GM1 Expression of Non-Hodgkin’s Lymphoma Determines Susceptibility to Rituximab Treatment

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Abstract

Immunotherapy with rituximab alone or in conjunction with chemotherapy has significantly improved the treatment outcome of B-cell lymphoma patients. Nevertheless, a subpopulation of patients does not respond to rituximab. The reason for treatment failure as well as the exact mechanism of action is still uncertain. The function of rituximab has long been associated with the partitioning of CD20 molecules to lipid microdomains. We now show that the extent of CD20 recruitment to lipid rafts correlates with response to rituximab. In addition, expression of the raft-associated sphingolipid GM1 on lymphoma cells is associated with the susceptibility of lymphoma cells to rituximab. Furthermore, we show substantially different GM1 expression in various primary non-Hodgkin’s lymphomas. Whereas chronic lymphocytic leukemia (CLL) cells have a low GM1 expression, marginal zone lymphoma cells exhibit much higher levels. Differences were not only detected among various lymphoma subgroups but also within one lymphoma subtype. Interestingly, whereas CLL cells from patients with high GM1 expression responded to rituximab, patients with low GM1 expressing CLL cells did not. These data show the importance of membrane microdomains in the effect of rituximab and may offer a predictive factor for the responsiveness of lymphoma cells to rituximab.

Introduction

The monoclonal antibody (mAb) rituximab directed against the cell-surface molecule CD20 of mature B cells has been proven to be successful in the treatment of a variety of B-cell malignancies (1–4). However, resistance against rituximab occurs and there is no prognostic marker to predict individual response (5). Rituximab has been shown to induce cell killing via antibody-dependent cellular cytotoxicity (ADCC; ref. 6), complement-dependent cytotoxicity (CDC; refs. 7, 8), and the induction of apoptosis (9, 10). However, the mechanism that renders rituximab very effective in vivo remains elusive. Some reports suggest CDC to be the major pathway that is used by rituximab to deplete human lymphoma cells in vitro and in vivo (11). On the other hand, expression of the complement inhibitors CD46, CD55, and CD59 did not predict treatment outcome (12). Other reports have proposed that the activity of rituximab in vivo depends on its interaction with immune cells, in particular through ADCC and phagocytosis (6, 13, 14). The fact that FcγRIIIa polymorphisms have been shown to be predictive for tumor clearance in follicular lymphomas supports the importance of ADCC in the action of rituximab (15).

Several studies have addressed the question whether CD20 expression level may be used to predict progression of disease and response to treatment with rituximab. This would be very suggestive especially for chronic lymphocytic leukemia (CLL), characterized by a low CD20 expression level and a general weak response to rituximab (8). However, in vivo studies with anti-CD20 antibodies showed no correlation between the density of CD20 molecules on the cell surface and response to rituximab (16). Thus, no reliable prognostic factor for the susceptibility of non-Hodgkin’s lymphoma to rituximab exists thus far.

On the molecular level, membrane microdomains have been shown to be associated with the function of rituximab. Binding of rituximab causes a rapid redistribution of CD20 molecules to detergent-resistant membranes termed lipid rafts (7). These specialized microdomains of the plasma membrane are highly enriched in sphingolipids and cholesterol (17). Membrane-associated events are involved in several biological cell functions such as cell signaling, cell adhesion, and protein sorting. Gangliosides are sialic acid–containing glycosphingolipids that are constituents of mammalian cell membranes. Increasing evidence suggests that gangliosides are not passive structural components of cell membranes but rather act as modulators for important biological processes such as proliferation, adhesion, differentiation, and inflammation (18–22). Furthermore, it has been shown that gangliosides play an important role in the formation and stabilization of lipid rafts (23, 24).

The ganglioside GM1 is widely used as a marker for lipid rafts (25, 26). In the present study, we analyzed the expression of GM1 in various non-Hodgkin’s lymphomas. Moreover, we asked whether differences in the GM1 expression of lymphoma cells influence CD20 recruitment to lipid rafts and, thus, the susceptibility of non-Hodgkin’s lymphomas to rituximab. Analyzing the GM1 expression level of more than 200 patient samples revealed a significant difference in the expression between various lymphoma subtypes. The different GM1 expression directly correlated with the responsiveness of lymphoma cells to rituximab. Whereas CLL and mantle cell lymphoma (MCL) cells with high GM1 expression responded to rituximab ex vivo, low GM1 expressing lymphoma cells did not.

