

# Anti-CD20- and B-cell Receptor-mediated Apoptosis: Evidence for Shared Intracellular Signaling Pathways<sup>1</sup>

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## ABSTRACT

Clinical administration of the anti-CD20 antibody IDEC-C2B8 can induce remission of low-grade B-cell lymphoma. Whereas it has been suggested that the main mechanisms of action are complement-mediated and antibody-dependent cell-mediated cytotoxicity, we demonstrate that monoclonal antibody IDEC-C2B8 is a strong inducer of apoptosis in CD20-positive B-cell lymphoma cell lines reflecting different stages of lymphomagenesis. Thus, CD20-dependent apoptosis was inducible in human surface IgM-positive Burkitt's lymphoma cell lines as well as in more mature surface IgM-negative B-cell lymphoma cell lines carrying the t(14;18) translocation. Furthermore, in Burkitt's lymphoma cell lines, we observed a striking correlation between anti-CD20- and B-cell receptor-mediated apoptosis with regard to sensitivity toward the apoptotic stimuli and the execution of the apoptotic pathway. Thus, induction of anti-CD20- or B-cell receptor-mediated apoptosis involved rapid up-regulation of the proapoptotic protein Bax. In addition, we show similar changes in the mRNA expression level of two early response genes, *c-myc* and *Berg36*, as well as activation of the mitogen-activated protein kinase family members p44 (extracellular signal-regulated kinase 1) and p42 (extracellular signal-regulated kinase 2) and activation of activator protein 1 (AP-1) DNA binding activity. These data support our hypothesis that both pathways are mediated in part by the same signal-transducing molecules. These results might help explain the resistance and regression of lymphomas to IDEC-C2B8 and give new insights in the signaling cascade after CD20 ligation.

## INTRODUCTION

CD20 antigen is expressed throughout B-cell differentiation from cytoplasmic  $\mu$ H chain ( $c\mu$ )-positive pre-B cells to all types of mature B cells. However, pro-B cells ( $c\mu^-$ ) and terminally differentiated B cells are CD20 negative (1). The CD20 molecule contains four transmembrane domains, suggesting that it functions as a membrane transporter or ion channel. There is evidence that CD20 is involved in transmembrane  $Ca^{2+}$  flux (2). Cross-linking of CD20 by anti-B1 antibodies inhibits cell cycle progression after B-cell activation, whereas the anti-CD20 antibody 1F5 is able to stimulate resting B cells to enter the  $G_1$  phase of the cell cycle. In contrast, all anti-CD20 antibodies inhibit immunoglobulin secretion (3–5). Although the function of the CD20 molecule is not completely known, these data suggest a regulatory function in B-cell proliferation and differentiation (4, 5). CD20 signaling is associated with the induction of Src family tyrosine kinases Lyn, Fyn, and Lck, but not Blk or Bruton's tyrosine kinase, which are involved in the modulation of signaling events after BCR<sup>3</sup> activation (6). Additional signaling pathways in-

volving activation of PLC- $\gamma$ 1 and PLC- $\gamma$ 2 have been described previously (7). Although these events after CD20 ligation are well characterized, further downstream events in the CD20 signaling cascade are not known.

Due to the broad expression pattern of the CD20 antigen throughout B-cell differentiation, expression on more than 90% of B-cell non-Hodgkin-lymphomas (8), and lack of antigen shedding (9), CD20 appeared to be an ideal target for immunotherapeutic approaches in the treatment of B-cell malignancies. Thus, a variety of antibodies have been used in clinical trials as naive antibodies (9) or radioimmunconjugates (10). The mouse-human chimeric antibody IDEC-C2B8 has recently been approved for clinical application (11, 12). This antibody recognizes the B1 epitope of the CD20 molecule. Thus far, two major effector mechanisms are hypothesized, CDC and ADCC (12); however, the role of CDC and ADCC for *in vivo* tumor regression has not been clearly shown. In patients treated with an i.v. infusion of IDEC-C2B8, a dose-dependent, rapid depletion of B cells could be observed (11). Although early B-cell depletion is observed in nearly all patients, only 40–60% show a clinical response of longer duration (13). In addition, a significant number of patients will have progressive disease during antibody therapy (14). This observation raised questions concerning the molecular and cellular mechanisms underlying tumor regression and the development of resistance to anti-CD20 treatment, underscoring the necessity to understand signaling events occurring after CD20 ligation as well as the function of the molecule. Because it had been reported that murine anti-CD20 antibodies can induce apoptosis after extensive cross-linking (15), we were interested in investigating the capacity of IDEC-C2B8 to induce apoptosis and the molecular mechanisms underlying this process. For this purpose, we took advantage of B-cell lymphoma models reflecting different stages of lymphomagenesis: induction of apoptosis was studied in human Burkitt's lymphoma cell lines, which are sIgM positive and reflect a more immature stage of differentiation. In addition, we analyzed more mature B-cell lymphoma cell lines carrying the t(14;18) translocation (16, 17), which is typical for follicular lymphoma, one of the main clinical targets of anti-CD20 treatment.

Apoptosis is a tightly regulated process of cell death, which is controlled by the interplay of a number of positive and negative regulatory factors including members of the Bcl-2 gene family (18). The execution of the death pathway is mediated by activated caspases (cysteinyll aspartate-specific proteinases; Ref. 19). Previous findings concerning B-cell apoptosis result from studies investigating the BCR-mediated cell death of B-cell lines. We and others have shown the involvement of Bcl-2 family members Bax- $\alpha$  and Bcl-x<sub>L</sub> and effector caspase-3 after BCR-mediated cell death (20–22). The cleavage of specific caspase target proteins like D4-GDI and transcription factor SP-1 was identified as a result of caspase-3-like activity (22). Another group of enzymes involved in mediating mitogenic or apoptotic signals is the MAPKs (for a recent summary, see Ref. 23). All three types of MAPKs, ERKs [p44 (ERK1) and p42 (ERK2)], c-Jun

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<sup>3</sup> The abbreviations used are: BCR, B-cell receptor; mAb, monoclonal antibody; sIgM, surface IgM; MAPK, mitogen-activated protein kinase; AP-1, activator protein 1; ERK, extracellular signal-regulated kinase; PLC, phospholipase C; CDC, complement-dependent cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; MFI, mean fluores-

cence intensity; p-NA, *p*-nitroanilide; PMSF, phenylmethylsulfonyl fluoride; PARP, poly-(ADP-ribose) polymerase; ERG, early response gene; PK, protein kinase; EpCAM, epithelial cell adhesion molecule; z-DEVD-fmk, z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH<sub>2</sub>F; Fc $\gamma$ R, Fc $\gamma$  receptor; ss, supershift; PTK, protein tyrosine kinase.

