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Mitochondria Connects the Antigen Receptor to Effector Caspases During B Cell Receptor-Induced Apoptosis in Normal Human B Cells[1]

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We have previously reported that CD40 stimulation sensitizes human memory B cells to undergo apoptosis upon subsequent B cell receptor (BCR) ligation. We have proposed that activation stimuli connect the BCR to an apoptotic pathway in mature B cells and that BCR-induced apoptosis of activated B cells could serve a similar function as activation-induced cell death in the mature T cell compartment. Although it has been reported that caspases are activated during this process, the early molecular events that link the Ag receptor to these apoptosis effectors are largely unknown. In this study, we report that acquisition of susceptibility to BCR-induced apoptosis requires entry of memory B cells into the S phase of the cell cycle. We also show that transduction of the death signal via the BCR sequentially proceeds through a caspase-independent and a caspase-dependent phase, which take place upstream and downstream of the mitochondria, respectively. Furthermore, our data indicate that the BCR-induced alterations of the mitochondrial functions are involved in activation of the caspase cascade. We have found both caspases-3 and -9, but not caspase-8, to be involved in the BCR apoptotic pathway, thus supporting the notion that initiation of the caspase cascade could be under the control of the caspase-9/Apaf-1/cytochrome *c* multimolecular complex. Altogether, our findings establish the mitochondria as the connection point through which the Ag receptor can trigger the executioners of apoptotic cell death in mature B lymphocytes. ***The Journal of Immunology*****, 1999, 163: 4655–4662.**

Apoptosis occurs during lymphopoiesis and immunopoiesis to maintain tolerance toward self (1) and to control the specificity of the immune response (2). It also contributes to regulate homeostasis of the immune system by controlling the size of the lymphocyte population (3–5).

The apoptotic process can be induced by direct cellular damages such as those caused by irradiation, but also by triggering surface receptors. Some of these, such as the death domain (DD)[3]-containing receptors, mainly function as apoptosis-inducing molecules (6). Others, like the Ag receptors and MHC class II molecules, only behave as such under certain strictly defined circumstances. Although the downstream events connected to the DD-containing receptors are becoming better understood, the intracellular death cascade linked to Ag receptors remains relatively obscure.

The efficiency of the DD-containing receptors at transducing death signals is partly due to their ability to rapidly form a multimolecular complex (death-inducing signaling complex or DISC) connected to their intracytoplasmic tail (7). This complex is constituted within seconds of ligand binding and induces death of the target cells within several hours. Most of the damages inflicted to the cell by the DD-containing receptors are due to the activation of a peculiar type of cysteine proteases, best known as caspases. These proteolytic enzymes act in a stepwise fashion and can behave as substrates for each other (8, 9). The initiator caspases (caspase-8 or -10) are recruited and activated within the DISC (10, 11). They in turn activate downstream effector caspases (6) that are responsible for the characteristic cellular alterations associated with apoptosis, such as phosphatidylserine (PS) exposure or DNA damage.

Most of our current knowledge of the apoptosis effectors recruited by the BCR comes from experiments conducted with transformed immature (12) or mature B cell lines (13, 14). These studies have established that, as for most apoptotic pathways described to date, the death signal transmitted through the BCR induces activation of effector caspases (caspase-3 in particular). Nevertheless, the cytoplasmic mediators that allow the BCR to switch from a positive to a negative regulatory function and that connect the Ag receptor to the executioners of apoptosis are presently unknown.

It has been proposed that BCR-induced death could be instrumental in censoring self-reactive mutants generated through V gene hypermutation in the germinal center (GC) (15–17). We have previously shown that susceptibility of mature B cells to BCR-induced apoptosis is not a specific feature of GC B cells, but that human memory B cells can also be sensitized by CD40L or anti-Ig Abs to undergo apoptosis upon subsequent challenge of the Ag receptor (18). We have thus hypothesized that all mature B cells become sensitive to BCR-induced killing provided that they have received the adequate activation stimulus.