Materials and Methods

Reagents, antibodies, and cell lines. The oligonucleotide DSP30 (TCGTGCTGTCTCGCTTCTTCGGCTCTTCCTTCGCC) was used at 2 μmol/L, single stranded, phosphorothioate stabilized, and synthesized by TIB Molbiol. Rituximab was purchased by Roche and used in the concentration indicated; interleukin-2 (IL-2) was obtained from PeproTech.
Cell lines were obtained from American Type Culture Collection or Deutsche Sammlung von Mikroorganismen und Zellkulturen and were maintained in RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin/streptomycin, 2 mM/L l-glutamine, and 0.05 mM/L 2-mercaptoethanol (Invitrogen). Human mAbs anti-CD10-PECy5, anti-CD20-PECy7, anti-CD20-PE, anti-CD23-PE, anti-CD45-FITC, anti-CD5-FITC, anti-CD38-PECy5, anti-CD-PECy7, and anti-CD19-PECy7, and appropriate isotype controls were purchased from Beckmann-Coulter. Cholera toxin subunit B-FITC was purchased from Molecular Probes.

Flow cytometric analysis. Peripheral blood or bone marrow aspirates were anticoagulated with sodium heparin. Further processing was done within 24 h. For lysis, 300 μL of bone marrow or 300 μL of peripheral blood were lysed with 2 mL of Versa-Lysis (Coulter) in rotating tubes for 10 min. After washing, cells were stained for CD19, CD20, CD5, CD23, CD38, FMC7, CD10, κ, λ, and GM1 with three-5-color combinations and analyzed by flow cytometry with the use of a Coulter Cyanic FC 500. The GM1 expression level in granulocytes was used as a staining control because GM1 was comparably expressed in granulocytes from different patients (data not shown). To determine the GM1 expression level of lymphoma cells in the same tube, separate gates were set on CD19-positive lymphoma cells and CD45+ granulocytes. Depicted is the ratio of GM1 expression in lymphoma cells relative to granulocytes.

Cells from cell culture were washed in PBS containing 2% FCS and incubated with saturating amounts of fluorochrome-conjugated antibodies. After 30 min at 4°C, cells were washed with PBS/2% FCS and analyzed by flow cytometry with the use of a Coulter Cyanic FC 500 cytometer.

Culture conditions of cell lines. Lymphoma cells were cultured in RPMI 1640, supplemented with 10% FCS, 100 units/mL penicillin/streptomycin, 2 mM/L l-glutamine, and 0.05 mM/L 2-mercaptoethanol (all from Life Technologies, Inc./Invitrogen). All tissue culture experiments were done in a fully humidified atmosphere with 5% CO2 at 37°C. Culture conditions for primary lymphoma cells. Primary lymphoma cells were cultured in RPMI 1640, supplemented with 10% FCS, 100 units/mL penicillin/streptomycin, 2 mM/L l-glutamine, and 0.05 mM/L 2-mercaptoethanol (Life Technologies/Invitrogen). All tissue culture experiments were done in a fully humidified atmosphere with 5% CO2 at 37°C. Cells were plated at 100,000 in a total volume of 200 μL in 96-well dishes and were grown for 1 to 3 d.

Isolation of B-cell CLL and MCL samples by immunomagnetic separation. After informed consent was obtained, peripheral blood was collected from patients with confirmed B-cell CLL (B-CLL) and MCL according to morphologic and immunophenotypic criteria. Patients were either untreated or had not received cytoreductive chemotherapy for at least 3 mo before blood collection. At the time of analysis, all patients were free of infectious complications.

Enrichment of B-CLL and MCL cells by immunomagnetic sorting has been described (27). In brief, mononuclear cells were first enriched by density gradient centrifugation over a Ficoll-Hypaque layer, and then 2% FCS was overlaid. After centrifugation for 3.30 h at 200,000 × g, mononuclear cells were collected from the interphase. Lymphocytes from different patients (data not shown). To determine the GM1 expression level of lymphoma cells in the same tube, separate gates were set on CD19-positive lymphoma cells and CD45+ granulocytes. Depicted is the ratio of GM1 expression in lymphoma cells relative to granulocytes.