NH<sub>2</sub>-terminal kinases (stress-activated PKs), and p38, modulate a distinct set of transcription factors and have been proposed to modulate BCR signaling. MAPK activity in B lymphocytes is shown for activation of different receptors as CD19, CD22, or CD40 (24–26). However, the role of MAPKs in the signaling processes of B cells is not yet clear, but activation of ERKs might be required for induction of BCR-mediated apoptosis, whereas stress-activated PK activity might mediate mitogenic signals (27).

We report that extensively cross-linked anti-CD20 antibody IDEC-C2B8 is a potent inducer of apoptosis in both of the lymphoma cell line models and that, among sIgM-positive Burkitt's lymphoma cell lines, only cell lines susceptible to BCR-mediated apoptosis were sensitive to anti-CD20-mediated apoptosis. Investigation of signaling events after antigen cross-linking further sustained these observations, revealing that both anti-CD20- and BCR-mediated apoptosis are dependent on caspase-3 and involve similar responses of the ERGs Berg36 and c-myc and up-regulation of bax- $\alpha$  as well as MAPK and AP-1 activation.

## MATERIALS AND METHODS

**Cells and Culture Conditions.** The CD20-expressing human Burkitt's lymphoma cell lines BL60-2 (22), Daudi, and BJAB; the sIgM-sensitive (BL41-s) and sIgM-resistant (BL41-r) subclones of BL41 (28); and the t(14;18)-positive cell lines K422 (Ref. 16; kindly provided by Rainer Haas, Heinrich Heine Universität, Düsseldorf, Germany) and SU-DHL-4 (Ref. 17; kindly provided by Linda M. Boxer, Stanford University School of Medicine, Stanford, CA) were cultured in RPMI 1640 (Seromed-Biochrom, Hamburg, Germany) at 37°C and 5% CO<sub>2</sub>. Culture medium was supplemented with 10% (v/v) heat-inactivated FCS, 1 mM sodium pyruvate, 2 mM glutamine, 10 mM HEPES (pH 7.4), 20 nM bathocuproine disulfonic acid, 50  $\mu$ M  $\alpha$ -thioglycerol, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. All supplements were purchased from Life Technologies, Inc. (Karlsruhe, Germany).

**Antibodies.** Anti-CD20-mediated apoptosis was induced using the following antibodies: (a) anti-CD20 mAb IDEC-C2B8 (generously provided by Dr. U. Riedel; Hoffmann-LaRoche, Freiburg, Germany); and (b) anti-CD20 antibody B9E9 (Coulter Immunotech, Hamburg, Germany). Cross-linking of soluble anti-CD20 antibody B9E9 was achieved using goat antimouse IgG F(ab')<sub>2</sub> antibody fragments (GAM; Dianova, Hamburg, Germany). BCR-mediated apoptosis was induced as described previously using goat antihuman IgM F(ab')<sub>2</sub> antibody fragments (Dianova; Ref. 28). The murine IgG1 antibody HD20 (kindly provided by Gerhard Moldenhauer; Deutsches Krebsforschungszentrum, Heidelberg, Germany) as well as a human anti-EpCAM IgG1 antibody (kindly provided by Torsten Dreier, Micromet GmbH, Martinsried, Germany) served as a control. All antibodies used in cell culture experiments were devoid of NaN<sub>3</sub>.

**[<sup>3</sup>H]Thymidine Incorporation.** Proliferation of cells in the absence or presence of immobilized or soluble anti-CD20 and control antibodies was determined by the use of [<sup>3</sup>H]thymidine incorporation. Ten thousand cells were incubated in 200  $\mu$ l of culture medium in 96-well flat-bottomed microtiter plates (Falcon; Becton Dickinson, Heidelberg, Germany). One  $\mu$ Ci of [<sup>3</sup>H]thymidine (Amersham, Braunschweig, Germany) per well was added, and cell cultures were continued for an additional 20 h. The cells were harvested onto glass filters (UniFilterTM-96, GF/C; Packard, Dreieich, Germany), and the incorporated radioactivity was measured using TopCount Counter (Canberra Packard TFM, Dreieich, Germany). All assays were performed in triplicate. Statistical analysis was performed using Student's *t* test.

**Immunofluorescence and Flow Cytometry.** Surface antigen expression was analyzed by flow cytometry using standard protocols. Cells were preincubated with 1% BSA (fraction V; Roth, Karlsruhe, Germany) and 1% human IgG (Venimmun; Cention, Marburg, Germany) in PBS for 20 min. Direct immunofluorescence staining was performed using FITC-conjugated mAbs against CD20 (Becton Dickinson). sIgM expression was detected indirectly by an anti-IgM antibody (Becton Dickinson) and a FITC-conjugated goat antimouse IgG antibody (Jackson ImmunoResearch; Dianova). Cells were washed twice and resuspended in 1  $\times$  PBS containing 1% BSA and 2  $\mu$ g/ml propidium iodide (Sigma, Deisenhofen, Germany) for gating on viable cells. Immunofluorescence

was determined using a FACScan flow cytometer and CELLQuest software (Becton Dickinson). Quantification of surface antigen expression was performed by determining the MFI.

**Induction and Analysis of Apoptosis.** Anti-CD20-mediated apoptosis was induced by two different methods. First, mAb IDEC-C2B8 was immobilized by coating the antibody (20  $\mu$ g/ml PBS) on non-tissue culture-treated 6-well plates (Falcon; Becton Dickinson) overnight at 4°C. For induction of apoptosis, the cell lines were incubated in these pretreated plates. Second, cells were incubated with 10  $\mu$ g/ml anti-CD20 antibody B9E9 for 30 min; subsequently, anti-CD20 antibody was cross-linked by 20  $\mu$ g/ml goat antimouse (GAM) antibody F(ab')<sub>2</sub> fragments. Control reactions were performed using the murine IgG1 antibody HD20 and a human anti-EpCAM IgG1 antibody. BCR-mediated apoptosis was induced with 1.3  $\mu$ g/ml goat antihuman IgM F(ab')<sub>2</sub> fragments. After induction of apoptosis, cells were stained with acridine orange (5  $\mu$ g/ml; Sigma) and observed by fluorescence microscopy. The number of cells revealing characteristic features of apoptosis (fragmented or condensed nuclei) were enumerated, and the percentage was determined per total of 100 cells. All assays were performed in triplicate. Statistical analysis was performed using Student's *t* test.

