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ORIGINAL ARTICLE

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Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells

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Abstract Anti-CD20 monoclonal antibodies have been successfully employed in the clinical treatment of non-Hodgkin's lymphomas in both unmodified and radiolabeled forms. Previous publications have demonstrated that the antitumor effects of unmodified anti-CD20 mAb are mediated by several mechanisms including antibody-dependent cellular cytotoxicity, complement-mediated cell lysis, and induction of apoptosis by CD20 cross-linking. In this report, we demonstrate induction of apoptosis by three anti-CD20 monoclonal antibodies [1F5, anti-B1, and C2B8 (Rituximab)]. The magnitude of apoptosis induction was greater with the chimeric Rituximab antibody than with the murine 1F5 and anti-B1 antibodies. Apoptosis could be enhanced with any of the antibodies by cross-linking with secondary antibodies (or Fc-receptor-bearing accessory cells). The signaling events involved in anti-CD20-induced apoptosis were investigated, including activation of protein tyrosine kinases, increases in intracellular Ca^{2+} concentrations, caspase activation, and cleavage of caspase substrates. Our results indicate that anti-CD20-induced apoptosis can be attenuated by PP1, an inhibitor of protein tyrosine kinases Lck and Fyn, chelators of extracellular or intracellular Ca^{2+}, and inhibitors of caspases, suggesting that anti-CD20-induced apoptosis may involve modulation of these signaling molecules. We also demonstrated that varying the expression of Bcl-2 did not affect the magnitude of anti-B1-induced apoptosis, possibly because of the sequestering effects of other Bcl-2 family members, such as Bad. These studies identify several of the signal-transduction events involved in the apoptosis of malignant B cells that transpire following ligation of CD20 by anti-CD20 antibodies in the presence of Fc-receptor-expressing cells or secondary goat anti-(mouse Ig) antibodies and which may contribute to the tumor regressions observed in mouse models and clinical trials.

Key words CD20 · Apoptosis · Mechanisms · Lymphomas · Immunotherapy

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Introduction

Ligation and cross-linking of many cell-surface receptors with monoclonal antibodies initiates signaling pathways that induce cell "suicide" programs and eventually lead to cell death. Cell-surface-receptor-mediated mechanisms that control apoptosis often act through a signal-transduction system that involves the stimulation of a receptor, the activation of protein kinase/phosphatase cascades, and the release of second messengers to up-regulate or suppress the transcription of specific genes.

Protein kinases and phosphatases that have been reported to be associated with receptor-mediated apoptosis include protein tyrosine kinases (PTK) Lyn, Lck, Blk, Abl, Btk; Jak kinases; PI-3 kinase; serine/threonine kinase p34cdc2 and protein tyrosine phosphatase 1C [4, 21, 55, 56, 59, 61–63]. Specific examples of PTK implicated in receptor-induced apoptosis include Lck in

T-cell-receptor-mediated apoptosis of T cells and Btk in ionizing-radiation-induced apoptosis of B cells [56, 59]. In contrast, activation of other PTK transduces cellular resistance to apoptosis, such as the suppression of apoptosis induced by growth-factor withdrawal and by chemotherapeutic agents in cells in which Abl is overexpressed [21].

Many surface receptors are coupled to Ca^{2+} signaling pathways by PTK, which activate phospholipase Cγ (PLCγ). Activated PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate producing inositol trisphosphate and diacylglycerol. Inositol trisphosphate promotes the release of Ca^{2+} from the endoplasmic reticulum (ER), and diacylglycerol activates protein kinase C [25]. Calcium has been strongly implicated as a second message in the induction of apoptosis in many model systems [19, 37, 38]. Possible targets impacted by intracellular Ca^{2+} elevations include downstream protein kinases and phosphatases, proteases, and endonucleases. The possible consequences of the activation of these targets include up-regulation or suppression of the transcription of specific genes, particularly genes that control the expression of Fas, Fas ligand, and related molecules [19, 37, 39].

Fas (CD95, APO-1) is a 48-kDa cell-surface antigen of the tumor necrosis factor/nerve growth factor receptor superfamily that can mediate apoptosis of a variety of cell types, including lymphocytes [41, 58]. Apoptosis induced by cross-linking cell surface receptors in a variety of cell types (e.g. the T cell receptor in T lymphocytes) has been shown to involve Fas-Fas-ligand (FasL) interactions [16, 27]. Cross-linking the T cell receptor (TCR) results in transient up-regulation of FasL in T lymphoma cells, and apoptosis induced by TCR cross-linking is markedly inhibited by either anti-Fas $F(ab')_2$ fragments (which are not cytotoxic) or soluble Fas-Fc [7, 18, 28]. These results indicate that, in T lymphoma cells, apoptosis induced by TCR activation results from the induction of FasL and its interaction with Fas, resulting in the activation of the Fas signaling pathway. Extensive studies of the Fas signaling pathway have revealed the crucial role of a cascade of proteases in Fas-mediated apoptosis, as initially described in studies of programmed cell death in the nematode, *C. elegans* [20]. Genetic analysis of apoptosis in *C. elegans* has revealed a number of gene products that regulate the cell death process including the CED-3 product, which is absolutely required for apoptosis in the nematode [20]. Molecular cloning of the *ced-3* gene revealed it to be a homologue of mammalian interleukin-1β-converting enzyme (ICE) [64]. Cross-hybridization with ICE cDNA and a search of the human genome database revealed at least 13 ICE homologues, which are divided into three subgroups (ICE-like, CPP32-like, and Ich1-like proteases), on the basis of their sequence homologies [1]. All of these cause apoptosis when overexpressed in cells. They appear to be cysteine proteases, containing conserved sequences for substrate binding and catalysis, which cleave their substrates at residues following aspartic acid and are therefore now designated as caspases (cysteine aspases).

Caspase activation and apoptosis appear to be modulated in many systems by expression of a protein known as Bcl-2. The Bcl-2 protein family contains at least 15 different members, which may be segregated into two categories: inhibitors of apoptosis (e.g. Bcl-2) and accelerators of apoptosis (e.g. Bax) [50]. The functional relationship between the Bcl-2 family of apoptotic modulators and the ICE family of proteases has not been completely resolved, though it appears that Bcl-2 functions upstream of the terminal caspase, caspase-3, since overexpression of Bcl-2 in Jurkat cells inhibited staurosporine-induced apoptosis and abrogated activation of caspase-3 [11]. However, Bcl-2 does not inhibit apoptosis mediated by all pathways (e.g. Fas-mediated apoptosis in Jurkat cells) [49].