Inhibition of functional rafts using methyl-β-cyclodextrin. Cells were incubated for 30 min with 10 mM methyl-β-cyclodextrin (MβCD; Sigma). After addition of rituximab in the concentration indicated, cells were incubated for 30 min at 37°C. After three washing steps, cells were incubated for 1 to 3 d and analyzed for the number of viable cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) assay.

Inhibition of sphingolipid synthesis. To inhibit GlcCer synthesis, the lymphoma cell line Nceb was cultured in the presence of 30 μM/L τ-threo-1-phenyl-2-decanoylamino-3-morpholinol-1-propanol (PDMP; Calbiochem) for 5 d (28).

Rituximab-mediated cytotoxicity assays. For rituximab-mediated cell death (apoptosis and CDC), 1 × 105 cells were resuspended in 300 μL of human serum (non–heat inactivated) and 700 μL of culture medium with or without 100 μg/mL rituximab at 37°C for 24 h. Dead and viable cells were discriminated by Annexin V/propidium iodide (PI) staining.

Annexin V/PI staining. Cells (1 × 105) were washed and resuspended in binding buffer [10 mM/L HEPES (pH 7.4), 140 mM/L NaCl, and 2.5 mM/L CaCl2] containing 1 μg/mL FITC-Annexin V. PI (1.25 μg/mL) was also added to the samples to distinguish between early apoptosis and secondary necrosis. Subsequently, cells were assessed by flow cytometry.

MTS assay. B cells were cultured in RPMI 1640 (Life Technologies/Invitrogen) supplemented with 10% FCS (PAA) or 30% autologous serum, 50 IU/mL penicillin/streptomycin, 2 mM/L l-glutamine, and 0.05 mM/L 2-mercaptoethanol (Life Technologies/Invitrogen). All tissue culture experiments were done at 37°C and 5% CO2 in a fully humidified atmosphere in 96-well plates at 1 × 105 cells (cell lines) or 5 × 104 primary lymphoma cells in a total volume of 200 μL for 1 to 3 d. At the end of the culture period, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay was done: 40 μL of a combined MTS/phenoxythanol solution (Promega) were added, and the absorbance at 490 nm (A490) was determined using an ELISA reader. Mean values were calculated from triplicate cultures. Data from individual experiments are presented as mean ± SD. We confirmed our results with the trypsin blue exclusion method in two independent samples.

Purification of raft fractions. Purification of raft fractions has been described (29). Cells (3 × 107) were lysed on ice in 1 mL of 1% Triton in MNE buffer [25 mM/L MES (pH 6.5), 150 mM/L NaCl, 5 mM/L EDTA, 50 mM/L NaF, and 1 mM/L sodium orthovanadate, and 10 mg/mL each of aprotinin, leupeptin, and pepstatin and the protease inhibitor mix Complete Mini (Roche)], dounced 10 times, and mixed with 1 mL of 85% sucrose made with MNE buffer. After transfer of the lysate to the centrifuge tube, 2 mL of 35% sucrose in MNE buffer were overlaid, then 1 mL of 5% sucrose in MNE was overlaid. After centrifugation for 3.30 h at 200,000 × g in a Beckman SW50.1 rotor, 400-μL fractions were collected from the top of the gradient. Protein concentration of each fraction was determined with the Bio-Rad protein assay kit, which is based on the Bradford dye binding procedure. Fractions were pooled or analyzed individually by SDS-PAGE and Western blotting.

Western blotting. Western blot analysis was done with the indicated antibodies following SDS-PAGE and transfer onto nitrocellulose paper (Schleicher and Schuell). They were hybridized with antibodies to CD20 (Novocastra), lyn (Santa Cruz Biotechnology), cholera toxin subunit B-HRP (Calbiochem), caspase-3 (Cell Signaling), and caspase-8 (R&D). All immunoblots were developed with the ECL system (Pierce).