**Measurement and Inhibition of Caspase Activity.** Caspase-3 activation was detected by Western blot analysis (see below). Caspase-3 family activity was measured using the ApoAlert CPP32 Assay Kit (Clontech, Heidelberg, Germany). After the CD20 antigen was triggered, 1  $\times$  10<sup>6</sup> cells were washed once in 1  $\times$  PBS and resuspended in 50  $\mu$ l of chilled cell lysis buffer. Thereafter, cells were incubated for 10 min on ice, followed by the addition of 50  $\mu$ l of 2 $\times$  reaction buffer containing the caspase substrate DEVD-p-NA to a final concentration of 50  $\mu$ M. On proteolytic cleavage of DEVD-p-NA by caspase activity, free p-NA was detected in a spectrophotometer at 400 nm.

Caspase activity was blocked using the cell-permeable irreversible caspase-3 family inhibitor z-DEVD-fmk (Calbiochem, Bad Soden, Germany) or the caspase-1 inhibitor ac-YVAD-cmk (Calbiochem). Samples were preincubated for 30 min using different concentrations of the caspase inhibitor. After this, apoptosis was induced, and cells were analyzed at 12 and 20 h as described above. Assays were performed in triplicate. Statistical analysis was performed using Student's *t* test.

**Protein Preparation and Immunoblotting.** Cells from different time points after the induction of apoptosis were washed once in 1  $\times$  PBS. Adherent cells were lysed directly on the cell culture plate. Cells were lysed for 30 min at 4°C [using lysis buffer containing 20 mM Tris-acetate (pH 7.4), 10 mM sodium glycerophosphate, 50 mM sodium fluoride, 5 mM Na PP<sub>i</sub>, 1% Triton X-100, 0.1 mM EDTA, 1 mM EGTA, and 0.2 mM PMSF] or for 5 min at 80°C [using lysis buffer containing 1% SDS, 10 mM Tris-HCL (pH 7.5), and 2 mM EDTA (pH 8.0)]. Samples were then centrifuged (14,000 rpm, 3 min). The amount of protein was measured by quantitative protein assay (Pierce, Rockford, IL). Protein samples (35  $\mu$ g/lane) were subjected to 10% or 12% SDS-PAGE and transferred onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). After Ponceau S staining, the filters were blocked [1% nonfat dry milk, 0.1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5)] and incubated with 1:1000 diluted primary antibodies [polyclonal anti-PARP (N-20), polyclonal anti-Ly-GDI (C-20), polyclonal anti-SP1 (PEP 2), polyclonal anti-Bcl-2 ( $\Delta$ C-21), polyclonal anti-Bcl-x<sub>L/S</sub> (S-18), polyclonal anti-Bad (N-20), and polyclonal anti-Bax (P19) (all from Santa Cruz, Heidelberg, Germany); polyclonal anti-caspase-3 (PharMingen, Hamburg, Germany); polyclonal anti-phospho-c-Jun (Ser<sup>63</sup>), polyclonal anti-phospho-p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>), and monoclonal anti-phospho-p44/p42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>; all from New England Biolabs, Beverly, MA)]. Thereafter, filters were incubated with horseradish peroxidase-conjugated secondary antibodies (dilution, 1:15000; Santa Cruz). Bands were visualized using the enhanced chemiluminescence system (Amersham).

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay.** Nuclear extracts were prepared as described previously (29). Briefly, 1  $\times$  10<sup>6</sup> cells were collected, washed with 10 ml of PBS, and pelleted by centrifugation. PBS was removed, and the cell pellet was resuspended in 400  $\mu$ l of cold buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF]. The cells were allowed to swell on ice for 15 min, after which 5  $\mu$ l of a 10% solution of NP40 (Boehringer, Mannheim, Germany) were added, and the tube was vortexed for 10 s. The homogenate was centrifuged for 30 s in a microfuge. The nuclear pellet was resuspended in 50  $\mu$ l of ice-cold buffer C [20 mM HEPES (pH 7.9), 0.4 M

NaCl, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF], and the tube was rocked at 4°C for 15 min. The nuclear extract was centrifuged for 5 min in a microfuge at 4°C, and the supernatant was frozen at -70°C. For electrophoretic mobility shift analysis, 5 µg of nuclear protein were incubated in 10 mM Tris (pH 7.5), 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM EDTA, 5% glycerol, 1 µg of BSA, and 1 µg of poly(dI-dC) with 20,000 cpm of radioactive <sup>32</sup>P-labeled probe for 15 min at room temperature, followed by separation of the DNA-protein complexes on 4% acrylamide gels. For super-shift analysis, 1 µg of antibody [c-Fos, c-Jun, and (c-Jun, JunB, and JunD)-specific antibody; all from Santa Cruz] was added to the nuclear extract and incubated for 15 min at 4°C before the addition of reaction buffer and labeled probe. The following double-stranded oligonucleotide was used as a probe: 5'-AGCTAGCATGAGTCAGACAC-3' and 5'-AGCTGTGTCTGACTCAT-GCT-3' (human Col-AP-1; Ref. 30). A double-stranded oligonucleotide with a mutated AP-1 consensus binding site served as a control.

**Northern Blot Analysis.** Total RNA was prepared using guanidinium isothiocyanate lysis and CsCl gradient centrifugation. Cells were lysed in a 4 M guanidinium solution [4 M guanidinium isothiocyanate, 20 mM sodium acetate (pH 5.2), 0.1 mM DTT, and 0.5% *N*-lauryl-sarcosine]. The viscosity of the solution was reduced by drawing the lysate 10 times through a 20-gauge needle. The cell lysate was layered on top of a 5.7 M CsCl cushion [5.7 M CsCl and 100 mM EDTA (pH 8.0)]. Thereafter, the tube was centrifuged for 18–22 h in an ultracentrifuge (Beckmann L-60; Beckmann, München, Germany) at 31,000 rpm (150,000 × *g*). After centrifugation, the supernatant was removed carefully, and the RNA pellet was dissolved, ethanol precipitated, and quantified spectrophotometrically. For Northern analysis, 10 µg of total RNA were subjected to gel electrophoresis on a 1.1% formaldehyde-1.2% agarose gel and transferred to a nylon membrane (Appligene, Heidelberg, Germany). After UV cross-linking, the membrane was prehybridized (ExpressHyb solution; Clontech) at 68°C for 30 min. The blots were hybridized with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled random prime-labeled DNA probes (c-myc, Berg36, and glyceraldehyde-3-phosphate dehydrogenase) overnight at 68°C. Membranes were washed for 40 min at room temperature in 2× SSC and 0.05% SDS and then washed for 40 min at 50°C in 0.5% SSC and 0.1% SDS.

