Development of Rituximab-Resistant Lymphoma Clones with Altered Cell Signaling and Cross-Resistance to Chemotherapy

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Abstract

Immunotherapy with rituximab (chimeric anti-CD20 monoclonal antibody, Rituxan), alone or in conjunction with chemotherapy, has significantly improved the treatment outcome of lymphoma patients. Via an elusive mechanism, a subpopulation of patients becomes unresponsive and/or relapses. To recapitulate various aspects of acquired resistance, rituximab-resistant (RR) clones were established from lymphoma lines and compared with parental cells. Surface CD20 expression was diminished in the clones. The clones neither responded to rituximab-mediated growth reduction or complement-dependent cytotoxicity nor underwent apoptosis in response to cross-linked rituximab. Rituximab failed to chemosensitize the RR clones, which exhibited constitutive hyperactivation of the nuclear factor-κB and extracellular signal-regulated kinase 1/2 pathways, leading to overexpression of B-cell lymphoma protein 2 (Bcl-2), Bcl-2–related gene (long alternatively spliced variant of Bcl-x gene), and myeloid cell differentiation 1 and higher drug resistance. Unlike parental cells, rituximab neither inhibited the activity of these pathways nor diminished the expression of resistant factors. Pharmacologic inhibitors of the survival pathways or Bcl-2 family members reduced the activity of these pathways, diminished antiapoptotic protein expression, and chemosensitized the RR clones. These novel in vitro results denote that continuous long-term rituximab exposure culminates in RR clones that do not respond to rituximab-mediated effects, have altered cellular signaling dynamics, and exhibit different genetic and phenotypic properties compared with parental cells. The data also reveal that although RR clones exhibit higher resistance to rituximab and cytotoxic drugs, these clones can be chemosensitized following treatment with pharmacologic inhibitors (e.g., dehydroxymethylepoxyquinomicin, bortezomib, PD098059) that target survival/antiapoptotic pathways. The findings also identify intracellular targets for potential molecular therapeutic intervention to increase treatment efficacy. The significance and potential clinical relevance of the findings are discussed. [Cancer Res 2007; 67(3):1270–81]

Introduction

The lymphatic cancers known as non–Hodgkin’s lymphoma (NHL) are steadily increasing in prevalence worldwide. Although NHLs initially respond to a variety of therapeutic modalities, they exhibit an unremitting relapsing nature and are essentially considered incurable. This pattern of inevitable failure of standard therapies is due to the emergence of drug-resistant variants that highlight the urgent need for the design of new treatment regimens. Monoclonal antibodies (mAb) targeted against specific surface markers that are less systematically toxic and less myelosuppressive have provided an alternative therapeutic approach. About 80% to 85% of NHLs are of B-cell origin and ~95% of these cells express surface CD20 (1, 2).

The B-cell–specific surface marker CD20 (3) does not circulate in the plasma as a free protein, which could potentially block antibody binding to the cells (4). It is also neither internalized upon antibody ligation (5) nor is shed from the cell surface (6)—properties that make CD20 an ideal target for immunotherapy of NHL. The chimeric mouse anti-human CD20 mAb rituximab (IgG1κ) binds with high affinity to CD20-expressing cells. It is the first Food and Drug Administration–approved mAb for NHL treatment (7).

Rituximab has been an important addition to the therapeutic armamentarium against low-grade follicular NHL (8). Furthermore, its usefulness alone or combined with chemotherapy is considered as first-line therapeutic option for other types of hematologic malignancies (9–11) improving patient survival. Its usage is also extended to other pathologic states culminating in long-lasting response (12–15). Rituximab exerts significant antitumor activity in vivo (1) via inhibition of cell proliferation or triggering multiple cell-damaging mechanisms, including antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity (CDC), and apoptosis (16, 17). It also augments the cytotoxic effects of drugs on drug-resistant NHL B-cells (18). Nonetheless, the contribution of these mechanisms on normal primary and malignant B-cells in vivo and the molecular mechanisms of rituximab action need to be defined.

We proposed that modifications of signaling pathways by rituximab may be crucial for its chemosensitizing attribute. To this end, we have recently reported that rituximab, via inhibition of nuclear factor-κB (NF-κB) and extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) pathways, reduces Bcl-xl. [B-cell lymphoma protein 2 (Bcl-2)–related gene (long alternatively spliced variant of Bcl-x gene)] expression and chemosensitizes NHL B-cells (19, 20). Activation of NF-κB and ERK1/2 pathways are emerging as major mechanisms of tumor cell drug resistance and induce their rapid proliferation. Thus, interruption of these pathways is a target for therapeutic intervention and may confer drug sensitivity (21, 22), which has proven successful in enhancing the apoptotic effects of tumor necrosis factor (TNF)-α and CPT-11 resulting in tumor regression in vivo (23). Inhibition of NF-κB and ERK1/2 pathways was shown by decrease in phosphorylation and kinase activities of the signaling molecules and reduced NF-κB and activator protein-1 (AP-1) DNA-binding ability concomitant with

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reduction in the expression of their common downstream target Bcl-xl (24).