The goal of this study was to elucidate the intracellular signaling pathways involved in transducing CD20 cross-linking with anti-CD20 antibodies to apoptosis of B lymphoma cells, a phenomenon that has recently been reported by us as well as other groups [17, 35, 54]. CD20 is a non-glycosylated 33 to 37-kDa phosphoprotein expressed on more than 95% of normal and neoplastic B cells. Current studies suggest that CD20 is a B cell surface protein with the capacity to serve as a calcium channel, initiate intracellular signals, and modulate cell growth and differentiation [57]. Ligation of CD20 with anti-CD20 mAb has been shown to activate serine/threonine and tyrosine protein kinases that are non-covalently associated with CD20, thereby inducing tyrosine phosphorylation of PLCγ. In addition, hyper-crosslinking CD20 with anti-CD20 monoclonal antibodies (mAb) plus a secondary goat anti-(mouse IgG) (GAM) antibody has been shown to mobilize calcium from intracellular stores [14, 15]. Previously we demonstrated that cross-linking CD20 with anti-CD20 mAb plus GAM- or Fc-receptor-bearing cells induced B cell death by apoptosis [54]. The data presented here indicate that a series of signaling events including protein tyrosine kinases, intracellular calcium and caspases are involved in this anti-CD20-induced apoptosis.

Materials and methods

Cells

The CD20-expressing human Burkitt's lymphoma cell line, Ramos, was obtained from the American Type Culture Collection (ATCC; Bethesda, Md.). The Ramos AW cell line was a kind gift from Dr. George Klein (Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden). The B lymphoma cell line SU-DHL4 and the T lymphoma cell line SupT-13 were from Dr. David Maloney and Dr. Claudio Anasetti respectively (Fred Hutchinson Cancer Research Center, Seattle). All cell lines were maintained in log-phase growth in RPMI-1640 medium supplemented with 12% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Antibodies and reagents

The anti-CD20 mAb 1F5 (IgG2a), the control anti-CD3 mAb 64.1 (IgG2a), and an anti-CD40 mAb (G28.5) were produced and purified as previously described [5, 12, 44, 45]. The anti-CD20 mAb B1 (IgG2a) was a gift from Dr. George Tidmarsh (Coulter Pharmaceuticals, Palo Alto, Calif.). The chimeric anti-CD20 mAb C2B8 (Rituximab) was purchased from Genentech (San Francisco, Calif.). The anti-CD3 mAb BC3 was from Dr. Claudio Anasetti. Other antibodies were purchased from the following commercial sources: $F(ab')_2$ fragments of a goat anti-(human cell surface IgM) Ab (anti-sIgM) from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pa.), $F(ab')_2$ fragments of a goat anti-(mouse IgG) (GAM) Ab and $F(ab')_2$ fragments of a goat anti-(human IgG) (GAH) from Pierce Chemical Co. (Rockford, Ill.), the anti-phosphotyrosine mAb 4G10 and a rabbit anti-[poly(ADP-ribose) polymerase] (PARP) antibody from Upstate Biotechnology (Lake Placid, N.Y.), the anti-caspase-3 and the anti-Bad mAb from Transduction Laboratories (Lexington, Ky.), a rabbit anti-Bcl-Xs antibody from Calbiochem (San Diego, Calif.), the anti-Bcl-2 mAb from Boehringer Mannheim (Indianapolis, Ind.), a fluorescein-isothiocyanate(FITC)-labeled anti-Fas mAb from Immunotech (Westbrook, Me.), and the anti-FasL mAb from PharMingen (San Diego, Calif.). Other reagents were obtained from the following sources: benzyloxycarbonyl-valyl-alanyl-aspartyl (*O*-methyl)-fluoromethylketone (zVAD-fmk) from Enzyme Systems Products, Inc. (Dublin, Calif.); herbimycin A, genistein, PP1, and ionomycin from Calbiochem; [ethylenebis(oxonitrilo)] tetraacetic acid (EGTA) from Sigma Chemical Co. (St. Louis, Mo.); and Bapta AM and Indo-1 from Molecular Probes (Eugene, Ore.).

Measurement of the cytoplasmic calcium concentration by Indo-1 loading

Calcium responses were measured using Indo-1 and a FACScan flow cytometer (FACScan; Becton Dickinson, San Jose, Calif.) as described [48]. Briefly, Ramos cells (5×10^6) were loaded with Indo-1 by incubation with its acetoxymethyl ester (3 μM) for 30 min at 37 °C in 2 ml complete RPMI-1640 medium. Cells were then washed and resuspended in fresh medium at a density of 10^6 cells/ml, stimulated with antibodies, and analyzed by flow cytometry.

Flow-cytometric analysis of apoptosis using FITC-labeled Annexin-V staining

Ramos cells were cultured with 10 μg/ml anti-CD20 mAb in the presence and absence of 50 μg/ml secondary goat anti-(mouse Ig) or goat anti-(human Ig) Ab and control Ab at 5×10^5 cells ml^{-1} $well^{-1}$ for 48 h. Apoptosis was assessed by staining with FITC-labeled Annexin-V and propidium iodide and performing flow-cytometric analysis (Becton Dickinson FACScan, Becton Dickinson, Mountain View, Calif.) according to the instruction manual from Becton Dicknson. Positive staining with FITC-labeled Annexin-V reflects a shift of phosphatidylserine from the inner to the outer layer of the cytoplasmic membrane, which occurs early in apoptosis. Annexin-V-positive and propidium-iodide-negative cells were scored as apoptotic cells.

Flow-cytometric analysis of apoptosis using propidium iodide staining

Flow-cytometric analysis of cellular DNA was performed following propidium iodide staining according to the method of Fried et al. [22]. Briefly, 10^6 Ramos cells were incubated with anti-CD20 mAb or control Ab in the presence or the absence of GAM, washed in PBS and then gently resuspended in 0.5 ml hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100, all from Sigma Chemical Co., St. Louis, Mo.). Samples were stored in the dark at 4 °C until flow-cytometric analysis of individual nuclei could be performed in a FACScan flow cytometer. The percentage of cells that were apoptotic was measured as described by Nicoletti et al. [40]. Briefly, cellular debris was excluded from analysis by raising the forward-scatter threshold, and the DNA content of intact nuclei was recorded on a logarithmic scale. Apoptotic cell nuclei containing hypodiploid DNA were enumerated as a percentage of the total population.

Flow-cytometric analysis of Fas by FITC-labeled mAb

Samples containing 2×10^5 cells were incubated with FITC-labeled anti-Fas mAb in staining buffer (RPMI-1640 medium + 5% fetal bovine serum + 0.1% sodium azide) for 45 min on ice. The cells were washed with ice-cold staining buffer and fluorescence was analyzed by a flow cytometer (Becton Dickinson and Co., Mountain View, Calif.).