## RESULTS

**Immobilized Anti-CD20 Antibody Inhibits Proliferation.** Anti-CD20 antibodies have been reported to inhibit proliferation (5). To determine the effect of the IDEC-C2B8 mAb on the proliferation of BL60-2 human Burkitt's lymphoma cells, extensive cross-linking of the CD20 surface molecules was achieved by immobilization of the antibody. Fig. 1A shows the time course of the antiproliferative effect of IDEC-C2B8. In comparison with the medium control and control antibodies, CD20-dependent inhibition of proliferation could be detected as soon as 2 h after incubation. Maximal inhibition (80% relative to the medium control;  $P < 0.00008$ ) was reached after 10–12 h of incubation. This contrasts with the soluble antibody (5–25 µg/ml), which showed only a slight decrease of proliferation (data not shown).

**Immobilized Anti-CD20 Antibody Induces Apoptosis.** Acridine orange staining of BL60-2 cells cultured on immobilized IDEC-C2B8 showed that the strong inhibition of proliferation was due to the induction of apoptosis (Fig. 1B). After 24 h, more than 70% of the cells demonstrated fragmented nuclei (IDEC-C2B8 versus HD20,  $P < 0.00002$ ). Similar results were generated by cross-linking of the mouse anti-CD20 antibody B9E9 on BL41-s cells using goat anti-mouse F(ab')<sub>2</sub> fragments and using immobilized mouse monoclonal anti-CD20 antibody B9E9 (data not shown). Again, only a weak increase with 15–20% apoptotic cells cultured in the presence of soluble anti-CD20 mAb (10 µg/ml) was detectable; the control antibodies HD20 and human anti-EpCAM IgG1 had no effect (data not shown). Furthermore, as a model for follicular lymphoma, the two t(14;18)-positive lymphoma cell lines, SU-DHL-4 and K422, were investigated. Both cell lines were sensitive to soluble and extensively cross-linked anti-CD20 antibody, with 20–35% apoptotic cells after

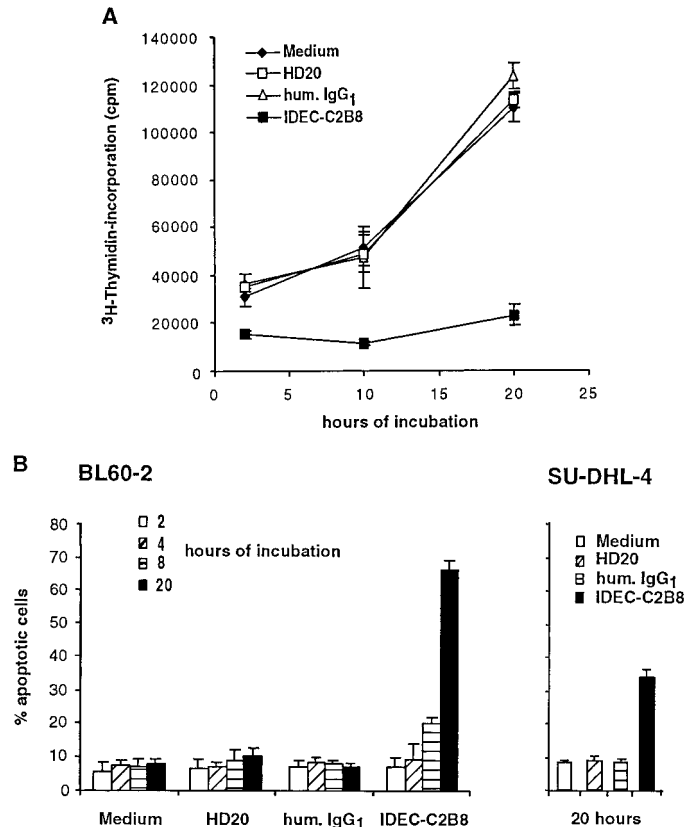


Fig. 1. Inhibition of proliferation and induction of apoptosis by anti-CD20 mAb IDEC-C2B8. A, BL60-2 cells were cultured in microtiter plates coated with 20 µg/ml IDEC-C2B8 (■). As a control, BL60-2 cells were incubated on uncoated plates (◆), and the plates were coated with the murine IgG1 antibody HD20 (□) and a human anti-EpCAM IgG1 antibody (△; *hum. IgG1*; all 20 µg/ml). After 2, 10, and 20 h, 1 µCi of [<sup>3</sup>H]thymidine was added per well, and the culture was continued for an additional 20 h. Results are the mean of triplicate measurements; error bars denote SDs. B, left panel, BL60-2 cells were incubated for 2 (□), 4 (▨), 8 (▩), and 20 (■) h in multiwell plates coated with IDEC-C2B8, mAb HD20, a human anti-EpCAM IgG1 antibody (*hum. IgG1*; all 20 µg/ml), or PBS alone (*Medium*). B, right panel, the t(14;18)-positive cell line SU-DHL-4 was incubated for 20 h in multiwell plates coated with IDEC-C2B8 (■), mAb HD20 (▨), a human anti-EpCAM IgG1 antibody (▩, *hum. IgG1*; all 20 µg/ml), or PBS (□) alone. At the indicated times, the percentage of apoptotic cells was determined by fluorescence microscopy after acridine orange staining. Results are the mean of triplicate measurements; error bars denote SDs.

incubation on immobilized IDEC-C2B8 for 20 h (Fig. 1B; IDEC-C2B8 versus HD20,  $P < 0.0003$ ; data not shown).