We have decided to explore two aspects of the death process connected to the BCR in normal human B cells: 1) the parameters

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[3] Abbreviations used in this paper: DD, death domain; AICD, activation-induced cell death; BCR, B cell receptor; BL, Burkitt lymphoma; $DiOC_6$, 3,3′ dihexyloxacarbocyanine iodide; DISC, death-inducing signaling complex; GC, germinal center; PARP, poly(ADP-ribose) polymerase; PS, phosphatidylserine; CD40L, CD40 ligand; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Z-IETD-fmk, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone; Z-LEHD-fmk, benzyloxycarbonyl-Leu-Glu-Mis-Asp-fluoramethylketone.

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that regulate the acquisition of their susceptibility to BCR-induced killing; 2) the molecular mechanisms responsible for execution of the apoptotic program. Although both GC and memory B cells are sensitive to BCR-induced killing, CD40L-stimulated memory B cells were chosen as the experimental model because of their lower tendency to enter spontaneous apoptosis in culture.

We report in this work that susceptibility of activated memory B cells to BCR-induced apoptosis relies on their entry in the S phase of the cell cycle. Execution of the BCR-induced apoptotic program is then divided into two phases: 1) an early caspase-independent step that leads to alteration of the mitochondrial permeability; 2) a later caspase-dependent step responsible for the ultimate manifestations of BCR-induced apoptosis. Our results demonstrate that the disruption of the mitochondrial function during the first phase initiates a downstream effector caspase cascade that involves activation of caspases-9 and -3.

Materials and Methods

Reagents and Abs

The trimeric human CD40L/leucine zipper fusion protein was kindly provided by Dr. R. Armitage (Immunex, Seattle, WA) and was used at 1 μg/ml throughout the study. F(ab′)$_2$ fragments of rabbit Abs anti-human IgM/G/A (H + L) were purchased from Jackson ImmunoResearch (West Grove, PA) and used at 5 μg/ml. Rabbit anti-human IgM Abs coupled to polyacrylamide beads were purchased from Bio-Rad (Richmond, CA) and used at 20 μg/ml. The agonistic anti-CD95/Fas mAb 7C11 (IgM) was purchased from Immunotech (Marseilles, France). Purified mouse IgM myeloma protein used as the isotypic control for mAb 7C11 was obtained from Sigma (St. Louis, MO). Both the 7C11 mAb and its isotypic control were used at 100 ng/ml. The mouse mAb directed against poly(ADP-ribose) polymerase (PARP) and the polyclonal rabbit Ab against caspase-3 were purchased from Biomol (Plymouth Meeting, PA) and PharMingen (San Diego, CA), respectively. The broad range caspase inhibitor Z-VAD-fmk was purchased from Bachem (Bubendorf, Switzerland). The caspase-8-specific inhibitor Z-IETD-fmk and the caspase-9-specific inhibitor Z-LEHD-fmk were purchased from Calbiochem (Nottingham, U.K.). Z-VAD-fmk, Z-LEHD-fmk, and Z-IETD-fmk peptides being dissolved in DMSO, control cultures were performed in the presence of the appropriate dilution of DMSO. The cell cycle blockers aphidicolin, deferoxamine, and mimosine were purchased from Sigma and used at 1 μg/ml, 300 μM, and 600 μM, respectively.

Cells

Purified tonsillar B cells were isolated as previously described (19). IgD-positive and IgD-negative B cells were separated using anti-IgD-coated SRBC, as described in detail by Feuillard et al. (20). Memory B cells were isolated from the IgD$^-$ population, as previously described (2). Experiments were conducted either on isolated memory B cells or on the Burkitt lymphoma cell line BL60. The choice of memory B cells to study the intracellular apoptotic pathway connected to the BCR in normal mature B cells was motivated by the fact that this subset is highly sensitive to BCR-induced apoptosis, but less prone to undergo spontaneous apoptosis than its GC counterpart.