Immunoblotting

Samples containing 10^6 cells were lysed for 30 min at 4 °C in 50 μl lysis buffer containing 1% NP-40, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 50 mM TRIS HCl (pH 8.0) and 1 mM phenylmethylsulfonyl fluoride. Debris was sedimented by centrifugation for 15 min at 12 000 *g*. Supernatant samples (30 μl) were mixed with 30 μl 2× Laemmli's SDS/polyacrylamide gel electrophoresis (PAGE) sample buffer (Bio-Rad, Hercules, Calif.), and heated for 5 min at 100 °C. Proteins were resolved at 150 V on 10%–12% gels and electrophoretically transferred to nitrocellulose membranes for 1 h at 100 V. Membranes were blocked for 1 h in PBS (pH 7.4) + 0.5% Tween-20 containing 5% nonfat dried milk. Blots were then probed for 1 h at room temperature with specific antibodies and then developed using a goat anti-(mouse Ig) or rabbit secondary antibody labeled with peroxidase (Amersham Corp, Arlington Heights, Ill.). Immunoreactive material was then revealed by enhanced chemiluminescence (ECL, Amersham Corp) according to the manufacturer's instructions.

Results

Apoptosis induced by anti-CD20 mAb

Apoptosis was readily induced in Ramos B cells under the conditions tested by all three anti-CD20 antibodies; however, the magnitude of apoptosis induction was significantly greater with the chimeric Rituximab antibody (30.1 ± 2.39%) than with the murine 1F5 (9.0 ± 0.38%) or anti-B1 (12.9 ± 0.48%) antibodies (Fig. 1). Apoptosis was minimal in control cells incubated with medium, or with the control 64.1 anti-CD3 antibody (1.74 ± 0.44%). Cross-linking any of the anti-CD20 antibodies with GAM (for 1F5 and B1) or GAH (for C2B8) secondary antibodies markedly enhanced the magnitude of apoptosis induced by all of the anti-CD20 antibodies, but did not affect cells incubated with the control antibody, 64.1 (Fig. 1).

Effects of protein kinases on apoptosis

Experiments carried out by other investigators [14] as well as our own unpublished results have demonstrated

that the CD20 molecule is noncovalently associated with protein kinases (Lyn, Fyn, Lck and 75/85 kinase). We initially investigated the activation of these kinases by phosphotyrosine immunoblotting of whole-cell lysates from Ramos cells. In agreement with a previous report by Deans et al. [14], we confirmed that CD20 cross-linking by anti-CD20 mAb 1F5 alone or 1F5 plus GAM increased tyrosine phosphorylation of substrates migrating at approximately 33, 48, 53, 56, 59, 69 and 82 kDa (Fig. 2), as well as substrates from 120 kDa to 197 kDa. Deans et al. [15] have shown that the protein bands with molecular masses of 53, 56 and 59 kDa are the protein tyrosine kinases, p56/53lyn, p56lck and p59fyn. Similar results were observed in Ramos cells cross-linked by another anti-CD20 mAb (B1 and B1 + GAM; data not shown).

To test whether these protein kinases are part of the cascade of events induced by CD20 cross-linking, which leads to apoptosis, we first exposed Ramos cells to the broad specificity tyrosine kinase inhibitors herbimycin A and genistein, prior to incubating cells with anti-CD20 mAb + GAM or anti-sIgM. Unexpectedly, these inhibitors augmented the induction of apoptosis in a dose-dependent manner in three separate experiments, rather than inhibiting it as anticipated (Table 1). Because the inhibitors genistein and herbimycin A inhibit different protein kinases to variable degrees [13], it is conceivable that they abrogated the messages transduced by putative anti-apoptotic kinases to a greater degree than those from the postulated pro-apoptotic kinases. The net result of such preferential inhibition would be an augmentation of anti-CD20-mediated apoptosis by the non-specific kinase inhibitors, as observed in our experiments. To avoid broad inhibition of protein kinases, we subsequently performed experiments with the more selective protein kinase inhibitor PP1, which specifically inhibits PTK Lck and Fyn. PP1 significantly decreased anti-CD20-induced apoptosis as shown in Table 1 ($P < 0.05$), suggesting that early signaling mediated by either Lck or Fyn is involved in anti-CD20-induced apoptosis. To support further the hypothesis that PTK is involved in anti-CD20-induced apoptosis, the effects of PP1 on phosphorylation of PTK Lck and Fyn was investigated by anti-phosphotyrosine immunoblotting of cell lysates. Reduced phosphorylation of p53–59 bands corresponding to Lck and Fyn was observed from cells treated with both anti-CD20 mAb and

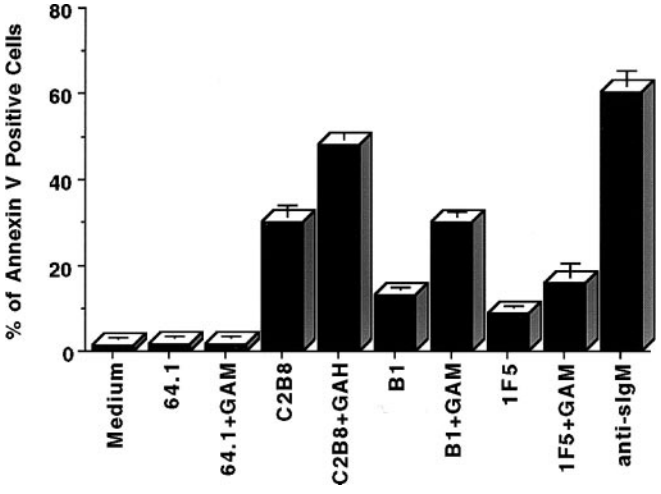

Fig. 1 Anti-CD20-induced apoptosis demonstrated by fluorescein-isothiocyanate(FITC)-labeled Annexin-V staining. 5×10^5 cells/ml were incubated with 10 μg/ml C2B8, B1 or 1F5 anti-CD20 antibodies or with the control 64.1 anti-CD3 antibody for 48 h in the presence or the absence of goat anti-(mouse IgG) (GAM) or goat anti-(human IgG) (GAH) cross-linker (50 μg/ml) and heat-inactivated serum. After the incubation, Cells were stained with FITC-labeled Annexin-V and propidium iodide and analyzed by flow cytometry. Annexin-V-positive and propidium-iodide-negative cells were quantified. Data are representative of five concordant experiments

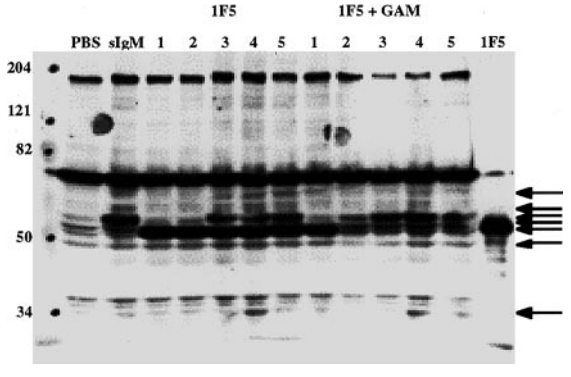

Fig. 2 Increased tyrosine phosphorylation in Ramos cells stimulated by 1F5. Ramos cells (10^7 cells/sample) were incubated with 50 μg/ml 1F5 at 4 °C for 30 min, washed and then incubated at 37 °C for 0, 1, 5, 15 and 30 min (*1*, *2*, *3*, *4*, *5*) with (*1F5 + GAM*) or without (*1F5*) 50 μg/ml GAM before lysis and analysis by Western immunoblotting using anti-phosphotyrosine mAb 4G10 on 10% reducing sodium dodecyl sulfate/polyacrylamide gels