Results

Rituximab mediates cell death in Nceb cells but not in DB cells. Rituximab has been shown to induce cell death in lymphoma cells via CDC, ADCC, and induction of apoptosis. However, resistance to rituximab occurs in vitro and in vivo. We investigated four different mantle cell, three diffuse large cell, two CLL, and two Burkitt lymphoma cell lines for their response to rituximab (Table 1). Rituximab-mediated cell death (apoptosis and CDC) was determined in the presence of non–heat-inactivated serum from a healthy donor. Lymphoma cells were incubated in the absence or presence of rituximab for 24 h. Cell death was assessed by fluorescence-activated cell sorting (FACS) analysis using Annexin V/PI staining. The results of two exemplary cell lines, the MCL cell line Nceb and the diffuse large B-cell lymphoma cell line DB, are depicted in Fig. 1. Nceb cells, but not DB cells, died after rituximab treatment. The antilymphoma effect of rituximab in
For this purpose, we investigated the effect of M\textsubscript{ibi}CD20 in Nceb cells to lipid rafts would be necessary for cell killing. A decrease in viable cells of >50% was observed. This apoptotic effect was completely abolished by preincubation with M\textsubscript{ibi}CD. Interestingly, within 5 minutes of rituximab treatment, Nceb cells showed a robust recruitment of CD20 molecules to lipid rafts. We isolated lipid rafts from Nceb and DB cells by sucrose gradient centrifugation. The raft fractions (fractions 1–4) and nonraft fractions (fractions 8–11) were pooled and subjected to Western blot analysis. The tyrosine kinase Lyn, known to be constitutively expressed in response to rituximab treatment in the susceptible Nceb cell line, was constitutively expressed in rituximab-resistant DB cells (Fig. 2C). In contrast, no cytotoxic effect, even at 100 μg/mL rituximab, was observed in the DB cell line.

### Table 1. GM1 expression but not CD20 expression correlates with susceptibility of lymphoma cell lines to rituximab

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NOTE: Surface expression of CD20 and GM1 of various cell lines was measured by FACS as described in Fig. 3. Susceptibility to rituximab of each cell line was determined. Cells were treated with 100 μg/mL rituximab for 24 h. Cells (10\textsuperscript{5}) in triplicates were used in a standard MTS assay. The number of +s signifies CD20 and GM1 surface expression. Cells were judged to be insensitive (–; induction of apoptosis <5%), weakly sensitive (+; induction of apoptosis <10%), moderately sensitive (++; induction of apoptosis <20%), or very sensitive (+++; induction of apoptosis >20%) based on the results of FACS analysis.

Lipid rafts are crucial for rituximab-mediated cell death. Rituximab has been shown to recruit CD20 molecules into lipid rafts (7). Moreover, it has been discussed whether CD20 recruitment to lipid rafts is essential for rituximab-mediated cell death. Because the two cell lines, DB and Nceb, responded differently to rituximab treatment, we asked whether the difference in response could be due to differences in CD20 raft recruitment. We isolated lipid rafts from Nceb and DB cells by sucrose gradient centrifugation. The raft fractions (fractions 1–4) and nonraft fractions (fractions 8–11) were pooled and subjected to Western blot analysis. The tyrosine kinase Lyn, known to be constitutively expressed in lipid rafts, served as a control (30). Both unstimulated DB and unstimulated Nceb did not exhibit any raft-associated CD20. Interestingly, within 5 minutes of rituximab treatment, Nceb cells showed a robust recruitment of CD20 molecules to lipid rafts. In contrast, little CD20 recruitment was observed in the rituximab-resistant DB cell line (Fig. 2A). We next asked whether the recruitment of CD20 in Nceb cells to lipid rafts would be necessary for cell killing. For this purpose, we investigated the effect of M\textsubscript{ibi}CD on the survival of Nceb cells after rituximab treatment. M\textsubscript{ibi}CD inhibits the function of lipid rafts by cholesterol depletion (17). Nceb cells were incubated with 10 mmol/L M\textsubscript{ibi}CD for 30 minutes. Cells were then treated with rituximab or untreated. After 24 hours, the percentage of viable cells was determined by MTS assay (Fig. 2B). On rituximab treatment, a decrease in viable cells of >50% was observed. This apoptotic effect was completely abolished by preincubation with M\textsubscript{ibi}CD. Therefore, the recruitment of CD20 to lipid microdomains on rituximab treatment is an initial event that is crucial for rituximab-mediated cell death.