Cultures

All cultures were made in RPMI 1640 supplemented with 10% selected heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% HEPES (all from Life Technologies, Grand Island, NY), except where indicated. Tonsillar B cell cultures were performed in two steps. First, B cell blasts were generated by stimulating B cells for 48 h with soluble trimeric CD40L at the density of 1×10^7 cells/well in six-well plates. Second, viable B cell blasts were recovered by density-gradient centrifugation and seeded at 1×10^5 cells/well in 96-well round-bottom microtiter plates in a final volume of 0.1 ml for the indicated times. We had observed that persistent CD40 stimulation not only failed to inhibit anti-Ig-induced apoptosis in B blasts, but rather decreased their spontaneous apoptosis (data not shown). Therefore, all secondary cultures of B blasts were established in the presence of CD40L, with or without addition of F(ab′)$_2$ fragments of rabbit anti-human Igs Abs. For the experiments conducted on the IgM-expressing Burkitt lymphoma cell line BL60, cells were seeded at 1×10^5 cells/well and cultured with or without immobilized rabbit anti-human IgM Abs. Percentages of specific cell death were calculated as follows: 100 × (apoptosis with anti-Ig Abs (%) − spontaneous apoptosis (%))/(100% − spontaneous apoptosis (%)). Intracellular ATP was depleted by incubating BL60 cells in glucose-free RPMI 1640 medium (Sigma) supplemented with 10% selected heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μM oligomycin, an inhibitor of F_0F_1-ATPases (21), to prevent production of ATP from both glycolysis and oxidative phosphorylation.

Measurement of apoptosis

Quantitation of apoptotic cells was made with: 1) The 3,3′ dihexyloxacarbocyanine iodide ($DiOC_6$) fluorochrome (Molecular Probes, Eugene, OR), which reveals disruption of the mitochondrial transmembrane potential ($\Delta\psi$m), as described by Zamzami et al. (22). In this assay, apoptotic cells are identified by their decreased $\Delta\psi$m ($DiOC_6^{low}$). 2) Biotinylated annexin V (Boehringer Mannheim, Mannheim, Germany) used according to the manufacturer's protocol to detect the translocation of PS from the inner side to the outer leaflet of the plasma membrane on apoptotic cells. The annexin V staining was revealed with FITC-conjugated avidin (Immunotech) used at 2.5 μg/ml.

Immunofluorescence stainings were analyzed on a FACScan flow cytometer using the Lysis II software (Becton Dickinson, San Jose, CA).

Western blot analysis

Briefly, 5×10^6 cells were pelleted, washed twice with PBS, and resuspended in 100 μl of lysis buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 2% 2-ME, supplemented with a protease inhibitor mixture (Boehringer Mannheim)). Samples were run on 8% (PARP) or 15% (caspase-3) SDS polyacrylamide gels. Following transfer to nitrocellulose membrane, the immunoblots were blocked by incubating with 0.5% BSA, Tris-buffered saline, and 0.1% Tween 20 (TBS-T), and probed overnight with the anti-PARP mAb or the anti-caspase-3 polyclonal Ab at a dilution of 1/2000 prepared in 0.5% BSA TBS-T. The immunoblots were then probed with HRP-conjugated goat anti-mouse Igs Abs (PARP) or goat anti-rabbit Ig Abs (caspase-3) (both from Amersham Life Science, Little Chalfont, U.K.) and developed using the ECL system (Pierce, Rockford, IL).

Measurement of caspase-3 enzymatic activity

The cell-permeable fluorigenic peptidic substrate PhiPhiLux-G_2D_2 (OncoImmunin, College Park, MD) containing the cleavage site DEVD was used to monitor caspase-3-like activity in intact cells. Cells were incubated with the substrate solution for 1 h at 37°C in the dark, according to the manufacturer's instructions. The fluorescence emission revealing the release of the fluorescent product was analyzed on a flow cytometer in the FL2 channel.