Table 1 Effects of protein kinase inhibitors on anti-CD20-induced apoptosis. *GAM* goat anti-(mouse IgG), *Anti-sIgM* goat anti-(human cell surface IgM)

Inhibitor	Apoptosis (%)		
	64.1 + GAM	B1 + GAM	Anti-sIgM
Herbimycin A (μg/ml)			
0	4[a]	17.4	17.1
0.125	4	19	22.2
0.25	5.5	29	21.3
0.5	7.4	36.6	24.3
Genistein (μg/ml)			
2.5	4.8	21.1	25
5	5.2	27.9	23.1
10	4.8	37.7	28.4
PP1 (μM)			
0	4.4 ± 1.27[b]	21.5 ± 3.03	ND
7	6.3 ± 1.87	14.7 ± 2.44	ND

[a] Percentage fragmented DNA detected by propidium iodide staining as described in Materials and methods. One of three concordant experiments is presented

[b] Percentage of fragmented DNA (average ± SD)

PP1 compared to cells treated with anti-CD20 mAb alone (data not shown).

Effects of calcium on apoptosis

Crossing-linking CD20 with anti-CD20 mAb plus GAM not only activates PTK and substrates of PTK like phospholipase Cγ (PLCγ), but also increases intracellular Ca^{2+} ($[Ca^{2+}]_i$) as has been reported by Deans et al. [15]. We independently confirmed these findings, and demonstrated that increased levels of $[Ca^{2+}]_i$ were induced in Indo-1-loaded Ramos cells by hyper-cross-linking them with various anti-CD20 mAb plus GAM (Fig. 3). Although the magnitude of the $[Ca^{2+}]_i$ flux induced by these anti-CD20 mAb was lower than that triggered by anti-sIgM, all three anti-CD20 mAb tested increased $[Ca^{2+}]_i$ above baseline $[Ca^{2+}]_i$ level and above levels of $[Ca^{2+}]_i$ detected in B cells treated with a negative control antibody (anti-CD3 mAb 64.1 + GAM; Fig. 3).

Elevated $[Ca^{2+}]_i$ levels have been reported to trigger apoptosis in many cell types [19]. Our own unpublished results as well as those of others [2, 32] indicate that apoptosis of Ramos cells induced by the calcium ionophore, ionomycin, and by anti-sIgM are mediated by elevations of $[Ca^{2+}]_i$. To test whether calcium is also involved in anti-CD20-induced apoptosis, we incubated Ramos cells in the presence and absence of the extracellular Ca^{2+} chelator EGTA and the intracellular Ca^{2+} chelator Bapta AM in the presence of anti-CD20 mAb B1 + GAM. Calcium chelation was found to significantly inhibit apoptosis of Ramos cells induced by anti-CD20 mAb + GAM (Fig. 4, $P < 0.01$). Similar results have been observed in apoptosis induced by other murine anti-CD20 mAb (1F5 and 2H7) and by anti-sIgM antibodies [54].

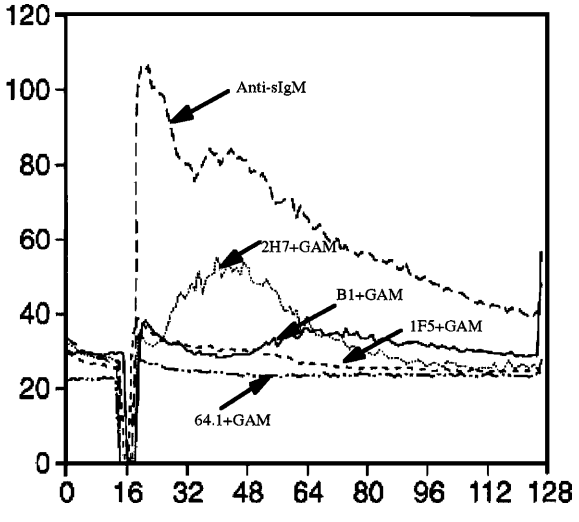

Fig. 3 Intracellular calcium mobilization initiated by anti-CD20 mAb plus GAM. mAb 1F5, B1 and 2H7 (50 μg/ml) were incubated with Indo-1-loaded Ramos cells for 20 min at 37 °C, then washed and resuspended. $F(ab')_2$ fragments of GAM (40 μg/ml) were added after the baseline was established. Anti-CD3 mAb 64.1 plus GAM was used as negative control, and anti-sIgM was used as a positive control

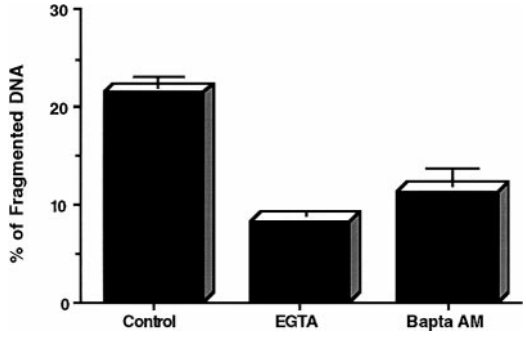

Fig. 4 Ca^{2+} chelators inhibit apoptosis induced by hypercross-linking CD20. 10^6 Ramos cells/ml were incubated with 10 μg/ml 64.1 (*Control*) or B1 anti-CD20 mAb + 50 μg/ml GAM in the presence or absence of 3 mM EGTA or 1.25 μM Bapta AM for 24 h. Cell nuclei were stained with propidium iodide and analyzed by flow cytometry as described in Materials and methods. Results are presented as anti-CD20-induced apoptosis minus non-specific-antibody-64.1-mediated apoptosis in the absence (*Control*) or presence of EGTA or Bapta AM. The data are representative of three experiments

Role of the Fas pathway in anti-CD20-induced apoptosis

Cross-linking the TCR on T lymphoma cells has been known to up-regulate the expression of FasL, which then interacts with induced or constitutively expressed Fas, thereby triggering apoptosis of T lymphoma cells [7, 18, 28]. Many B lymphoma cells, including Ramos cells, have been found to express low levels of Fas constitutively on the cell surface [6]. Since anti-CD20 mAb can induce homotypic adhesion [57], we investigated whether the Fas-FasL interactions are involved in anti-CD20-induced apoptosis. We first examined whether crosslinking of CD20 by B1 + GAM could affect the expression levels of Fas. As shown in Fig. 5, increased expression of Fas was observed in Ramos cells treated by anti-CD40 mAb, which was used as a positive control since it has been reported by several groups that anti-CD40 up-regulates Fas expression [3, 24, 52, 53]. Up-regulation of Fas was also observed in Ramos cells treated by anti-CD20 mAb + GAM (Fig. 5). We then investigated the role of the Fas-FasL pathway in apoptosis of Ramos cells incubated with anti-CD20 mAb + GAM using an anti-FasL mAb to block binding of FasL to Fas. The percentage of fragmented DNA was 5.2 ± 1.30% compared to 5.3 ± 1.27% from cells treated with 64.1 + GAM ($P > 0.05$), and 23.9 ± 4.21% compared to 27.6 ± 0.96% from cells treated with B1 + GAM ($P > 0.05$) in the absence and presence of the anti-FasL mAb. The same anti-FasL mAb inhibited apoptosis in the T lymphoma cell line SupT-13, as measured by a decrease in the percentage of