**Increase of the Bcl-2 Gene Family Member Bax during Anti-CD20-mediated Apoptosis.** Recently, it was reported that extensively cross-linked murine anti-CD20 mAbs are able to induce B-cell apoptosis (15). However, the mechanisms leading to apoptosis by these antibodies are not known. Because members of the Bcl-2 family have been shown to be involved in different processes of B-cell apoptosis, we studied the protein expression of the proapoptotic members Bax and Bad and the antiapoptotic members Bcl-2 and Bcl-x<sub>L</sub> after induction of anti-CD20-mediated apoptosis. The investigated BL60-2 cells demonstrated significant Bax up-regulation after 2 h of stimulation, followed by a decrease in protein expression level after 6–8 h (Fig. 2A). No significant changes of the other Bcl-2 family members investigated were observed.

**Proteolytic Cleavage of PARP and SP1.** The execution of the apoptotic pathway requires caspase activity. An indicator for the involvement of distinct caspases is the proteolytic cleavage of defined target proteins (19). To show this, we studied protein expression of the caspase target proteins SP1, which is cleaved into a  $M_r$  68,000 and  $M_r$  45,000 fragment on sIgM-mediated B-cell apoptosis (22), and PARP

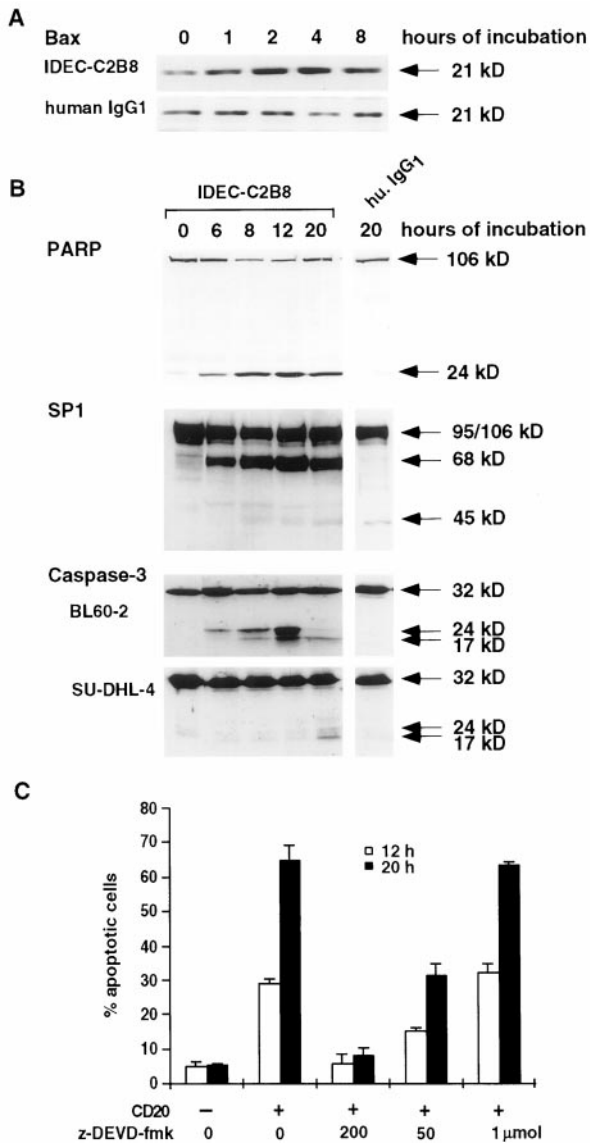


Fig. 2. Effects of CD20 triggering on Bax expression and caspase activity. *A*, up-regulation of Bax expression on extensive CD20 cross-linking. BL60-2 cells were cultured in anti-CD20 antibody (20  $\mu\text{g}/\text{ml}$ ) or human anti-EpCAM IgG1 antibody (human IgG1; 20  $\mu\text{g}/\text{ml}$ ) coated multiwell plates. At the indicated times, the cells were harvested, whole cell lysates were prepared and electrophoretically separated (35  $\mu\text{g}/\text{lane}$ ), and Western blot analysis was performed using the indicated antibody and horseradish peroxidase-conjugated secondary antibody. *B*, time course of PARP, SP1 (shown for BL60-2), and caspase-3 (shown for BL60-2 and SU-DHL-4) cleavage during anti-CD20-mediated apoptosis. The experimental procedure is described above (*A*). Arrows, full-length proteins and cleavage products (see text). *C*, dose-dependent inhibition of anti-CD20-mediated apoptosis by z-DEVD-fmk, a specific inhibitor of the caspase-3 family. BL60-2 cells were preincubated with various concentrations of z-DEVD-fmk as indicated in anti-CD20 antibody-coated multiwell plates. After 12 ( $\square$ ) and 20 ( $\blacksquare$ ) h of incubation, the number of fragmented or condensed nuclei per 100 cells was determined by fluorescence microscopy after acridine orange staining. Error bars denote SDs.

(Fig. 2*B*). The detection of the larger SP1 cleavage fragment of  $M_r$  68,000 was possible as early as 4–6 h after induction of apoptosis. Six h after CD20 triggering by immobilized IDEC-C2B8, PARP degradation was detectable. Fig. 2*B* shows the cleavage of PARP using an antibody recognizing the  $M_r$  24,000 cleavage product. No cleavage products were detectable after incubation of the cells with the control antibodies.

**CD20-induced Apoptosis Activates Caspase-3.** The results mentioned above indicated the involvement of caspase-3 family in anti-CD20-induced apoptosis. Indeed, activation of caspase-3 was detectable in the analyzed cell lines BL60-2 and SU-DHL-4 by Western blot

analysis after extensive cross-linking of IDEC-C2B8. Cleavage of the  $M_r$  32,000 procaspase-3 generated the p24 intermediate autocatalytically cleaved to active caspase-3 (Fig. 2*B*). Measurement of caspase-3 family activity by analysis of cleavage of the caspase-substrate DEVD-p-NA underlined the observed time course of activation (data not shown). Furthermore, inhibition of caspase-3 by the cell-permeable irreversible caspase-3 family inhibitor z-DEVD-fmk inhibited apoptosis in a dose-dependent manner, with almost complete inhibition at 200  $\mu\text{M}$  (Fig. 2*C*;  $P < 0.0001$ ). In contrast to these results, the interleukin 1 $\beta$ -converting enzyme inhibitor ac-YVAD-fmk did not block apoptosis (data not shown), suggesting that caspase-1 is not involved in anti-CD20-mediated apoptosis.