The superior efficacy of cyclophosphamide-doxorubicin-vincristine-prednisone (CHOP) + rituximab compared with CHOP alone in elderly diffuse large B-cell lymphoma patients was reported, where the combination therapy resulted in higher rates of complete remission and survival (2) and also increases overall survival in aggressive Bcl-2–positive and Bcl-2–negative patients (25). Despite its well-established clinical efficacy, a subpopulation of patients, via an elusive mechanism, does not respond to rituximab and/or acquires resistance upon long-term rituximab therapy (16, 17). Based on previous reports, we hypothesized that development of rituximab resistance may be related to failure of tumor cells to respond to rituximab-mediated signaling. Further, the unresponsiveness of the cells to drug therapy (alone or combined with rituximab) may be due to hyperactivation of survival signaling pathways and up-regulation of resistant factors. Due to challenges in obtaining patient-derived specimens for analysis, and to recapitulate various aspects of acquired rituximab-resistant (RR) situations, rituximab-refractory clones were generated (26). Using a battery of functional and biochemical assays, representative clones were compared with parental cells to examine alterations in rituximab-mediated effects and to examine the above hypotheses. Different RR clones have also been established and analyzed (27). The following objectives were investigated: (a) phenotypic and functional properties of RR clones (e.g., differences regarding CD20 surface expression, proliferation, CDC, cross-linked rituximab-mediated apoptosis); (b) chemosensitivity of the clones and chemosensitization by rituximab; (c) activation status of ERK1/2 and NF-κB pathways; (d) expression of Bcl-2 family members; and (e) effects of various inhibitors of survival pathways on reversal of chemoresistance. The results are concordant with hypotheses and reveal that RR clones display different biochemical and functional properties compared with wild-type (WT) cells. These differences and potential clinical significance of the observations are discussed.

Materials and Methods

Cell Lines and Clones

CD20+ human Burkitt's lymphoma B-cell lines Daudi and Ramos were obtained from the American Type Culture Collection (Bethesda, MD). For the generation of RR clones, WT cells were grown in the presence of stepwise increasing concentrations of rituximab (5–20 μg/mL for 10 weeks). Single cells were then subjected to three consecutive rounds of limiting dilution analysis (26). Single cells were propagated and maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; ref. 28). Clones were supplemented with 20 μg/mL rituximab weekly and grown in rituximab-free medium at least 1 week before analysis. Cultures were incubated in controlled atmosphere incubator at 37°C with saturated humidity at 0.5 × 10^6/mL.

Reagents

Paclitaxel, cisplatin, etoposide (VP-16), Adriamycin, and vincristine were purchased from Sigma (St. Louis, MO) and were diluted in DMSO. The concentration of DMSO did not exceed 0.1% in any experiment. Mouse anti-human CD20 mAb (IDEC Pharmaceuticals, San Diego, CA) was purchased from DAKO (Carpinteria, CA). Rabbit anti–p-ERK1/2 (Thr185/Tyr187) antibody, mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti–Bcl-2 anti–Bcl-xL and anti–myeloid cell differentiation 1 (Mcl-1) mAbs were purchased from Sigma (St. Louis, MO) and were diluted in DMSO. The concentration of DMSO did not exceed 0.1% in any experiment. Mouse phosphorylated inhibitor of NF-κB (Ser32/36) and antiactin mAbs were obtained from Imgenex (San Diego, CA); ref. 28).

Surface CD20 Expression

Cells (2 × 10^6) were washed twice with ice-cold 1× PBS and stained with 1 μg mouse anti-human CD20 mAb (IDEC Pharmaceuticals, San Diego, CA) or isotype control (pure IgG1; 20 min on ice, light protected). Then, the cells were washed twice with ice-cold 1× PBS, stained with FITC-labeled secondary antibody (30 min on ice, light protected), and subjected to fluorescence-activated cell sorting (FACS) analysis.

Immunoblot Analysis

Cells (10^7) were grown either in complete medium or in complete medium supplemented with various inhibitors. Cells were lysed at 4°C in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl] supplemented with one tablet of protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN). A detergent-compatible protein assay kit (Bio-Rad, Hercules, CA) was used to determine protein concentration. An aliquot of total protein lystate was diluted in an equal volume of 2× SDS sample buffer, boiled for 10 min, and cell lysates were electrophoresed on 12% SDS-PAGE gels. Western blot was carried out as described (28). The relative intensity of bands, hence, the relative alterations in protein expression, was assessed by densitometric analysis of digitized images using public domain NIH image program.1

Assessment of Apoptosis

DNA fragmentation assay. Percentage of apoptotic cells was determined by evaluation of propidium iodide–stained preparations of cells using an EpicXL flow cytometer. Cellular debris was excluded from analysis by raising the forward scatter threshold, and DNA content of the intact nuclei was recorded on a logarithmic scale (29). Percentage apoptosis is represented as percentage of hypodiploid cells accumulated at sub-G0 phase of the cell cycle.

Evaluation of active caspase-3 levels. Levels of active caspase-3 were evaluated with FITC-labeled anti–active caspase-3 mAb (PharMingen, San Diego, CA; ref. 28).

Assessment of Viable Cell Recovery

Viable cell recovery was assessed using standard 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay kit (Roche) that measures metabolic activity of viable cells (30). Percentage cell recovery was calculated using background-corrected reading as follows: % cell recovery = [(absorbance of sample wells / absorbance of untreated cells)] × 100.

Electrophoretic Mobility Shift Analysis

The DNA-binding abilities were evaluated using biotin-labeled oligonucleotide AP-1 (5′-CGCTTGGTAATCCGGAA-3′; ref. 31) and NF-κB (5′-AGTGGAGGGACTCTCCGGGC-3′) probes (32) using an electrophoretic mobility shift assay (EMSA) kit (Panomics, Inc., Redwood City, CA) according to the manufacturer’s instructions. Ten micrograms of nuclear extracts were subjected to denaturing 5% PAGE and were developed (19, 20).

Immunocomplex Kinase Assay

The kinase activity of IKK and MAP/ERK kinase 1/2 (MEK1/2) was assessed by their ability to phosphorylate IκB-α (Ser53/56) and MAPK kinase substrate 4 (Thr185/Tyr187) using a slightly modified version of previous methods (19, 20, 33).