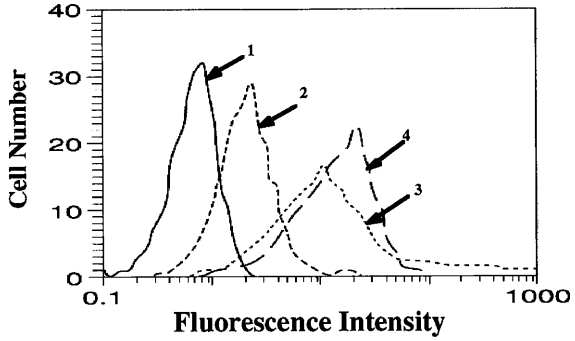

Fig. 5. Up-regulation of Fas by cross-linking CD20. 10^6 Ramos cells were incubated with phosphate-buffered saline, 10 μg/ml anti-CD40 mAb (G28.5) or 10 μg/ml B1 + 50 μg/ml GAM for 24 h. After the stimulation, 2×10^5 cells were incubated with staining buffer or FITC-labeled anti-Fas mAb for 45 min at 4 °C. Cells were washed before being analyzed by flow cytometry. *1* Cells + PBS + staining buffer; *2* Cells + PBS + FITC-anti-Fas; *3* Cells + B1 + GAM + FITC-anti-Fas; *4* Cells + anti-CD40 + FITC-anti-Fas

fragmented DNA induced by an anti-CD3 mAb BC3 from 56.8 ± 1.1 to 38.4 ± 4.8 ($P < 0.01$). These results indicated that apoptosis induced by CD20 cross-linking with the anti-B1 antibody was not inhibited by anti-FasL mAb, suggesting that the Fas-FasL pathway may not contribute to anti-CD20-induced apoptosis even though anti-CD20 mAb + GAM up-regulate the expression of Fas.

Effects of caspases on apoptosis

Since the initial report that Fas-induced apoptosis is mediated by a cascade of ICE-like proteases, multiple confirmatory reports have emerged implicating caspases in apoptosis induced by multiple stimuli including ionomycin and cross-linking of cell-surface receptors such as sIgM [1, 2]. In view of the pervasive influence of caspases in apoptosis pathways, we explored their involvement in anti-CD20-induced apoptosis. In initial experiments, we assessed cleavage of the classic "death substrate" of caspases, namely, poly(ADP-ribose) polymerase (PARP) [25] following CD20 cross-linking. Cell lysates from cells treated by control and anti-CD20 antibodies were separated by SDS-PAGE, transferred to membranes, and probed by Western blotting using anti-PARP antibodies. As shown in Fig. 6, the PARP precursor (116 kDa) was present in cells treated with either control or anti-CD20 antibodies. However, the activated PARP fragment (85 kDa) was present only in samples from cells treated with anti-CD20 antibodies, but not in samples treated with the negative control antibody. PARP cleavage was markedly enhanced by CD20 hyper-crosslinking (using GAH for chimeric C2B8 or GAM for murine anti-CD20 mAb B1; Fig. 6). These results are consistent with our previously published results showing that hyper-crosslinking CD20

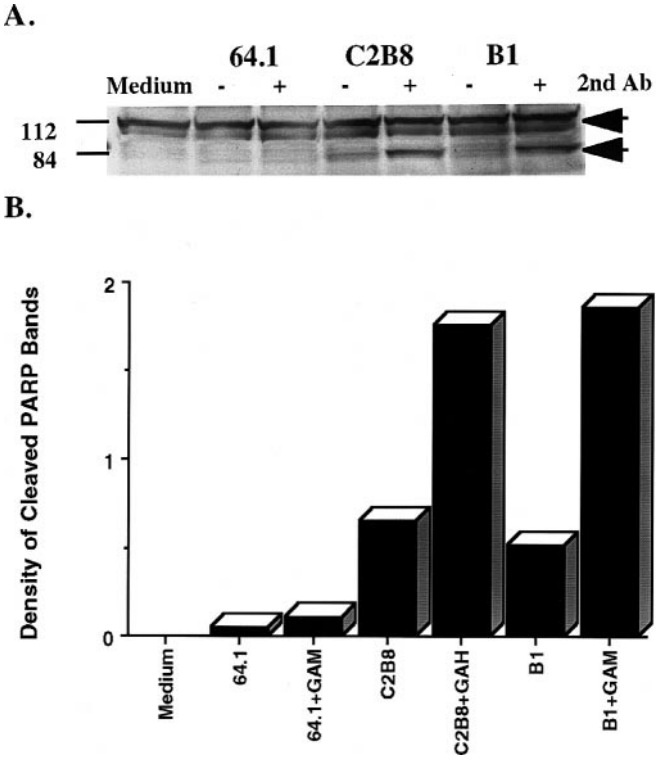

Fig. 6A, B Cleavage of poly(ADP-ribose)polymerase (PARP) induced by cross-linking CD20 in Ramos cells. Ramos cells (10^6 cells/ml) were incubated with 10 μg/ml C2B8 or B1 anti-CD20 antibodies or with the control 64.1 anti-CD3 antibody for 48 h in the presence or the absence of GAM or GAH cross-linker (50 μg/ml) and heat-inactivated serum. **A** The expression of PARP was determined by immunoblotting with an anti-PARP antibody. The results of one of two concordant experiments are shown. **B** The density of cleaved PARP (85 kDa) was detected by a densitometer

with GAM (or Fc-receptor-bearing cells) markedly augments apoptosis of Ramos cells (Fig. 1 and [54]). These experiments suggest that hyper-crosslinking CD20 induces apoptosis of Ramos cells through activation of the caspase cascade, similar to apoptosis induced by many other stimuli.