**Correlation between Anti-CD20- and BCR-mediated Apoptosis.** In all experiments studying CD20-dependent cell death in Burkitt's lymphoma cell lines, we used BCR-mediated apoptosis as a positive control for apoptosis sensitivity of the investigated cell lines. Surprisingly, we observed a strict correlation between anti-CD20- and BCR-mediated apoptosis in the investigated Burkitt's lymphoma cell lines. This led us to perform experiments comparing BCR- and CD20-mediated induction of apoptosis. CD20-dependent apoptosis occurred in the BCR-sensitive Burkitt's lymphoma cell lines BL60-2 and BL41-s, but not in the resistant variant BL41-r. Cells resistant to BCR-mediated apoptosis were clearly resistant to CD20-mediated apoptosis. There was no correlation between antigen expression level and sensitivity or resistance to the apoptotic stimuli. The results are summarized in Table 1; the number of apoptotic cells after anti-CD20- and anti-BCR-mediated apoptosis and the MFI of CD20 and IgM expression are indicated.

**Similar Changes of c-myc and Berg36 RNA Expression Level after CD20 and BCR Cross-linking.** Due to the fact that most of the molecular events described above occur during the final execution phase of apoptosis, we compared changes of the ERGs c-myc and Berg36 after anti-CD20- and BCR-mediated apoptosis. c-myc has been shown to be down-regulated after BCR-mediated apoptosis (31), and Berg36 has been shown to be up-regulated during B-cell apoptosis induced by calcium signaling (32). Shortly after the induction of both signaling events, the expression level of c-myc mRNA decreased, whereas the expression level of Berg36 mRNA increased in BL60-2 cells (Fig. 3, *A* and *B*). In contrast to these changes observed after extensive cross-linking of CD20 antigen, the c-myc expression level remained nearly unaltered, and Berg36 mRNA showed only a weak increase in the presence of soluble IDEC-C2B8 (10  $\mu\text{g}/\text{ml}$ ; Fig. 3*C*). These data support our observation of a correlation between BCR- and anti-CD20-mediated apoptosis in sIgM-positive cell lines.

Table 1 Correlation between anti-CD20- and BCR-mediated apoptosis

The indicated cell lines (BL60-2, sIgM-sensitive subclone of BL60; BL41-s, sIgM-sensitive subclone, BL41-r, sIgM-resistant subclone of BL41) were incubated in the presence of immobilized IDEC-C2B8 or soluble anti-sIgM F(ab')<sub>2</sub> fragments. After 20 h, cells were stained with acridine orange, and the number of apoptotic cells per 100 cells was determined by fluorescence microscopy. Cell surface antigen expression was determined by immunofluorescence staining. The MFI of CD20 or sIgM staining is shown.

Cell line	Antigen expression (MFI)		Apoptosis sensitivity	
	CD20	IgM	Anti-CD20	Anti-IgM
BL60.2	265.2	11.2	++++	++++
BL41-s	435.7	39.2	++	++
BL41-r	114.1	22.2	–	–
Daudi	90.4	29.2	–	–
BJAB	309.4	12.4	–	–
++++	>40%	Apoptotic cells		
+++	30–40%			
++	20–30%			
+	10–20%			
–	0–10%			

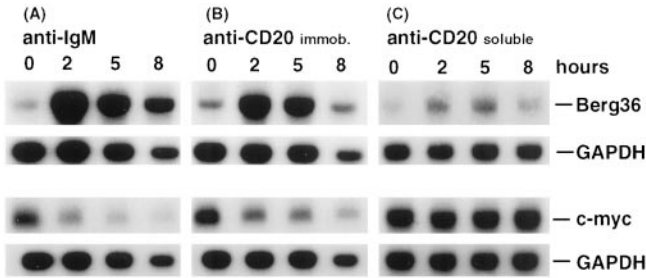


Fig. 3. Effects of CD20 and sIgM triggering on mRNA expression of c-myc and Berg36. BL60-2 cells were cultured in the presence of soluble (10  $\mu$ g/ml) or immobilized IDEC-C2B8 (20  $\mu$ g/ml) or soluble anti-sIgM F(ab')<sub>2</sub> fragments (1.3  $\mu$ g/ml). At the indicated times, total RNA was prepared, and Northern blot analysis of total RNA (10  $\mu$ g/lane) was performed. Blots were hybridized with c-myc-, Berg36-, and glyceraldehyde-3-phosphate dehydrogenase-specific radioactive labeled DNA probes. BL60-2 cells were incubated in the presence of (A) soluble anti-sIgM F(ab')<sub>2</sub> fragments, (B) immobilized (anti-CD20 immob.) anti-CD20 antibody, and (C) soluble anti-CD20 antibody. The decrease of c-myc-expression and the strong increase of Berg36 expression after incubation of BL60-2 with anti-IgM F(ab')<sub>2</sub> fragments or immobilized IDEC-C2B8 are shown.

**Induction of p44/p42 MAPK Activity and AP-1 DNA Binding Activity.**

Signaling events after CD20 ligation downstream from activation of PLC- $\gamma$  are not known. In contrast, BCR activation is known to induce functional relevant PLC- $\gamma$ -dependent p44/p42 MAPK-activation (25) and increased AP-1 activity (33). Therefore, we focused on comparison of MAPK and AP-1 activation after CD20 and BCR triggering. After culturing BL60-2 cells on immobilized IDEC-C2B8, a strong induction of p44/p42 phosphorylation, which correlates with induction of activity, was detectable using an anti-phospho-p44/p42 MAPK mAb. Comparable with p44/p42 MAPK induction after BCR ligation, the phosphorylation of p42 (ERK2) was more pronounced, as described previously for BCR-mediated apoptosis (Fig. 4A; Ref. 25). Furthermore, soluble anti-CD20 antibody also showed a weak induction of p44/p42 phosphorylation. Modulation of p44/p42 activity might therefore not only be induced by extensive CD20 cross-linking. The cell lines BJAB and Daudi, which are both resistant to anti-CD20- and BCR-mediated apoptosis, showed

different levels of basal phosphorylated p44/p42 MAPK that remained unaltered on extensive CD20 or BCR cross-linking (Fig. 4A). Because no changes in c-jun or p38 MAPK phosphorylation were detected by use of phospho-specific antibodies (data not shown), these MAPKs might not be involved in the signaling events leading to the induction of apoptosis after CD20 or sIgM triggering. In addition, the sIgM-negative t(14;18)-positive cell line SU-DHL-4 also revealed p44/42 activation following CD20 triggering, suggesting that p44/42 activity can be regulated by CD20 in the absence of the sIgM (Fig. 4A).