Results

Susceptibility of mature B cells to BCR-induced apoptosis is correlated with their cycling potential

We have previously demonstrated that both GC (19) and memory B cells (18) acquire susceptibility to BCR-induced apoptosis upon primary stimulation via CD40. We thus postulated that activation stimuli induce a rewiring of the Ag receptor to an apoptotic pathway in mature B cells. This led us to propose that BCR-induced apoptosis in the mature B cell lineage fulfills a function similar to that ascribed to AICD in the T cell compartment.

It has been demonstrated that the susceptibility of mature T cells to Ag receptor-induced apoptosis is acquired upon their entry into the S phase of the cell cycle (23). CD40L-activated and resting memory B cells, which are respectively sensitive and resistant toward BCR-induced apoptosis, also differ by their cycling status (18). We thus examined whether the acquisition of susceptibility toward BCR-induced killing in normal mature B cells could also rely on their entry in a particular phase of the cell cycle. To address this issue, different cell cycle blocking agents were tested for their capacity to inhibit the proapoptotic effect of anti-Ig Abs on activated mature B cells. Mimosine and deferoxamine were used to arrest cells in the G_1 phase of the cycle, while aphidicolin was used to arrest cells in the S phase of the cell cycle. Isolated memory B cells were first stimulated for 48 h with CD40L in the presence or absence of either aphidicolin, deferoxamine, or mimosine. For secondary cultures, viable B blasts were restimulated with CD40L, in

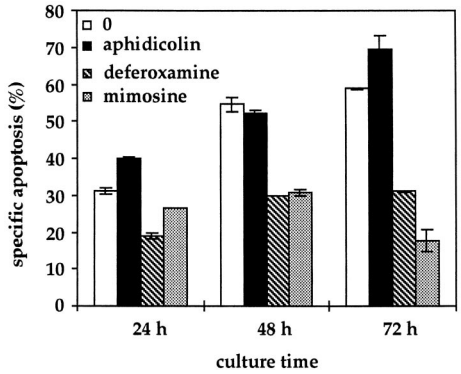

FIGURE 1. The susceptibility of CD40L-activated B cells to BCR-induced apoptosis is correlated with their entry into the S phase of the cell cycle. Isolated memory B cells were stimulated with CD40L for 48 h and next recultured with CD40L, in the presence or absence of $F(ab')_2$ fragments of anti-Ig Abs. Cultures were further supplemented or not with the indicated cell cycle blockers. When added, the blocking agents were present throughout the primary and secondary cultures. The proportions of apoptotic cells were estimated at the indicated time points using the cell-permeable fluorescent dye $DiOC_6$. Data are presented as means ± SD of the percent specific apoptosis calculated from duplicate determinations. Representative of three independent experiments.

the presence or absence of $F(ab')_2$ fragments of rabbit anti-human Ig Abs for 24, 48, and 72 h. When added, the cell cycle blockers were present throughout the primary and the secondary cultures. Estimation of the mitochondrial transmembrane potential with the fluorescent dye $DiOC_6$ was used to monitor the proportions of apoptotic cells at the end of the secondary cultures. None of the cell cycle blockers used induced any significant rise in the proportions of apoptotic cells per se. As illustrated by Fig. 1, aphidicolin does not inhibit the anti-Ig-driven apoptosis of CD40L-activated blasts, whatever the time point considered. By contrast, blockade of the cells in the G_1 phase of the cycle with deferoxamine or mimosine strongly impairs their capacity to undergo apoptosis following triggering of the BCR. Mimosine, for example, caused more than a 50% reduction in the levels of specific apoptosis at 72 h. Therefore, the susceptibility of mature B cells to Ag receptor-induced death is acquired as they enter the S phase of the cell cycle. This finding suggests that the cycling potential of mature B cells is one of the parameters that regulates their threshold of sensitivity to BCR-induced apoptosis.