In subsequent experiments, we assessed the role of upstream caspases in PARP cleavage using the caspase inhibitor zVAD. As shown in Fig. 7A, zVAD inhibited the cleavage of PARP induced by anti-CD20 mAb + GAM and by anti-sIgM. Since it has been reported that caspase-3 is one of the immediate upstream caspases responsible for PARP cleavage [39], we also tested the activity of caspase-3 and the effect of zVAD on the activation of caspase-3 in anti-CD20-induced apoptosis. Ramos cells treated with B1 + GAM or anti-sIgM contained much less of the intact caspase-3 precursor molecule than control cells, suggesting that caspase-3 was activated in cells stimulated with either B1 + GAM or anti-sIgM and degraded to smaller fragments that are not recognized by the anti-caspase-3 precursor antibody used in the assay (Fig. 7B). This interpretation was supported by subsequent studies demonstrating that zVAD markedly inhibited degradation of the caspase-3

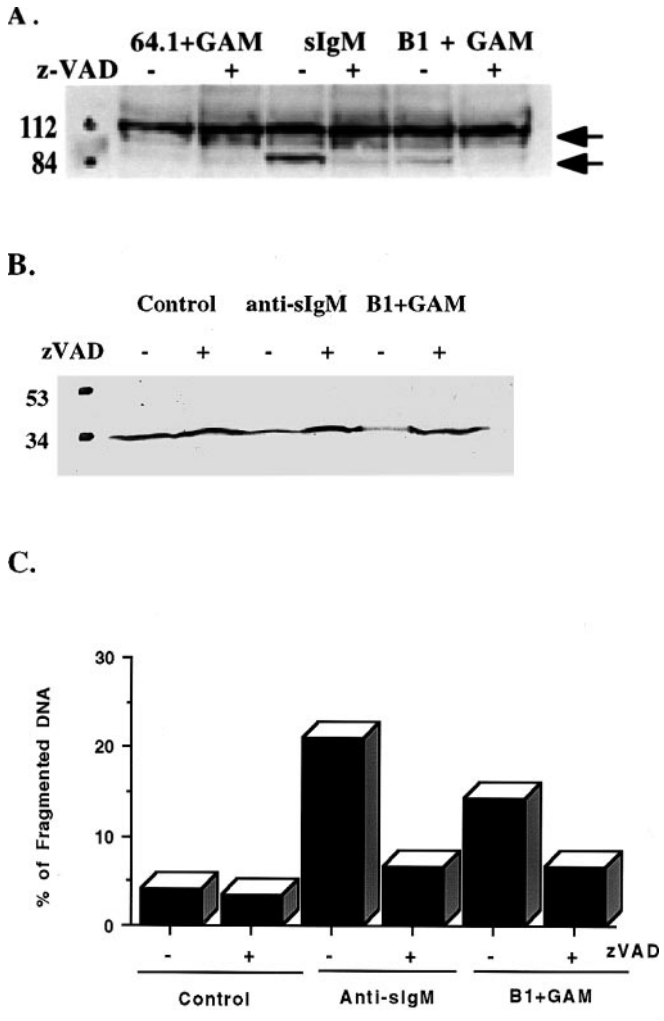

Fig. 7A–C Benzyloxycarbonyl-valyl-alanyl-aspartate (zVAD) inhibits anti-CD20-induced PARP cleavage, caspase-3 processing and apoptosis in Ramos cells. Ramos cells (10^6 cells/ml) were cultured for 24 h with 10 μg/ml 64.1 (*Control*) or B1 + 50 μg/ml GAM or with 10 μg/ml anti-sIgM antibodies in the presence or absence of 80 μM zVAD. The expression of PARP and its cleavage product (*arrow*) was determined by immunoblotting with an anti-PARP antibody (**A**), the expression of the caspase-3 precursor was determined by immunoblotting with an anti-caspase-3 precursor mAb (**B**), and the apoptosis was assessed by propidium iodide staining of cell DNA followed by flow cytometry analysis (**C**). Data are representative of three experiments

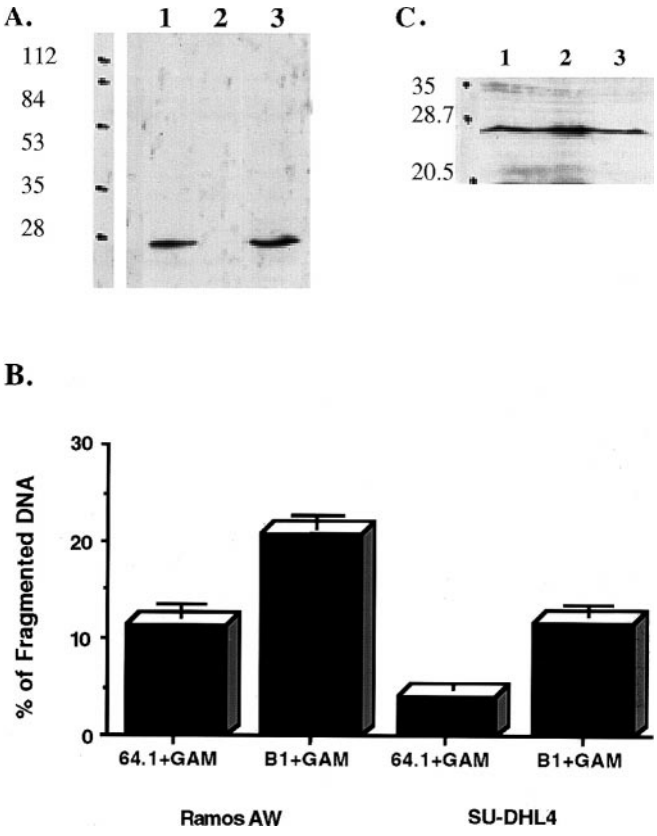

Fig. 8 **A** Expression of Bcl-2 in Ramos AW cells. 2×10^6 cells were lysed by 50 μl lysis buffer. The expression of Bcl-2 was determined by immunoblotting with an anti-Bcl-2 mAb. *Lane 1* Ramos AW, *lane 2* Ramos, *lane 3* positive control (Raji). **B** Apoptotic effect of anti-CD20 mAb on Ramos AW and SU-DHL4 cells. 10^6 Ramos AW or SU-DHL4 cells were incubated with 10 μg/ml 64.1 or B1 in the presence of 50 μg/ml GAM for 24 h. Cell nuclei were stained with propidium iodide and analyzed by flow cytometry. Data are representative of three experiments. **C** Expression of Bad in Ramos AW and SU-DHL4 cells. 2×10^6 cells were lysed by 50 μl lysis buffer. The expression of Bad was determined by immunobloting with an anti-Bad mAb. *Lane 1* Ramos AW, *lane 2* SU-DHL4, *lane 3* positive control (MCF-7)

precursor in Ramos cells treated with either B1 + GAM or anti-sIgM (Fig. 7B). Concordant activation of caspase-3 and PARP in Ramos cells undergoing anti-CD20-induced apoptosis and inhibition of this activation by zVAD strongly support the contention that the classic caspase cascade is utilized in anti-CD20-induced apoptosis. In order to confirm further the role of caspases in anti-CD20-induced Ramos cell apoptosis, we analyzed DNA fragmentation in cell samples treated with zVAD from the experiment shown in Fig 7B. As shown in Fig. 7C, zVAD inhibited DNA fragmentation induced by either anti-CD20 mAb + GAM or anti-sIgM antibodies.