To confirm the effect of M\textsubscript{ibi}CD on the molecular level, we analyzed the rituximab-mediated CD20 recruitment to lipid rafts in the presence of M\textsubscript{ibi}CD. As expected, M\textsubscript{ibi}CD treatment inhibited the rituximab-mediated raft recruitment of CD20 (Fig. 2C).

Rituximab has been shown to induce cell killing by the induction of apoptosis (10, 11). Because CD20 was recruited to membrane microdomains after rituximab treatment in the susceptible Nceb cells, we next asked whether caspases, mediators of the apoptosis pathway, are also recruited to this membrane compartment. Rituximab-susceptible Nceb and rituximab-resistant DB cells were treated with rituximab and raft fractions were isolated. Interestingly, procaspase-8 and procaspase-3 recruitment to lipid rafts was only observed in the susceptible Nceb cell line but not in the rituximab-resistant DB cells (Fig. 2D), indicating the importance of this molecular event in rituximab-mediated cell death.

GM1 expression correlates with susceptibility of lymphoma cells to rituximab \textit{in vitro}. The expression of CD20 molecules on lymphoma cells is still discussed as being involved in the responsiveness to rituximab. To test the hypothesis that CD20 expression levels are responsible for differences in CD20 recruitment and, subsequently, altered responsiveness to rituximab, we measured the CD20 expression levels by FACS analysis. Compared with Nceb cells, DB cells showed a significantly higher CD20 expression (Fig. 3A), although they did not respond to rituximab treatment. Because differences in CD20 recruitment to lipid rafts correlated with response to rituximab, we next asked whether differences in the expression of the raft-associated ganglioside GM1 could be observed between the two cell lines. Nceb cells exhibited a substantially higher GM1 expression compared with DB cells (Fig. 3A). To confirm the hypothesis that GM1 expression correlates with sensitivity to rituximab, we investigated GM1 and CD20 expression in those cell lines, in which the response to rituximab was initially investigated (Table 1). No correlation between CD20 expression and sensitivity to rituximab was observed. In contrast, GM1 expression and response to rituximab correlated in all the B-cell lymphoma cell lines investigated. To further show the importance of GM1 expression in rituximab-mediated cell death,
Nceb cells were treated with the glycosphingolipid synthesis inhibitor PDMP. It has been shown that PDMP down-regulates sphingolipid expression (31). Treatment of Nceb cells with the sphingolipid inhibitor PDMP resulted in a marked reduction in GM1 expression as compared with untreated cells (Fig. 3B). Analyzing the effect of rituximab-mediated cell death in PDMP-treated Nceb cells, a significant decrease in susceptibility to rituximab-mediated cell death was observed (Fig. 3C). After rituximab treatment of non–PDMP-treated Nceb cells, <30% of the cells were viable. In contrast, PDMP treatment antagonized the function of rituximab-mediated cell death. Thus, alterations in GM1 expression directly influence the susceptibility of lymphoma cells to rituximab-mediated cell death.

**GM1 expression varies in primary lymphoma cells.** It is well known that the effect of rituximab differs in various non-Hodgkin’s lymphoma subtypes. For example, CLL cells exhibit a lower sensitivity to rituximab than marginal zone lymphomas. Because we observed a correlation between susceptibility to rituximab and GM1 expression in various lymphoma cell lines, we asked whether this could also be observed in primary lymphoma cells. To this end, we analyzed the GM1 expression of lymphoma cells from a total of 217 patients with indolent lymphomas. FACS analysis of GM1 in CD19+ lymphoma cells was done from blood and bone marrow. Figure 4A indicates the number of patients with different lymphomas analyzed. CLL samples accounted for >50% of all patient samples. GM1 levels of B-cell lymphomas from 34 marginal zone lymphomas, 20 follicular lymphomas, 11 immunocytomas, and 22 MCLs were analyzed by FACS staining. Figure 4B shows the GM1 expression of a typical patient with CLL, MCL, and marginal zone lymphoma in a histogram. The patient with CLL exhibited a much lower GM1 level than did the patient with MCL or marginal zone lymphoma. The average GM1 expression levels of all 217 patients analyzed are shown in Fig. 4A. Interestingly, there was a significant difference in GM1 expression between most non-Hodgkin’s lymphoma subgroups as determined by Student’s t test (GM1 expression CLL/follicular lymphoma, \( P = 0.014 \)). Of note, differences were also observed within the subgroup of patients with CLL, with some patients exhibiting high levels and some with low GM1 expression levels (data not shown; Fig. 5A). The analysis of polyclonal B cells revealed a GM1 expression that was distinct from the expression of monoclonal B cells in most of the samples analyzed. The average GM1 expression of polyclonal B cells from 15 healthy donors indicated expression levels that were higher than those of CLL, follicular lymphoma, and immunocytoma, but lower than those of MCL and marginal zone lymphoma (Fig. 4A).