Quantitative Real-time PCR

Samples were analyzed in triplicate with iQ SYBR Green Supermix using iCycler Sequence Detection System (Bio-Rad). Total RNA was extracted

from 10^6 cells for each condition with 1 mL per sample of STAT6-60 reagent and quantified by 3.12 NanoDrop ND-1000 spectrophotometer. Total RNA (3 μg) was reverse to first-stranded cDNA for 1 h at 42°C with 200 units SuperScript II reverse transcriptase and 20 μmol/L random hexamer primers. Amplification of 2.5 μL of cDNAs was done using gene-specific primers. Internal control for equal cDNA loading in each reaction was assessed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Amplicons were resolved by 2% gels for confirmation and were of expected size. Percentages of expression of each molecule were calculated with the assumption that control samples were considered as 100%.

**Statistical Analysis**

Assays were set up in triplicates, and results were expressed as mean ± SD. Statistical analysis and P values were calculated by a two-tailed paired t test with a 95% confidence interval for determination of significance of differences between treatment groups (P < 0.05, significant). ANOVA was used to test significance among the groups using InStat 2.01 software.

**Results**

**Phenotypic and Functional Properties of the RR Clones**

Diminished surface CD20 expression and failure to respond to rituximab-mediated inhibition of cell growth and apoptosis following cross-linking. WT cells and Ramos-RR1 and Daudi-RR1 clones were stained with isotype control (pure IgG1; Fig. 1A, gray lines) or FITC-labeled anti-CD20 mAb (IgG1 subtype; solid black lines) and were subjected to FACS analysis. As measured by mean fluorescence intensity, WT cells show significant CD20 surface expression whereas the clones exhibit ~40% to 50% reduction in surface CD20 (Ramos, 46.8 ± 14.0 versus 264 ± 9.6; Daudi, 346 ± 11.4 versus 156.4 ± 10.2). Similar results were obtained in multiple independent experiments with other Ramos-RR and diffuse large B-cell lymphoma RR clones (data not shown), suggesting that continuous rituximab treatment results in diminished, but not complete loss of, surface CD20 expression on RR clones.

To assess the ability of RR clones to respond to growth-inhibitory effects of rituximab, both the WT and the clones were either left untreated or treated with a predetermined concentration of rituximab for WT cells (20 μg/mL, 24 h; ref. 34). An aliquot (10^6) of cells was used in a standard XTT viability assay. Rituximab exerts an inhibitory effect on the WT cells (Ramos, 27%; Daudi 46%), whereas it fails to reduce the growth of RR clones (Ramos-RR1, 98%; Daudi-RR1, 106%; Fig. 1B, top). Higher concentrations (50 or 100 μg/mL) or longer exposure time (up to 48 h) of rituximab did not alter the phenotype of the clones (Fig. 1B, bottom); thus, 20 μg/mL rituximab (24 h) was used in subsequent studies. Results of the XTT assay were confirmed by trypan blue dye exclusion and FACS (data not shown), which essentially showed that rituximab fails to induce apoptosis or inhibit cell growth in RR clones. Untreated RR clones exhibited homotypic aggregation that remained unaffected by rituximab. In contrast, WT cells grew as suspended nonaggregated cells, and rituximab caused them to form clumps (Fig. 1C). These results suggest that whereas rituximab efficiently lowers the growth rate of the WT cells, it fails to reduce the growth of RR clones even at higher concentrations.

Next, we assessed the ability of cross-linked rituximab to induce apoptosis in RR clones. WT cells and the clones were pretreated with an optimal concentration of cross-linked rituximab (50 μg/mL antihuman immunoglobulin + 20 μg/mL rituximab, 24 h; ref. 35) and were subjected to apoptosis assay. DNA fragmentation assay for apoptosis detection in subsequent experiments was confirmed by measuring active caspase-3 levels (ref. 28; data not shown). Neither rituximab (Ramos-RR1, 11.0 ± 0.8%; Daudi-RR1, 6.7 ± 0.6%) nor the antihuman immunoglobulin (Ramos-RR1, 10.2 ± 1.1%; Daudi-RR1, 12.8 ± 0.5%) alone efficiently killed the cells. However, the combination of the two agents (cross-linked rituximab) induced significant levels of apoptosis in Ramos (29.2 ± 2.4%) and Daudi (32.8 ± 2.1%) WT cells, whereas cross-linked rituximab moderately killed RR clones (Fig. 1D), suggesting that clones have developed higher threshold (Ramos-RR1, 2.86-fold; Daudi-RR1, 2.56-fold) and, unlike the WT cells, do not efficiently respond to cross-linked rituximab-mediated apoptosis.

**Failure to respond to CDC.** The ability of rituximab to mediate CDC in RR clones compared with the WT cells was assessed by analyzing the percentage of dead cells that were treated with human AB serum as source of complement (5% or 10%, 24 h). As shown in Fig. 1E, WT cells exhibited modest sensitivity to the cytotoxic effects of AB serum (as a function of serum concentration; Ramos, 15.5 ± 1.3%; Daudi, 14.3 ± 0.9%), an effect that was significantly augmented in the presence of rituximab (Ramos, 36.9 ± 1.5% versus 11.0 ± 0.8%; Daudi, 29.2 ± 1.6% versus 6.2 ± 0.6%). There was augmentation of cytotoxicity with 10% serum than with 5%. However, compared with WT cells, the clones were less sensitive to human AB serum, and rituximab failed to enhance their sensitivity (Ramos-RR1, 14.2 ± 1.6% versus 7.8 ± 0.6%; Daudi-RR1, 11.6 ± 2.4% versus 6.6 ± 1.1%). Increasing serum concentration (15%) neither enhanced their sensitivity nor augmented rituximab-mediated CDC of the clones (data not shown). These results show that WT cells are sensitive to CDC, which is enhanced by both serum levels and rituximab treatment, whereas long-term rituximab exposure is accompanied by higher CDC resistance in the clones (Ramos-RR1, 2.6-fold; Daudi-RR1, 2.5-fold). Moreover, rituximab fails to augment CDC.

