytotoxic effects in B cells from some CLL patients, regardless of apoptotic or non-apoptotic mechanisms.

To conclude, our findings indicate that membrane lipids are crucially implicated in the survival of CLL B cells following treatment with depleting monoclonal antibodies. Clearly, these data provide new insights into understanding CLL physiopathology, and may help to predict the efficiency of related therapies.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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