Effects of Bcl-2 on anti-CD20-induced apoptosis

Bcl-2 has been reported to inhibit apoptotic cell death induced by many, but not all, stimuli. In order to investigate the role of Bcl-2 in anti-CD20-induced apoptosis, we first assayed the expression of Bcl-2 in Ramos cells. The results from Western blotting, using an anti-Bcl-2 mAb as a probe, indicated there was no detectable Bcl-2 expression in Ramos cells compared to a positive control cell line. However, Ramos AW cells, produced by infection of Ramos cells with Epstein-Barr virus, expressed high levels of Bcl-2 (Fig. 8A). We then tested whether hyper-crosslinking CD20 on Ramos AW cells with anti-CD20 + GAM could induce apoptosis in spite of the expression of high levels of Bcl-2. As shown in Fig. 8B, Ramos AW cells were readily induced to undergo programmed cell death by hyper-crosslinking CD20 (Fig. 8B; $P < 0.01$), suggesting that Bcl-2

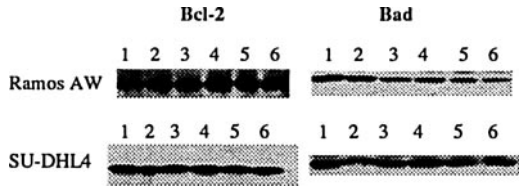

Fig. 9 Effects of anti-CD20 mAb on the expression of Bcl-2 and Bad. 5×10^5/ml Ramos AW or SU-DHL4 cells were incubated with 10 μg/ml B1or C2B8 anti-CD20 mAb or with the control 64.1 anti-CD3 mAb for 48 h in the presence or the absence of GAM or GAH cross-linker (50 μg/ml). Expression of Bcl-2 or Bad was determined by immunoblotting with an anti-Bcl-2 or anti-Bad mAb. *1* 64.1, *2* B1, *3* C2B8, *4* 64.1 + GAM, *5* B1 + GAM, *6* C2B8 + GAH

expression does not prevent anti-CD20-induced apoptosis. Another B lymphoma cell line, SU-DHL4, which contains a t(14;18) translocation that activates the Bcl-2 proto-oncogene [34], was also sensitive to anti-CD20-induced apoptosis (Fig. 8B). Similar findings have been reported in anti-CD19 and anti-CD22-mediated apoptosis of Ramos AW cells [10]. Since it has been suggested that pro-apoptotic members of the Bcl-2 family such as Bad and Bcl-Xs may inhibit the function of anti-apoptotic proteins such as Bcl-2 by sequestering them [50], we investigated whether pro-apoptotic members of Bcl-2 family were expressed in Ramos AW and SU-DHL4 cells by Western blotting using anti-Bad and anti-Bcl-Xs antibodies as probes. Our results indicated that Bad was expressed in both Ramos AW and SU-DHL4 cells (Fig. 8C), suggesting that the simultaneous expression of Bad in these Bcl-2 expressing cells may negate the antiapoptotic effect of Bcl-2 in these cells lines. No detectable Bcl-Xs was found in either Ramos AW or SU-DHL4 cells (data not shown).

To investigate whether the sensitivity of Bcl-2-positive cells to anti-CD20-induced apoptosis derives from the down-regulation of Bcl-2 and/or up-regulation of Bad by anti-CD20 mAb in these cells, we examined the expression levels of Bcl-2 and Bad in both Ramos AW and SU-DHL4 cells after stimulation of these cells by anti-CD20 mAb. No significant changes in Bcl-2 and Bad expression were observed in either Ramos AW or SU-DHL4 cells treated with anti-CD20 mAb compared to cells treated with negative control mAb (Fig. 9), suggesting that the sensitivity of these Bcl-2-positive cells to anti-CD20-induced apoptosis does not derive from changes in the relative ratio of Bcl-2 to Bad.

Discussion

Anti-CD20 mAb have been effectively employed for the immunotherapy of B cell lymphomas in multiple recent clinical trials in either unmodified form or conjugated to radionuclides [30, 31, 45, 47]. Complement-mediated cell lysis, antibody-dependent cellular cytotoxicity (ADCC), and the emission of β particles from radioisotopes have been implicated as antitumor mechanisms responsible for these therapeutic results [26, 46, 47]. However, accumulating evidence from in vitro studies [12, 57], animal tumor models [23, 51], and early clinical trials [8, 43] suggest that a significant portion of the tumoricidal effect of anti-CD20 mAb may be mediated by mechanisms independent of complement, ADCC, or radioactive emissions. In this report, we confirm and extend previous observations that CD20 ligation with monoclonal antibodies transduces intracellular signals leading to programmed cell death [17, 35, 54]. We show that apoptosis can be induced by any of the three anti-CD20 monoclonal antibodies tested (anti-B1, 1F5, or C2B8), but that the murine antibodies (anti-B1 and 1F5) are less potent in inducing apoptosis than the chimeric C2B8 antibody (Rituximab). This difference in potency merits comment since it has led to disagreements among investigators. The assays used in this manuscript and depicted in Fig. 1 have been optimized to demonstrate anti-CD20-induced apoptosis. Less-sensitive assays or assays carried out for only 18–24 h rather than for 48 h as in Fig. 1 frequently fail to demonstrate significant apoptosis with the murine 1F5 and anti-B1 antibodies unless GAM crosslinking is performed, as reported in our early experiments [54] and those of others [35]. Furthermore, the ease of apoptosis induction is dependent on the differentiation stage of the B cell line studied, the cell culture density, the incubation time and other culture conditions. In contrast, the signal delivered by the chimeric C2B8 antibody appears to be considerably stronger, permitting detection of apoptosis even after shorter incubation periods and in less-sensitive assays. The explanation for the enhanced potency of the chimeric antibody is not clear, though it is possible that the human Fc region interacts more effectively with Fc receptors expressed on lymphoma cell lines, permitting some functional crosslinking even in the absence of a secondary crosslinking antibody. Alternatively, C2B8 may bind to a different portion of the extracellular loop of CD20 than 1F5 or anti-B1, which may be more effective at transducing the apoptotic signal.

The significance of the enhanced apoptotic effect of GAM or GAH hyper-crosslinking of anti-CD20 antibodies in vitro may be questioned, since administration of GAM or GAH reagents in vivo is not feasible. However, we have previously published results demonstrating that the same enhancement of anti-CD20-induced apoptosis occurs when "physiological" crosslinking with Fc-receptor bearing accessory cells is used [54]. Since lymphoma-bearing organs possess large numbers of Fc-receptor-expressing cells (macrophages, monocytes, dendritic cells), augmentation of anti-CD20-induced apoptosis should also occur in vivo. This enhancement of apoptosis is expected to be more marked for chimeric antibodies possessing human Fc regions; however, such an enhancing effect also has been shown for murine anti-CD20 antibodies [54].