**Failure of rituximab to chemosensitize the RR clones.** Augmentation of the cytotoxic effects of drugs is an established
property of rituximab (18). To assess the ability of rituximab to chemosensitize RR clones, WT cells and the clones were pretreated with rituximab, subsequently treated with various concentrations of paclitaxel (0.1–10 nmol/L), and subjected to apoptosis assay. Paclitaxel was used as a representative drug; similar results were obtained with VP-16 and cisplatin (data not shown). Rituximab significantly augmented the apoptotic effect of paclitaxel in WT Ramos and Daudi cells in a concentration-dependent manner (range 45–58% apoptosis; Fig. 1F, left). However, RR clones had higher resistance to paclitaxel, and rituximab was incapable of augmenting paclitaxel-induced apoptosis (Fig. 1F). Higher concentrations of rituximab (2.5- to 5-fold) failed to sensitize the clones (Fig. 1F, right). These results suggest that whereas rituximab efficiently chemosensitizes WT cells, it is incapable of chemosensitizing the clones, which suggests higher resistance of RR clones to rituximab-mediated chemosensitization.

Development of Higher Drug Resistance in RR Clones

Because RR clones were not chemosensitized by rituximab, their sensitivity against a battery of drugs, including paclitaxel, vincristine, VP-16, Adriamycin, and cisplatin, was examined. Compared with WT cells, which exhibit moderate sensitivity to these drugs in a concentration-dependent manner, clones exhibited higher apoptosis threshold to these drugs, albeit to varying degrees. As such, Ramos-R1R1 showed 1.54-fold (136%), 1.42-fold (130%), 2.3-fold (158%), 1.8-fold (145%), 1.41-fold (120%) and Daudi-R1R1 showed 1.63-fold (139%), 1.66-fold (140%), 1.94-fold (149%), 2.97-fold (167%), 1.95-fold (149%) resistance to paclitaxel, vincristine, Adriamycin, VP-16, and cisplatin, respectively, compared with their respective WT cells (Fig. 2A). Prolonged incubation time (48 h) did not significantly augment drug cytotoxicity in the clones (data not shown), suggesting that compared with WT cells, RR clones exhibit higher (1.41- to 2.97-fold) drug resistance. Functional analysis of the multidrug resistance (MDR) pump showed the existence of functional MDR pump in both WT cells and the clones. Further, RR clones exhibited no functional impairment of MDR pump (±rituximab; data not shown), suggesting that higher drug resistance of the clones is independent of the MDR pump.

Overexpression of Bcl-2, Bcl-xL, and Mcl-1 in RR Clones

RR clones did not respond to rituximab-mediated chemosensitization and exhibited higher drug resistance. Previous findings have established Bcl-xL as an important resistant factor (19, 20); thus, we evaluated Bcl-xL levels and other antiapoptotic Bcl-2 family members in the clones. Total RNA was extracted and converted to first stranded cDNA, which was subjected to real-time quantitative PCR (qPCR) analysis. RR clones exhibited increased expression of Bcl-2 (Ramos-R1R1, 3.6-fold; Daudi-R1R1, 3.2-fold), Bcl-xL (Ramos-R1R1, 8.2-fold; Daudi-R1R1, 4.2-fold), Mcl-1 (Ramos-R1R1, 3.4-fold; Daudi-R1R1, 2.8-fold) at the transcription level (Fig. 2B). Immoblotting showed that clones exhibit higher Bcl-2, Bcl-xL, and Mcl-1 protein levels (~2.3- to 5.0-fold) compared with WT cells. Interestingly, expression levels of these proteins remained unaffected by rituximab treatment of the clones, whereas Bcl-xL levels in WT cells were reduced (Fig. 2C). Notably, expression levels of other proapoptotic and antiapoptotic factors (Bcl-xS, Bfl-1/A1, Bad, Bax, Bid, Bak, c-IAP-1, c-IAP-2, survivin, XIAP) were similar in WT cells and RR clones (±rituximab; data not shown). These results show that clones express higher levels of protective factors, which may explain their unresponsiveness to rituximab-mediated chemosensitization and higher drug resistance. Also, rituximab was unable to reduce the levels of resistant factors, suggesting that signaling pathways in the clones are no longer responsive to rituximab.

Hyperactivation of the ERK1/2 and NF-κB Signaling Pathways in the RR Clones

The above findings suggest that the dynamics of the cellular signaling pathways are altered in the clones. Rituximab inhibits ERK1/2 and NF-κB pathways leading to reduced DNA-binding ability of AP-1 and NF-κB transcription factors in WT cells (19, 20). Thus, we examined the activation status of these pathways in the clones. Whole-cell extracts of WT cells and the clones (±rituximab) were subjected to immunoblotting for components of NF-κB and ERK1/2 pathways. The phosphorylation-dependent state of IKK, IκB-α, and ERK1/2 was higher in the clones (~3.2- to 4.8-fold) than in WT cells. Basal levels of these signaling molecules remained unaffected in WT and RR clones (±rituximab, data not shown). Rituximab significantly reduces the phosphorylation of these molecules in WT cells (19, 20), an effect that is not observed in clones (Fig. 3A), suggesting that molecular switches responsible for rituximab-mediated dephosphorylation of these molecules are no longer operative in clones.