**GM1 expression in CLL and MCL cells correlates with susceptibility to rituximab.** Because different GM1 expression levels were detected in the 122 CLL patients tested, we asked whether differences in response to rituximab could be observed in dependence on the GM1 status. Samples from 14 CLL patients were analyzed by flow cytometry for the expression of the CD20 antigen and the sphingolipid GM1. In Fig. 5A, the CD20 and GM1 expression levels of the 14 patients analyzed are documented. The induction of apoptosis by rituximab was investigated after 48 hours by Annexin V/PI staining and MTS assay (Fig. 5B; data not shown). The antileukemic effect of rituximab strongly correlated with GM1 expression because only those CLL cells with a high GM1 expression level responded to rituximab. In contrast, CLL cells with low GM1 expression showed only little, if any, susceptibility to rituximab (Fig. 5B). No correlation between response to rituximab and CD20 expression was observed (Fig. 5A and B).

Due to limited case numbers of leukemic MCL, we had access to samples from two patients. Blood samples from these two patients were immunoseparated to >95% purity based on the content of monoclonal CD19+ B cells. Again, cells were analyzed by flow cytometry for the expression of the CD20 antigen and the sphingolipid GM1. Both samples exhibited a comparable CD20 expression, whereas patient 2 displayed a much higher expression level for GM1 (Fig. 5C). MCL cells were cultured in the absence or presence of rituximab. After 48 hours, apoptotic cells were determined by FACS using Annexin V/PI staining and MTS assay. Although CD20 was expressed comparably, MCL cells derived from patient one did not respond to rituximab treatment, whereas ~50% of the cells from patient 2 died (Fig. 5D). These results suggest that GM1 expression, rather than CD20 expression, correlates with responsiveness to rituximab in further types of non-Hodgkin’s lymphoma.
Discussion

In the present study, we show that expression of the sphingolipid GM1 correlates with response to rituximab in various non-Hodgkin’s lymphomas. Furthermore, not only in vitro expression but also ex vivo GM1 expression differs between various lymphoma subtypes and, more importantly, even within one lymphoma subgroup. Analysis of apoptosis induction in CLL and MCL cells revealed that only lymphoma cells with high GM1 expression, but not low expressing lymphoma cells, responded to rituximab (Fig. 5B and D). On the molecular level, susceptibility of lymphoma cells to rituximab correlated with the recruitment of CD20 and procaspase-8 and procaspase-3 molecules to lipid rafts. Together, these data may offer a predictive factor for the treatment response of non-Hodgkin’s lymphomas to rituximab.