Because one of the functional consequences of ERK activation is activation of AP-1, the DNA binding activity of AP-1 was measured in BL60-2 cells by electrophoretic mobility shift assays with nuclear protein extracts after CD20 or BCR triggering. After extensive cross-linking of CD20 as well as BCR ligation, a rapid increase of AP-1 DNA binding activity was detected using the AP-1 consensus binding sequence (Fig. 4B). This increase was more pronounced after BCR triggering. A supershift analysis revealed predominantly fos and, to a weaker extent, junB and junD but almost no c-jun as components of the AP-1 complex (Fig. 4C). Again, soluble anti-CD20 antibody was also able to induce AP-1 DNA binding activity, although to a much lesser degree in comparison with the extensively cross-linked antibody (Fig. 4B).

**DISCUSSION**

Promising clinical results were obtained in the treatment of indolent lymphoma using the chimeric anti-CD20 antibody IDEC-C2B8 (13). The rapid B-cell depletion in nearly all treated patients might be a result of CDC and ADCC, which were both shown for IDEC-C2B8 *in vitro* (12). However, there is no evidence for CDC or ADCC being the main effector mechanisms *in vivo* (11, 34).

It was reported that mAbs directed against B-cell surface receptors with strong signaling activity such as CD19 were the most effective antibodies in an *in vivo* B-cell lymphoma therapy model (34). These results suggested that the signaling activity of the antigen might be responsible for lymphoma inhibition rather than effector cell recruit-

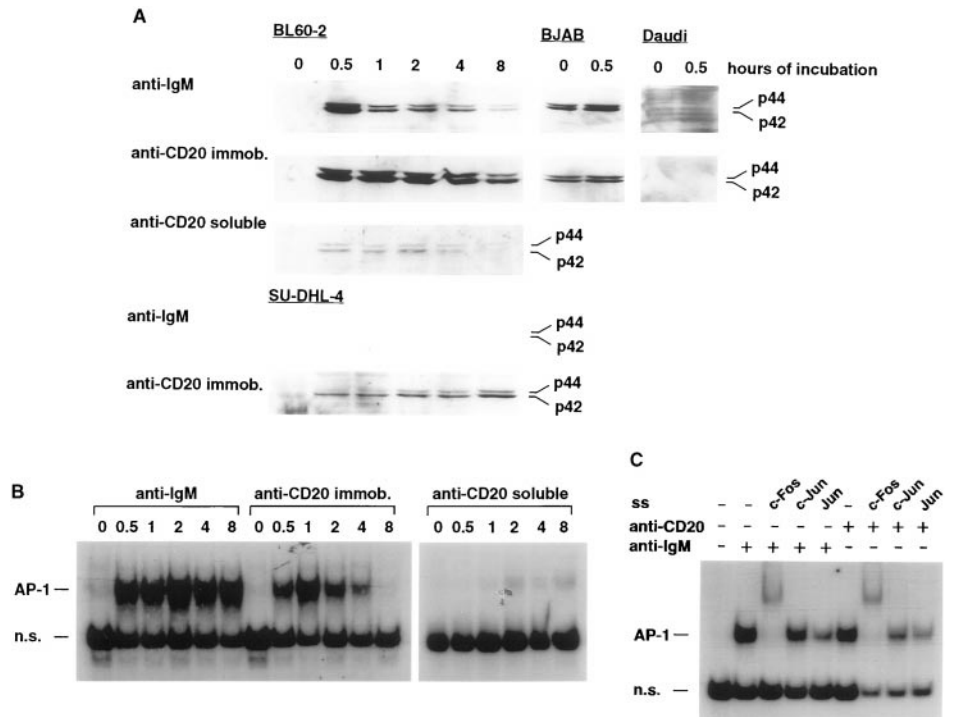


Fig. 4. Induction of p44/p42 MAPK and AP-1 DNA binding activity. A, induction of p44/p42 MAPK activity. BL60-2, BJAB, Daudi, and SU-DHL-4 cells were cultured in the presence of immobilized (20  $\mu$ g/ml) or soluble (10  $\mu$ g/ml) IDEC-C2B8 or anti-sIgM F(ab')<sub>2</sub> fragments (1.3  $\mu$ g/ml). At the indicated times, the cells were harvested, whole cell lysates were prepared and electrophoretically separated (35  $\mu$ g/lane), and Western blot analysis was performed using a phospho-specific anti-p44/p42 MAPK antibody. B, induction of DNA binding activity of AP-1. Nuclear extracts were prepared at the indicated times from BL60-2 cells cultured in the presence of immobilized (20  $\mu$ g/ml) or soluble IDEC-C2B8 (10  $\mu$ g/ml) or anti-sIgM F(ab')<sub>2</sub> fragments (1.3  $\mu$ g/ml) and analyzed by electrophoretic mobility shift assay. Extracts were incubated with radioactive labeled probes containing the AP-1 consensus binding sites. The position of the AP-1-DNA complex is indicated. For supershift analysis (C), nuclear extracts prepared 30 min after incubation with immobilized IDEC-C2B8 or anti-sIgM F(ab')<sub>2</sub> fragments were preincubated with specific antibodies for c-Fos (ss, c-Fos), c-Jun (ss, c-Jun), or c-Jun, JunB, and JunD (ss, Jun). The same amount of protein (4  $\mu$ g) was loaded onto each lane.

ment. Indeed, antiproliferative effects were also reported for IDEC-C2B8 (35). In contrast, recently published data show the importance of Fc $\gamma$ R-positive cells for antibody-mediated antitumor activity (36). The mechanisms of antitumor activity of IDEC-C2B8 are not clear; therefore, it was our aim to study effects of anti-CD20 mAb IDEC-C2B8 on human lymphoma cell lines.