To ascertain the observed hyperphosphorylation results in increased activity of NF-κB and ERK1/2 pathways, immunocomplex kinase assays were done to assess IKK and MEK1/2 kinase activities of the clones (±rituximab) using IκB-α peptide (IKK substrate) and MAPK kinase substrate-4 (ERK1/2 substrate). Untreated clones showed significantly increased kinase activities as shown by increased ability of the lysates to phosphorylate their specific substrates, whereby rituximab did not reduce the IKK and MEK1/2 kinase activities. This phenomenon was not observed by IκB-α peptide S32/36A (data not shown). In contrast, in WT cells, rituximab diminishes the kinase activity of IKK and MEK1/2 (Fig. 3B); thus, increased phosphorylation of signaling molecules culminates in higher kinase activity of these pathways in the clones. Next, alterations in the DNA-binding ability of NF-κB and AP-1 in the clones were examined. Biotin-labeled oligonucleotides probes comprising the NF-κB DNA-binding probe (IKK substrate) and MAPK kinase substrate-4 (ERK1/2 substrate). Untreated clones showed significantly increased kinase activities as shown by increased ability of the lysates to phosphorylate their specific substrates, whereby rituximab did not reduce the IKK and MEK1/2 kinase activities. These findings have established Bcl-xL as an important resistant factor (19, 20); thus, we evaluated Bcl-xL levels and other antiapoptotic Bcl-2 family members in the clones. Total RNA was extracted and converted to first stranded cDNA, which was subjected to real-time quantitative PCR (qPCR) analysis. RR clones exhibited increased expression of Bcl-2 (Ramos-R1R1, 3.6-fold; Daudi-R1R1, 3.2-fold), Bcl-xL (Ramos-R1R1, 8.2-fold; Daudi-R1R1, 4.2-fold), Mcl-1 (Ramos-R1R1, 3.4-fold; Daudi-R1R1, 2.8-fold) at the transcription level (Fig. 2B). Immoblotting showed that clones exhibit higher Bcl-2, Bcl-xL, and Mcl-1 protein levels (~2.3- to 5.0-fold) compared with WT cells. Interestingly, expression levels of these proteins remained unaffected by rituximab treatment of the clones, whereas Bcl-xL levels in WT cells were reduced (Fig. 2C). Notably, expression levels of other proapoptotic and antiapoptotic factors (Bcl-xS, Bfl-1/A1, Bad, Bax, Bid, Bak, c-IAP-1, c-IAP-2, survivin, XIAP) were similar in WT cells and RR clones (±rituximab; data not shown). These results show that clones express higher levels of protective factors, which may explain their unresponsiveness to rituximab-mediated chemosensitization and higher drug resistance. Also, rituximab was unable to reduce the levels of resistant factors, suggesting that signaling pathways in the clones are no longer responsive to rituximab.

Figure 2. Higher drug resistance and overexpression of the antiapoptotic Bcl-2 family members in the RR clones. A, cells (2 × 10^6) were either left untreated or treated with various concentrations of paclitaxel (1.0, 10, and 20 μg/mL), Adriamycin (0.5, 1.0, and 2.0 μg/mL), cisplatin (DDP: 1.0, 10, and 20 μg/mL), vincristine (0.1, 0.5, and 1.0 μg/mL), and VP-16 (1.0, 10, and 20 μg/mL) for 18 h. Then, the cells were stained with propidium iodide solution, and apoptosis was assessed by FACS. Samples were set up in duplicates. Columns, mean (n = 2); bars, SD. (Fold drug resistance was measured using the highest percentage of apoptosis induced by the highest concentration of the drug in the WT cells as 100% and calculating the required drug concentration to achieve the same level of apoptosis in the clones.) B, total RNA of various culture conditions was extracted from 10^7 cells (as indicated) and converted to cDNA. CDNA (2.5 μg) was used in qPCR analysis to determine the transcript levels. Levels of GAPDH were confirmed for equal loading. Columns, mean of triplicate samples; bars, SD. C, whole-cell extracts (40 μg) of WT cells and RR clones (±20 μg/mL rituximab, 24 h) were subjected to immunoblotting for protein levels. Levels of β-actin were used for equal loading (n = 2). *, P < 0.05, significant compared with control.
Rituximab-induced decrease in NF-κB and AP-1 DNA-binding ability was only observed in WT cells. The specificity of EMSA was corroborated using appropriate controls (Fig. 3C). Specific inhibitors (DHMEQ and PD098059) preferentially reduced NF-κB and AP-1 DNA-binding abilities. Collectively, these results show that NF-κB and ERK1/2 pathways are constitutively hyperactivated in RR clones and denote the inability of rituximab to negatively regulate the activities of these pathways in the clones unlike WT cells. Hyperactivation of these pathways will lead to enhanced transcription of their respective antiapoptotic target genes, leading to higher drug resistance of the clones.

Chemosensitization of RR Clones by Pharmacologic Inhibitors of ERK1/2 and NF-κB Pathways

The NF-κB and ERK1/2 pathways are hyperactivated in RR clones, leading to overexpression of Bcl-2, Bcl-xL, and Mcl-1, all of which are unaffected by rituximab. This prompted us to investigate whether inhibition of these pathways or Bcl-2 members can reverse chemoresistance. Because these pathways have higher activities in RR clones, higher concentrations of inhibitors were required for chemoresistance. Because these pathways have higher activities in RR clones and denote the inability of rituximab to negatively regulate the activities of these pathways in the clones unlike WT cells, hyperactivation of these pathways will lead to enhanced transcription of their respective antiapoptotic target genes, leading to higher drug resistance of the clones.

Because inhibitors efficiently chemosensitized the clones, we assessed their effect on expression of the resistant factors. As depicted by qPCR inhibitors, mRNA levels of Bcl-2, Bcl-xL, and Mcl-1 were reduced by 1.2- to 4.8-fold and 1.2- to 6.6-fold (Fig. 5A), and immunoblots showed 1.25- to 3.3-fold and 1.1- to 3.3-fold decreases in their protein levels in Ramos-RR1 and Daudi-RR1, respectively (Table 1B). The ability of inhibitors to significantly chemosensitize the RR clones (1.5- to 5.1-fold) suggests that inhibition of NF-κB and ERK1/2 pathways can avert chemoresistance and rituximab resistance in clones to subtoxic drug concentrations.