Ag receptor-mediated apoptosis of activated mature B cells involves activation of caspases

We next studied the signaling elements involved during the effector phase of BCR-induced apoptosis in activated memory B cells. To explore the caspase dependency of this apoptotic pathway, we compared the impact of the broad range caspase inhibitor Z-VAD-fmk on the apoptosis of CD40L-activated memory B cells driven either by anti-Ig or anti-Fas Abs. Two types of assays were used to evaluate the levels of apoptosis: first, estimation of the disruption of the mitochondrial transmembrane potential revealed by the $DiOC_6$ fluorochrome; second, examination of the membrane PS exposure, revealed by the binding of annexin V. As shown in Fig. 2*A*, Z-VAD-fmk inhibits in a dose-dependent fashion the PS externalization subsequent to anti-Ig treatment, whereas it only marginally affects the loss of mitochondrial transmembrane potential. On average, a 200 μM concentration of Z-VAD-fmk causes a 74% reduction of the specific apoptosis levels as estimated by annexin V binding, whereas it only induces a 19% reduction in the levels of specific apoptosis as determined by $DiOC_6$ staining. Because

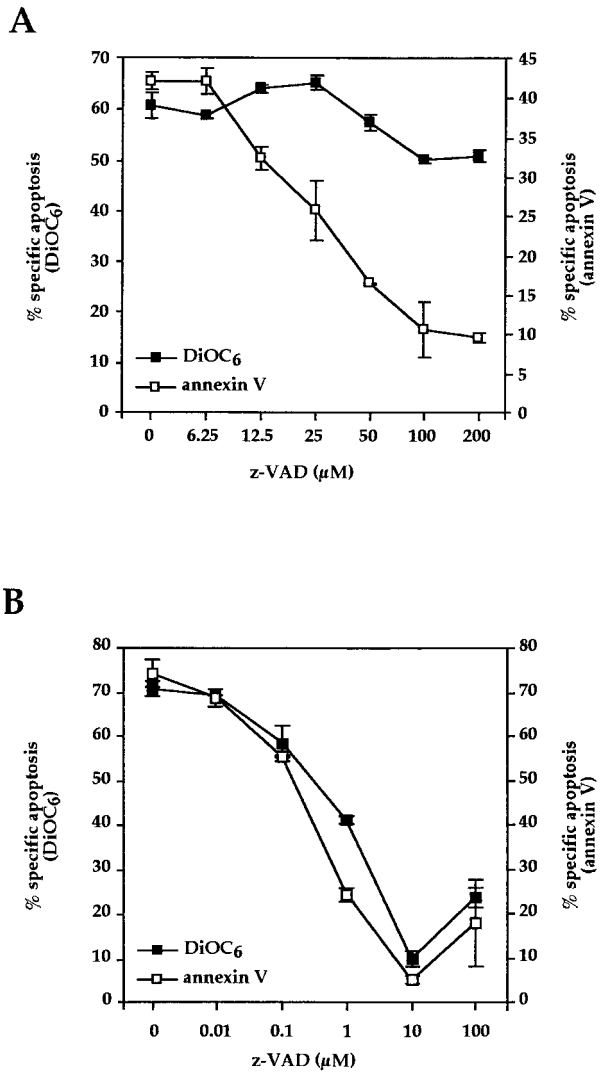

FIGURE 2. The broad spectrum caspase inhibitor Z-VAD-fmk inhibits the membrane, but not the mitochondrial alterations consecutive to BCR ligation in memory B blasts. CD40L-induced memory B blasts were recultured with CD40L, with or without $F(ab')_2$ fragments of anti-Ig Abs (*A*), with either the anti-Fas mAb 7C11 or its isotypic control (*B*). Both types of cultures were made in the presence or absence of serial dilutions of the Z-VAD-fmk peptide or the equivalent dilutions of DMSO. Both the mitochondrial and membrane alterations were estimated after 72 h of culture by $DiOC_6$ staining (*left y-axis*) and annexin V binding (*right y-axis*), respectively. Data are presented as means ± SD of the percent specific apoptosis calculated from duplicate determinations. Representative of four independent experiments.