Immobilized or extensively cross-linked anti-CD20 antibody IDEC-C2B8 resulted in potent induction of apoptosis in both of the B-cell lymphoma cell line models investigated. Thus induction of apoptosis was observed in the t(14;18)-positive lymphoma cell lines SU-DHL-4 and K422 as well as in the Burkitt's lymphoma cell lines BL60-2 and BL41 (Fig. 1B). In contrast, soluble antibody led only to a weak induction of apoptosis. *In vivo* cross-linking of anti-CD20 mAb might be achieved through Fc $\gamma$ R-positive cells (15). It has been demonstrated recently that BALB/c nude mice bearing a xenotransplanted human Burkitt's lymphoma could be cured by IDEC-C2B8 application, whereas nearly no tumor regression could be observed in FcR $\gamma$ -/- *nu/nu* mice (36). Our findings might reflect this observation. In our experimental system, extensive cross-linking of the antibody is leading to strong induction of apoptosis in the absence of effector cells. Because extensive antibody cross-linking is shown to enforce the signaling activity of the recognized antigen (see above, compare soluble and extensively cross-linked antibody; Ref. 37), this mechanism might occur in the presence of Fc $\gamma$ R-positive cells through Fc-Fc $\gamma$ R interaction in addition to effector cell-related mechanisms.

Postulating that induction of apoptosis contributes to the prolonged remission of lymphomas after treatment with IDEC-C2B8, we investigated key regulators of programmed cell death. Rapid induction of apoptosis could be observed after extensive CD20 cross-linking with a significant increase in Bax protein expression (Fig. 2A). Furthermore, CD20-dependent apoptosis involved activation of caspase-3 activity, resulting in cleavage of the caspase-3 family target proteins PARP and SP1 (22, 38), and could be completely abrogated by inhibitors of caspase 3 (Fig. 2, B and C).

During BCR-triggered apoptosis, constitutively expressed Bcl-2 and Bcl-x<sub>L</sub> protein levels remained unaltered, whereas a strong induction of Bax- $\alpha$  was detectable in the human Burkitt's lymphoma cell line BL41 (39). Furthermore, overexpression of Bax- $\alpha$  sensitized BL41 cells to BCR-mediated apoptosis (21). A similar pattern of Bcl-2 family gene expression is described in this work for anti-CD20-mediated apoptosis. This suggests that, as in BCR-mediated apoptosis, up-regulation of Bax might be an early key event in CD20-mediated apoptosis.

Dysregulated expression of Bcl-2 gene family members has been reported in different malignancies and is believed to contribute to the pathogenesis of these tumors and to influence the response to treatment (40–42). It is therefore conceivable that dysregulated Bcl-2 family protein expression in lymphomas (43), resulting in the inability to undergo apoptosis on certain stimuli, might contribute to resistance to IDEC-C2B8. The slower onset of apoptosis induction in the t(14;18)-positive cell lines compared with the Burkitt's lymphoma cell lines could be explained by the high level of bcl-2 expression detectable in t(14;18)-positive cell lines.

One of the most interesting findings in our study is the observation that there is a tight correlation between anti-CD20- and BCR-mediated apoptosis (Table 1) with regard to the sensitivity of the Burkitt's lymphoma cell lines studied. Both t(14;18)-positive lymphoma cell lines, which showed no sIgM expression, were sensitive to anti-CD20-mediated apoptosis. Among the Burkitt's lymphoma cell lines, all of which showed sIgM expression, only cell lines sensitive to BCR-mediated apoptosis were sensitive to anti-CD20-mediated apoptosis. Recently, it has been suggested that PTKs are involved in

anti-CD20-mediated apoptosis (15). CD20 is associated with the Src family PTKs Lyn, Fyn, and Lck (6, 44), which are also activated after BCR activation (45). Furthermore, CD20 signaling appeared to involve, in part, phosphorylation substrates similar to those observed after sIgM stimulation (7).

These data and the observation of induction of anti-CD20-mediated apoptosis in Burkitt's lymphoma cells susceptible to sIgM-mediated apoptosis could be interpreted as the result of similar signaling events after both stimuli. To support this hypothesis, we compared the changes of two ERGs, c-myc and Berg36, in Northern blot analysis (Fig. 3). Down-regulation of c-myc has been shown to be critically involved in the process of BCR-mediated apoptosis (31), and an increase of Berg36 RNA has been shown to occur after calcium ionophore-induced apoptosis in Burkitt's lymphoma cell lines (32). Similar kinetics were seen after sIgM induction and extensive CD20 cross-linking with a strong induction of Berg36 and a decrease of c-myc. The down-regulation of c-myc RNA does not differ from previously published results showing c-myc mRNA induction after anti-CD20 stimulation (46) because these data were generated using antibody 1F5, which is, in contrast to all known anti-B1 anti-CD20 antibodies (including IDEC-C2B8), able to activate B cells (3, 4).

Furthermore, BCR- and CD20-mediated signaling was able to induce activation of the MAPKs p44/p42 (Fig. 4). MAPK p44/p42 activity was shown to mediate BCR-induced apoptosis in WEHI 231 B cells (25). Activation of the p44/p42 MAPKs by the BCR is dependent on activation of PLC- $\gamma$ 2 (47). CD20- and BCR-initiated signaling activate PLC- $\gamma$ 1 and PLC- $\gamma$ 2 (7, 48), which might explain the similar pattern of MAPK activation. Because activation of PLC- $\gamma$ 2 after BCR signaling requires Syk activity (49), and this PTK is not directly associated with the CD20 molecule (6), anti-CD20-mediated PLC- $\gamma$ 2 activation might differ from that observed after BCR signaling. Furthermore, it was shown that BCR-mediated ERK activation involves the RAS/Raf-1/MAPK/ERK kinase signaling pathway (50, 51). It will be important to determine the function of this signaling pathway after CD20 ligation. The similar composition of the induced AP-1 complex after CD20 and BCR ligation (Fig. 4, B and C) underlines the similarity of the signaling pathways after extensive CD20 and BCR ligation. As described previously for BCR-mediated apoptosis, this complex is predominantly composed of c-fos (33). Nevertheless, in the more mature t(14;18)-positive B-cell lymphoma cell lines, anti-CD20-mediated apoptosis occurs independent of the presence of sIgM-associated signaling events. Possibly, only a subgroup of the more immature lymphoma cells is sensitive to anti-CD20-mediated apoptosis, whereas sensitivity in the more mature t(14;18)-positive B-cell lymphoma cells is a more general phenomenon.

We propose that IDEC-C2B8 treatment failure might thus be explained not only by lack or modulation of CD20 antigen expression but also by dysregulated signaling and execution pathways of apoptosis. The involvement of p44/p42 MAPK and AP-1 in CD20 signaling may contribute to new insights in the signaling cascade after CD20 ligation, and it will be interesting to further investigate a functional relationship between CD20- and BCR-mediated signaling.

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