The chemoprotective role of the overexpressed Bcl-2, Bcl-xL, and Mcl-1 in clones was further confirmed by pretreatment with 2MAM-A3 (38), which chemosensitized the clones at levels comparable with those achieved by rituximab. In WT Ramos, 2MAM-A3 augmented paclitaxel cytotoxicity by 1.96-fold (13.6 ± 1.3% → 26.7 ± 2.2%), which was 4.71-fold (8.42 ± 2.1% → 39.7 ± 2.4%) in Ramos-RR1. A similar pattern was observed in Daudi (WT, 1.98-fold; RR1, 3.2-fold; Fig. 5C). These findings support our contention that overexpression of antiapoptotic Bcl-2 members upon prolonged rituximab treatment protects the cells against

![Figure 3.](cancerres.aacrjournals.org) Hyperactivation of the NF-κB and ERK1/2 survival pathways in the RR clones. After overnight growth in RPMI 1640 + 1% FBS, RR clones were washed and grown in complete medium ± rituximab (20 μg/mL, 24 h). A, total cell lysates (40 μg) were subjected to Western blot analysis using phosphospecific antibodies for various components of the NF-κB and ERK1/2 pathways. B, the kinase activity of the IKK complex using k-B peptide (amino acids 1-50 including S32/36) and ERK1/2 using MAPK kinase substrate 4 (amino acids 172-192) using immunocomplex kinase assay. C, after overnight growth in RPMI 1640 + 1% FBS, cells were washed and were grown in complete medium (+ rituximab, DHMEQ, or PD098059). Ten micrograms of nuclear lysates were subjected to EMSA (n = 2; refs. 19, 20).
drug-induced apoptosis and that their functional impairment is critical for chemosensitization.

Discussion

This is the first report on the establishment of B-NHL RR clones, which exhibit a different phenotypic profile compared with WT cells. Using various biochemical and functional assays, compared with WT cells, RR clones express lower levels of surface CD20 and do not respond to growth inhibition by rituximab, rituximab-mediated CDC, or cross-linked rituximab-induced apoptosis. Further, RR clones are not chemosensitized by rituximab and exhibit higher drug resistance (1.41- to 2.97-fold). Two major survival pathways (NF-κB and ERK1/2) are constitutively hyperactivated in the clones, leading to overexpression of resistant factors (Bcl-2, Bcl-xL, Mcl-1; ~2.3- to 5.0-fold). Pharmacologic inhibition of Bcl-2 family members (by 2MAM-A3; NF-κB by DHMEQ, bortezomib), and ERK1/2 pathways (by PD098059) averts the drug-resistant phenotype, and the clones undergo apoptosis in response to low concentrations of various drugs (Fig. 6).

Reducing the proliferation rate of tumor cells is postulated as one of the potential modes of action of rituximab (16, 17). Rituximab treatment of WT cells reduces their growth rate (28); however, the clones grew at similar rates as WT cells and rituximab (even at 2.5- to 5.0-fold higher concentrations) was incapable of inhibiting their growth (Fig. 1B) consistent with the higher growth rate and progressive nature of relapsed lymphomas, suggesting that RR clones have lost the ability to undergo rituximab-mediated growth reduction possibly through a defective ceramide acid sphingomyelinase (A-SMase) pathway (39). Compared with WT cells, RR clones exhibit higher resistance to CDC. Rituximab pretreatment significantly enhanced CDC in WT cells—an effect that was not noticed in the clones. Increasing serum concentrations enhanced rituximab-mediated CDC in WT cells but not in clones (Fig. 1D), consistent with our preliminary findings that show increased expression of complement inhibitors on the clones (data not shown). Further studies are warranted to delineate the role of complement inhibitors in CDC resistance of the RR clones. Cross-linked rituximab induced significant apoptosis (Fig. 1E) and cytostasis (data not shown) on WT cells. Neither induction of apoptosis nor growth inhibition was observed on treatment of the clones with cross-linked rituximab, suggesting that RR clones have developed higher threshold in response to biological effects of cross-linked rituximab. The above effects were independent of

Figure 4. Chemosensitization of the RR clones by chemical inhibitors. Cells (2 × 10⁶) were either left untreated or pretreated with (A) DHMEQ (WT, 10 μg/mL; clones, 20 μg/mL), (B) bortezomib (WT, 4 μmol/L; clones, 8 μmol/L), or (C) PD098059 (WT, 15 μg/mL; clones, 30 μg/mL) for 2 h. Cells were then incubated with paclitaxel (1.0, 10, and 20 μg/mL), Adriamycin (ADR; 0.5, 1.0, and 2.0 μg/mL), cisplatin (1.0, 10, and 20 μg/mL), vincristine (0.1, 0.5, and 1.0 μg/mL), and VP-16 (1.0, 10, and 20 μg/mL) for an additional 18 h and subjected to DNA fragmentation assay. The samples were set up in duplicates. Columns, mean of two independent experiments; bars, SD. *, P < 0.05, significant compared with drug treatment alone.
rituximab concentration as higher (2.5- to 5-fold) concentrations of rituximab failed to reverse the phenotype of the clones (Fig. 1).