The main objective of this manuscript is to elucidate the fundamental intracellular mechanisms responsible for apoptosis induced in malignant human B cells after

CD20 ligation. In this report, we provide evidence that CD20 ligation triggers PTK (Lck or Fyn) activation leading to increased intracellular calcium levels, caspase activation, and ultimately, apoptosis. PTK are known to be non-covalently associated with CD20 and to be activated by crosslinking CD20 with anti-CD20 mAb, leading to downstream activation of PTK substrates, such as PLCγ [14, 15]. The effects of these early and immediate signaling events on apoptosis are not fully understood and appear to vary for different PTK and for different cell lines, with generation of either apoptosis-promoting or apoptosis-inhibiting effects [25]. This complexity was confirmed in our investigations using protein kinase inhibitors of broad specificity, such as herbimycin A and genistein, which were shown to augment rather than inhibit anti-CD20-induced apoptosis. In contrast, more selective inhibitors such as PP1, an inhibitor of PTK Lck and Fyn, suppressed anti-CD20-induced apoptosis, suggesting that these CD20-associated pathways may be involved in mediation of B cell apoptosis caused by CD20 crosslinking.

Studies in our laboratory and others [14] have convincingly demonstrated that activation of CD20-associated PTK induces increased levels of intracellular calcium in anti-CD20-crosslinked cells, presumably via activation of phospholipase C by activated Lck and Fyn. Resting cells typically maintain an intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) of 0.05–0.2 μM against an extracellular concentration of approximately 1 mM. Various organelles, such as the endoplasmic reticulum and mitochondria, also store Ca^{2+} at levels higher than the cytoplasmic levels. The maintenance of these gradients is achieved by compartmentalization and by the action of a number of Ca^{2+}-transporting ATPases located in the plasma and organelle membranes. The release of Ca^{2+} from intracellular stores or the influx of Ca^{2+} from the extracellular media often occurs as part of a signaling pathway in which Ca^{2+} plays a role as a second messenger in the regulation of diverse cellular activities [9]. The involvement of increased intracellular Ca^{2+} levels in apoptosis has been reported in many cell types stimulated by different cell death-promoting factors [19]. Our experiments support the hypothesis that anti-CD20-induced apoptosis is mediated by increases in $[Ca^{2+}]_i$ since (1) the death process could be mimicked by a calcium ionophore, which releases Ca^{2+} from a variety of intracellular stores and also increases Ca^{2+} influx from the extracellular media; (2) hyper-crosslinking CD20 was shown to increase $[Ca^{2+}]_i$ levels; (3) the increase in $[Ca^{2+}]_i$ preceded DNA fragmentation; and (4) the chelation of extracellular or intracellular Ca^{2+} with EGTA or Bapta AM, respectively, inhibited DNA degradation.

Caspases have been shown to play an important role in apoptosis in many different systems. Our results demonstrate that the signaling pathways triggered by CD20 crosslinking eventually activate the cascade of caspases in an analogous fashion to that observed in other apoptotic systems, though the details of this process are only partially understood. The best-characterized pathway leading to apoptosis is the Fas-FasL system, which has been implicated in apoptosis induced by many stimuli including protracted TCR ligation in T lymphoma cells [7, 18, 28]. In this model system, crosslinking of the TCR up-regulates Fas ligand, which binds to the Fas receptor, thereby triggering a complicated sequence of molecular interactions, ultimately culminating in apoptosis. We originally expected that the Fas-FasL interaction might be involved in anti-CD20-induced apoptosis in Ramos cells and hypothesized that crosslinking CD20 up-regulated the expression of Fas and/or FasL through activation of PTK and increased $[Ca^{2+}]_i$, and that the subsequent Fas-FasL interaction triggered the classic cascade of caspases leading to apoptotic cell death. Our results, however, indicate that this hypothesis might not be correct, since an anti-FasL mAb, which was used to block the Fas-FasL interaction, did not inhibit anti-CD20(B1)-induced apoptosis, although up-regulation of Fas was observed after crosslinking CD20. The precise mechanism by which increased levels of intracellular calcium induced by CD20 hypercross-linking mediate apoptosis therefore requires further study. It has been shown that Ca^{2+} can cause DNA degradation through Ca^{2+}-dependent endonucleases in nuclei [38]; however, little is known about its role during the cytosolic phase of apoptosis. The connection between Ca^{2+} and caspases was not clear until recently. A study by Juin et al. [29] indicates that adding Ca^{2+} to normal cytosolic extracts can activate caspase-3 and eventually triggers nuclear apoptosis in a cell-free system. Our experimental results indicate that Ca^{2+} chelators can inhibit anti-CD20-induced cleavage of PARP (data not shown). All these studies suggest that increased intracellular Ca^{2+} induced by CD20 crosslinking may activate caspases directly or through downstream signal events.

The Bcl-2 family of proteins has been shown to play a role in apoptosis upstream of terminal caspases. The expression of Bcl-2 inhibits apoptosis in many cell types induced by many, but not all, stimuli [50]. Our data show that anti-CD20 + GAM can induce apoptosis in Bcl-2-expressing cell lines, (e.g. Ramos AW and SU-DHL4 cells) as readily as in cell lines lacking Bcl-2 expression (Ramos), suggesting that Bcl-2 does not play an important role in regulating apoptosis induced by CD20 crosslinking. Similar observations of Bcl-2-independent apoptosis have been reported in anti-CD19 and anti-CD22-mediated apoptosis of Ramos AW cells and in other apoptotic systems [10, 33, 42, 60]. However, a second possible explanation is that expression of other Bcl-2 family members may antagonize the effects of Bcl-2 in Ramos AW and SU-DHL4 cells, negating the effects of Bcl-2. It has been reported that some proapoptotic members of the Bcl-2 family like Bad, Bcl-Xs and Bik inhibit the function of antiapoptotic proteins like Bcl-2 by sequestering these proteins [50]. Our finding that Bad is also expressed in Ramos AW and SU-DHL4 cells may explain their susceptibility to apoptosis mediated by

anti-CD20 + GAM despite expression of Bcl-2. It is believed that the ratio of expression of proapoptotic to antiapoptotic members of the Bcl-2 family strongly influences the likelihood that a cell will activate the program for apoptotic cell death when confronted with an appropriate stimulus. Therefore, we investigated the effects of CD20 crosslinking on the expression of two prominent members of the Bcl-2 family (Bcl-2 and Bad) in Ramos AW and SU-DHL4 cells. Our data indicate that crosslinking CD20 with anti-CD20 mAb did not significantly change the expression levels of either Bcl-2 or Bad (Fig. 9) in Ramos AW or SU-DHL4 cells compared with levels of constitutive expression. Therefore, it is unlikely that the sensitivity of these Bcl-2-expressing cells to anti-CD20-induced apoptosis results from changes in the ratio of Bcl-2 to Bad. These experiments do not exclude potential effects on other members of the Bcl-2 family, however. It is anticipated that further studies dissecting the pathways mediating anti-CD20-induced apoptosis of lymphoma cells may permit clinical perturbations of signaling pathways and augmentation of the efficacy of anti-CD20 antibody therapy of B cell lymphomas.

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