Although antibody-induced CD20 recruitment to lipid rafts is documented, the importance of this molecular event is still uncertain. Whereas complement-mediated lysis by anti-CD20 mAbs was shown to correlate with the segregation into lipid rafts, the same group reported that CD20-induced lymphoma cell death was independent of raft redistribution (7, 32). However, these observations were made using different CD20 antibodies. In contrast to the anti-CD20 mAb B1, rituximab treatment resulted in CD20 raft recruitment. The rituximab-induced raft recruitment of CD20 was accompanied by CDC-mediated eradication of lymphomas. In contrast, no CDC-mediated cell death was observed on treatment with the anti-CD20 mAb B1 (7). Thus, different CD20 antibodies have different effects on the molecular level in vitro and in vivo (33, 34). Therefore, studies using rituximab are necessary to explain the mechanism of lymphoma eradication in patients.
As shown by previous studies, rituximab treatment results in CD20 recruitment to lipid microdomains. With the two exemplary cell lines, which respond differently to rituximab, we show that the amount of CD20 recruitment to lipid rafts is crucial for the effect of rituximab. In the rituximab-resistant cell line DB, only a small amount of CD20 was recruited to lipid rafts, whereas in the rituximab-sensitive cell line Nceb, CD20 was recruited substantially to lipid rafts (Fig. 2A). Furthermore, procaspase-8 and procaspase-3 recruitment to lipid rafts was also observed only in rituximab-susceptible Nceb cells but not in the resistant DB cell line. The fact that molecules of the apoptosis pathway are selectively recruited to membrane microdomains following rituximab treatment offers new insights into the mechanism of rituximab-mediated cell death and highlights the importance of the induction of apoptosis. Recently, it has been shown that rituximab results in a rapid and transient increase in acid sphingomyelinase activity and concomitant cellular ceramide generation induced in rituximab-sensitive cell lines (35). The fact that most acid sphingomyelinase activity was contained in lipid rafts supports the importance of membrane microdomains in rituximab-mediated cell death. Furthermore, inhibition of caspase-8 using carboxobenzoxy-Val-Ala-Asp-fluoromethylketone inhibits ceramide formation and subsequent apoptosis (36). This underlines the necessity of CD20 and procaspase-8 recruitment to lipid rafts for rituximab-mediated cell death. Together, these data suggest that the recruitment of the death-inducing signaling complex as indicated by caspase-8 recruitment to lipid rafts is an early event that mediates rituximab-induced cell death. In case of raft recruitment of the death-inducing signaling complex (rituximab-susceptible cells), downstream signaling events such as caspase-3 activation, acid sphingomyelinase activity, and ceramide release are initiated.

The importance of CD20 raft recruitment was further documented by the use of the raft-inhibiting detergent MβCD. When Nceb cells were treated with rituximab in the presence of MβCD,

**Figure 3.** GM1 expression, but not CD20 expression, correlates with susceptibility of lymphoma cell lines to rituximab. A, surface expression of CD20 and GM1 on DB and Nceb cells was determined by FACS. Cells (1 x 10⁶) were stained with 1 μg of anti-CD20 mAb-PE (IgG1 subtype; solid black lines), isotype control (pure IgG1; gray lines), or 1 μg of cholera toxin subunit B-FITC, and then analyzed by FACS. B, Nceb cells were treated with PDMP (30 μmol/L) or left untreated. GM1 expression was measured by FACS as described in A. C, PDMP-treated and untreated Nceb cells were incubated with 100 μg/mL rituximab for 24 h or left untreated, as indicated, in the presence of 30% non–heat-inactivated human serum. Cells were analyzed by FACS analysis with a standard Annexin V/PI staining.

As shown by previous studies, rituximab treatment results in CD20 recruitment to lipid microdomains. With the two exemplary two cell lines, which respond differently to rituximab, we show that the amount of CD20 recruitment to lipid rafts is crucial for the effect of rituximab. In the rituximab-resistant cell line DB, only a small amount of CD20 was recruited to lipid rafts, whereas in the rituximab-sensitive cell line Nceb, CD20 was recruited substantially to lipid rafts (Fig. 2A). Furthermore, procaspase-8 and procaspase-3 recruitment to lipid rafts was also observed only in rituximab-susceptible Nceb cells but not in the resistant DB cell line. The fact that molecules of the apoptosis pathway are selectively recruited to membrane microdomains following rituximab treatment offers new insights into the mechanism of rituximab-mediated cell death and highlights the importance of the induction of apoptosis. Recently, it has been shown that rituximab results in a rapid and transient increase in acid sphingomyelinase activity and concomitant cellular ceramide generation induced in rituximab-sensitive cell lines (35). The fact that most acid sphingomyelinase activity was contained in lipid rafts supports the importance of membrane microdomains in rituximab-mediated cell death. Furthermore, inhibition of caspase-8 using carboxobenzoxy-Val-Ala-Asp-fluoromethylketone inhibits ceramide formation and subsequent apoptosis (36). This underlines the necessity of CD20 and procaspase-8 recruitment to lipid rafts for rituximab-mediated cell death. Together, these data suggest that the recruitment of the death-inducing signaling complex as indicated by caspase-8 recruitment to lipid rafts is an early event that mediates rituximab-induced cell death. In case of raft recruitment of the death-inducing signaling complex (rituximab-susceptible cells), downstream signaling events such as caspase-3 activation, acid sphingomyelinase activity, and ceramide release are initiated.