Rituximab binds to B-cell–restricted cell surface CD20, thus exerting its effects. Various mechanisms are postulated for rituximab resistance including transient CD20 down-regulation (40), loss of CD20 (41), and circulating CD20 (42). Substantial CD20 resistance including transient CD20 down-regulation exerting its effects. Various mechanisms are postulated for the significance of reduced CD20 expression in clones requires further investigation as several lines of evidence suggest that the biological effects of rituximab therapy is autonomous of the intensity of surface CD20 even in vivo (40). Thus, failure of rituximab to exert its biological effects on RR clones may be independent of diminished CD20. It may be due to the activation status of the clones and/or aberrant cellular signaling as deregulation of the signaling pathways or aberrant expression of signaling molecules contribute to acquired chemoresistance (42, 43). Analysis of the signaling pathways in the clones revealed hyperactivation of ERK1/2 and NF-κB pathways leading to overexpression of their downstream resistant factors Bcl-2, Bcl-xL, and Mcl-1, and the exhibition of higher drug resistance (1.41- to 2.97-fold) concordant with the protective role of Bcl-2 family members (44–46), suggesting that the selective pressure applied by prolonged rituximab treatment has censored for cells that express higher levels of antiapoptotic proteins which have lost the capacity to undergo apoptosis in response to various stimuli. The possibility of the preexistence of resistant cells in the native culture is not ruled out. Clearly, rituximab has not altered the biological properties of 100% of the cells. However, the resistant subclones in native culture will dominate the sensitive population on long-term rituximab treatment as the sensitive cells will be eliminated over time. However, there is no unequivocal evidence, as yet, that this is the dominant mechanism in vivo. Because drugs use apoptosis as a means of exerting their effects, drug-resistant tumors develop cross-resistance to apoptosis induced by structurally and functionally distinct stimuli, including immunotherapy and vice versa. Thus, as NHL cells develop resistance to rituximab, they may also develop cross-resistance to drugs and the immune system, consistent with our observation that drug-resistant RR clones also exhibit higher resistance to TNF-related apoptosis-inducing ligand (TRAIL) and anti-Fas agonistic antibody (47). Rituximab pretreatment of WT cells, not the clones, efficiently sensitized them to drugs and TRAIL (47).

Unlike WT cells (19, 20), rituximab was incapable of inhibiting hyperactivated NF-κB and ERK1/2 pathways in the clones. Thus, it is logical to speculate that constitutive hyperactivation of these pathways confer higher drug resistance (21, 22); hence, their inhibition could potentially avert the chemoresistance, prompting us to evaluate the chemosensitizing effects of specific inhibitors of NF-κB and ERK1/2 pathways as well as bortezomib (Velcade; ref. 37). DHMEQ is a unique inhibitor of NF-κB acting at the level of nuclear translocation, completely inhibits NF-κB DNA-binding ability, inhibits the growth of human hormone-refractory prostate and bladder cancer cells, and induces apoptosis at high concentrations (36, 48). PD098059 exerts its effects by specifically binding to inactive form of MEK1/2 and prevents its activation by Raf-1, thus inhibiting ERK1/2 activation (33). Bortezomib is approved for the treatment of multiple myeloma and mantle cell lymphoma.

### Table 1. Chemosensitization of the RR clones by chemical inhibitors

#### A. Chemosensitization

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inhibitor</th>
<th>Medium</th>
<th>Paclitaxel (20 μg/mL)</th>
<th>Adriamycin (2 μg/mL)</th>
<th>VP-16 (20 μg/mL)</th>
<th>Cisplatin (20 μg/mL)</th>
<th>Vincristine (1 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ramos-RR1</td>
<td>Control</td>
<td>7.60 ± 1.4</td>
<td>20.4 ± 3.0</td>
<td>14.3 ± 3.3</td>
<td>10.3 ± 1.1</td>
<td>19.0 ± 0.7</td>
<td>15.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>DHMEQ</td>
<td>10.1 ± 2.1</td>
<td>46.5 ± 2.2</td>
<td>32.4 ± 2.9</td>
<td>53.0 ± 2.6</td>
<td>61.2 ± 2.1</td>
<td>53.5 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Bortezomib</td>
<td>9.62 ± 2.4</td>
<td>46.5 ± 1.8</td>
<td>26.3 ± 1.8</td>
<td>52.6 ± 3.1</td>
<td>43.8 ± 3.2</td>
<td>51.1 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>PD098059</td>
<td>10.8 ± 0.9</td>
<td>36.0 ± 2.3</td>
<td>36.5 ± 0.8</td>
<td>31.6 ± 2.8</td>
<td>41.6 ± 1.2</td>
<td>36.4 ± 1.8</td>
</tr>
<tr>
<td>Daudi-RR1</td>
<td>Control</td>
<td>3.8 ± 2.2</td>
<td>14.9 ± 3.2</td>
<td>12.4 ± 2.5</td>
<td>11.8 ± 0.9</td>
<td>25.7 ± 3.1</td>
<td>14.2 ± 1.7</td>
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<tr>
<td></td>
<td>DHMEQ</td>
<td>4.9 ± 1.6</td>
<td>42.0 ± 1.9</td>
<td>40.5 ± 3.1</td>
<td>32.0 ± 2.2</td>
<td>36.6 ± 0.9</td>
<td>36.6 ± 2.3</td>
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<tr>
<td></td>
<td>Bortezomib</td>
<td>5.3 ± 2.7</td>
<td>42.8 ± 1.8</td>
<td>32.4 ± 2.8</td>
<td>39.4 ± 1.8</td>
<td>43.8 ± 1.3</td>
<td>32.7 ± 0.9</td>
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<tr>
<td></td>
<td>PD098059</td>
<td>6.6 ± 3.4</td>
<td>32.6 ± 2.6</td>
<td>29.9 ± 1.4</td>
<td>32.1 ± 2.4</td>
<td>39.7 ± 0.8</td>
<td>28.7 ± 1.6</td>
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</table>

#### B. Fold enhancement of apoptosis

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inhibitor</th>
<th>Paclitaxel</th>
<th>Adriamycin</th>
<th>VP-16</th>
<th>Cisplatin</th>
<th>Vincristine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ramos-RR1</td>
<td>DHMEQ</td>
<td>2.3</td>
<td>2.3</td>
<td>5.1</td>
<td>3.2</td>
<td>3.5</td>
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<tr>
<td></td>
<td>Bortezomib</td>
<td>2.3</td>
<td>1.8</td>
<td>5.1</td>
<td>2.3</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>PD098059</td>
<td>1.8</td>
<td>2.6</td>
<td>3.1</td>
<td>2.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Daudi-RR1</td>
<td>DHMEQ</td>
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<td>3.3</td>
<td>2.7</td>
<td>1.4</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Bortezomib</td>
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<td>2.6</td>
<td>3.3</td>
<td>1.7</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>PD098059</td>
<td>2.2</td>
<td>2.4</td>
<td>2.7</td>
<td>1.5</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE: (A) RR clones (2 × 10⁶ per treatment) were either left untreated or pretreated with DHMEQ (20 μg/mL), bortezomib (8 μmol/L), or PD098059 (30 μg/mL) for 2 h. Cells were then incubated with paclitaxel (20 μg/mL), Adriamycin (2.0 μg/mL), cisplatin (20 μg/mL), vincristine (1.0 μg/mL), and VP-16 (20 μg/mL) for an additional 18 h and subjected to DNA fragmentation assay. Samples were set up in duplicates and the results are presented as mean ± SD of two independent experiments. In all cases, statistically significant values (P < 0.05) were obtained by the combination compared with drug and/or inhibitor treatment alone. (B) Fold enhancement of apoptosis by treatment of RR clones with inhibitors based on the data from part A.
treatment of multiple myeloma and has significant single-agent activity against certain subtypes of NHL (49). These inhibitors efficiently sensitized the RR clones to structurally and functionally distinct drugs, including topoisomerase II inhibitor, DNA alkylating agents, and microtubule poisons, albeit to varying degrees (Table 1). The inhibitors also reduced Bcl-2, Bcl-xL, and Mcl-1 levels, further suggesting that deregulated signaling culminates in overexpression of antiapoptotic proteins in RR clones, which then leads to higher drug resistance. The chemoprotective role of Bcl-2 family members was confirmed by using 2MAM-A3, a specific inhibitor that binds to the hydrophobic groove formed by the highly conserved BH1, BH2, and BH3 domains, thus impairing the function of Bcl-2, Bcl-xL, and Mcl-1 (38). 2MAM-A3 efficiently chemosensitized the clones, further attesting that higher expression of resistant factors protects RR clones from drug-induced apoptosis. Hence, aberrations in the normal dynamics of cellular survival pathways upon continuous rituximab exposure contribute to acquired rituximab and/or drug resistance, whereas interruption of these pathways leads to chemosensitization of RR clones. Thus, functional impairment of antiapoptotic proteins is critical for reversion of chemoresistance.

The nature of the molecular cues that trigger the aberrant activation of survival pathways in the clones, hence their altered phenotype, are unclear at present. In WT Daudi, rituximab induces a rapid and transient increase in A-SMase activity parallel with cellular ceramide generation in lipid rafts. These cells externalize both ceramide and A-SMase, which colocalize with CD20. Also, rituximab-induced growth inhibition may be mediated through a ceramide-dependent pathway (39). Preliminary observations suggest that rituximab-induced A-SMase translocation and ceramide generation at cell surface are reduced in clones (50). Because microdomains serve as signaling platforms, these data suggest that rituximab resistance in clones is partly due to faulty mobilization of the signaling molecules to lipid rafts and a crippled ceramide/A-SMase pathway. These possibilities are currently under scrutiny.

The present findings are the first report on the establishment of an in vitro model of RR NHL clones, which shows that repeated rituximab exposure results in loss of the ability of rituximab to regulate molecular switches leading to constitutive hyperactivation of survival pathways, overexpression of resistant factors, and increased apoptosis threshold. Accordingly, rituximab fails to exert antilymphoma effects and RR clones develop higher drug resistance, which may explain the treatment-refractory and the aggressive nature of clinical rituximab-resistant and drug-resistant NHL. Figure 6 shows the potential mechanisms of RR. The observed drug resistance and rituximab resistance of RR clones mimic those previously observed (27). However, RR clones are still

Figure 5. Inhibition of the expression of antiapoptotic factors by chemical inhibitors. RR1 clones were either left untreated or treated with DHMEQ (20 μg/mL), bortezomib (8.0 μmol/L), and PD098059 (30 μg/mL). A. 2.5 μg cDNA was used in qPCR using gene-specific primers. Levels of GAPDH were confirmed for equal loading. Samples were set up in duplicates. Columns, mean (n = 2); bars, SD. B. Total cell lysates (40 μg) were subjected to immunoblotting for Bcl-2, Bcl-xL, and Mcl-1. Levels of α-actin were used for equal loading (n = 2). C. Role of antiapoptotic Bcl-2 members in chemosensitization. Cells were either left untreated or pretreated with 2MAM-A3 (WT, 15 μg/mL; clones, 35 μg/mL, 7 h). The cells were then washed, treated with paclitaxel (10 nmol/L, 18 h), and subjected to DNA fragmentation assay. Samples were set up in duplicates. Columns, mean of two independent experiments; bars, SD. *P < 0.05, significant compared with paclitaxel alone.
amenable to chemotherapy using specific molecular targeting of the components of deregulated pathways. Our studies identify several such targets for potential molecular intervention in the treatment of rituximab-resistant and drug-resistant NHL. Analysis of dynamics of survival pathways in patient-derived specimens may also serve as biomarkers in choosing treatment options. Such patients may be suitable candidates for alternative (e.g., targeted therapy) over conventional regimens. Studies are under way to validate our *in vitro* findings with freshly derived RR specimens and to establish RR mouse model.

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**References**


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