The importance of CD20 raft recruitment was further documented by the use of the raft-inhibiting detergent MβCD. When Nceb cells were treated with rituximab in the presence of MβCD,

**Figure 4.** GM1 expression varies in primary lymphoma cells. A, the average GM1 levels of all 217 patients and 15 healthy donors grouped by lymphoma subtype were measured by FACS. The number of patients for each subtype is indicated below. Y-axis, GM1 expression level of B lymphoma cells relative to granulocytes; bars, SE. B, lymphoma cells were stained anti-CD19-PE and cholera toxin subunit B-FITC. Cells were gated on CD19⁺ lymphoma cells. FACS analysis of a typical patient with CLL, MCL, and marginal zone lymphoma (MZL).
no raft recruitment was observed and neither CDC nor apoptosis was induced (Fig. 2B and C). This documents the necessity of CD20 raft recruitment for the function of rituximab, at least for CDC and induction of apoptosis. Monovalent Fab fragments of rituximab have also been shown to be sufficient to recruit CD20 molecules to lipid rafts (37). This emphasizes that cross-linking of two adjacent epitopes by rituximab is not necessary for CD20 raft recruitment. Moreover, these data suggest that Fab fragments stabilize a conformation of CD20 that exhibits a higher affinity for rafts. The higher-affinity association of CD20 to rafts might create a suitable environment for CD20 activation, providing close proximity to raft-localized kinases (38).

Using non–heat-inactivated serum from a healthy donor, we are able to detect CDC and apoptosis, but not ADCC. The major effect of CDC and apoptosis compared with ADCC for rituximab-mediated cell death has been shown by Di Gaetano and colleagues. In their study, rituximab prevented death in all mice treated with rituximab after i.v. inoculation of the murine T lymphoma cell line EL4 retrovirally transfected with the human CD20 cDNA. The depletion of natural killer cells, mononuclear cells, and T cells did not affect the therapeutic effect of rituximab (11). Therefore, at least in this system, ADCC was dispensable for the function of rituximab. In contrast, Clynes and colleagues suggest that ADCC is the central effector mechanism of rituximab. In a s.c. xenograft model of lymphoma, the FcγR chain necessary for signaling from the activating FcγRI and FcγRIII receptors, present on the surface of macrophages, neutrophils, and natural killer cells, has been shown to be required for the full therapeutic activity of rituximab (6). One explanation for the differences observed in the two models could be the use of different lymphoma cells. It is also possible that the site of the tumor determines the mechanism by which rituximab can kill tumor cells. It might be possible that effector cells invade solid lymphoid tumors and kill lymphoma cells. Leukemic or hypervasculated tumors might be more susceptible to CDC. Both mechanisms, the intrinsic properties of different B cells and their localization within a protective microenvironment, have been shown to play a role in the susceptibility of non-Hodgkin’s lymphomas in vivo (39).

Our data provide evidence for a predictive factor for the response of lymphoma cells to rituximab. Starting with the observation that CD20 raft recruitment is a key event following rituximab treatment, we correlated the amount of raft-translocated CD20 with response. Whereas recruitment of CD20 to lipid rafts was in line with treatment response, cell-surface expression levels of CD20 showed no correlation. Interestingly, the expression levels of the ganglioside GM1, widely used as a marker for lipid rafts, correlated with response to rituximab (Table 1). The importance of GM1 expression was further underlined by inhibiting the sphingolipid synthesis in Nceb cells. This resulted in a reduced GM1 expression and, subsequently, in a marked reduction of rituximab-mediated cell death (Fig. 2A). Therefore, the observed decrease in rituximab susceptibility is due to a direct reduction in GM1 expression. Gangliosides are not passive structural components of cell membranes. It has been shown that they modulate important biological processes such as proliferation, adhesion, differentiation, and inflammation (18–22). Furthermore, gangliosides play an important role in the formation and stabilization of lipid membrane microdomains. The reduced rituximab-induced CD20 recruitment in the low-expressing DB cell line suggests a role for the ganglioside GM1 in raft formation (Fig. 2A).


GM1 Expression of Non-Hodgkin's Lymphoma Determines Susceptibility to Rituximab Treatment


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