this slight inhibition of loss of $\Delta\psi$m could only be detected at the latest culture point (72 h), it is evocative of a secondary positive feedback mechanism whereby activated caspases would amplify the mitochondrial damages. These observations suggest that alteration of the plasma membrane integrity subsequent to BCR ligation is dependent on activation of caspases, while disruption of the mitochondrial potential is not. In striking contrast, the loss of $\Delta\psi$m and PS externalization induced by the anti-Fas mAb 7C11 are both caspase-dependent events, because they are inhibited by Z-VAD-fmk (Fig. 2*B*). Besides, a concentration of Z-VAD 20-fold lower than that required to block the BCR-induced membrane alterations is sufficient for complete inhibition of anti-Fas-induced PS exposure. Altogether, these findings suggest that in activated memory B cells, the Fas death pathway is more critically dependent on

caspase activation than the BCR death pathway. Nevertheless, to confirm the involvement of caspases in the BCR apoptotic pathway, studies were then undertaken to directly assess the caspase activity following engagement of the BCR on CD40L-induced memory B blasts. There are at least 10 caspases identified to date, but the effector caspase-3 has been shown to be activated in response to multiple apoptotic stimuli (6, 9). Moreover, the involvement of caspase-3 in the BCR-induced apoptotic pathway has been documented in several B cell lymphoma models (13, 14). Therefore, we examined whether engagement of the BCR on CD40L-activated memory B cells could induce the cleavage of the typical caspase-3 substrate PARP. For this purpose, CD40L-stimulated memory B blasts were recultured with CD40L in the presence or absence of anti-Ig Abs, with the caspase inhibitor Z-VAD-fmk (200 μM) or the appropriate dilution of DMSO as a negative control. Analysis of the PARP protein was performed by Western blot in cell lysates prepared from cells recovered 72 h after the onset of the secondary cultures. As shown in Fig. 3, the full-length 116-kDa form of PARP is present in all four samples tested. Spontaneous cleavage of PARP occurs to some extent in untreated blasts (receiving neither anti-Ig Abs nor Z-VAD-fmk), as visualized by a faint 85-kDa band on the gel. Nevertheless, the 85-kDa cleavage fragment of PARP is only clearly detectable in anti-Ig-treated B cells. This assertion is further supported by the densitometry analysis of the 116- and 85-kDa bands (*lower panel*), which indicates that the ratio of the cleaved form vs the uncleaved form of PARP is at least 3 times higher in anti-Ig-treated blasts than in untreated blasts. We next used the cell-permeable fluorigenic substrate PhiPhilux-G_2D_2 to confirm the involvement of a caspase-3-like activity in BCR-induced apoptosis. CD40L-activated memory B blasts were recultured with CD40L, in the presence or absence of anti-Ig Abs, with or without Z-VAD-fmk. Memory B blasts treated with the agonistic anti-Fas mAb 7C11 were used as positive control because caspase-3 has been shown to be involved in the Fas

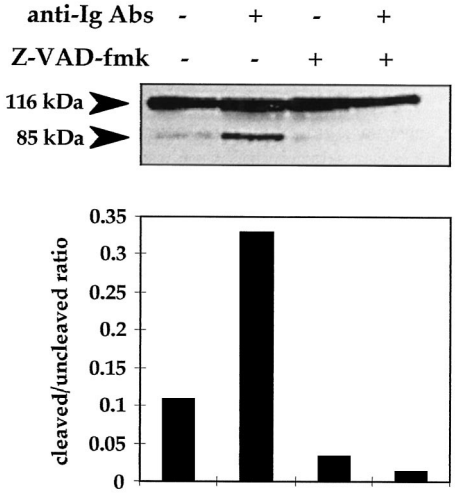

FIGURE 3. PARP cleavage is induced upon BCR ligation on CD40L-induced memory B blasts. Viable CD40L-induced memory B blasts were recultured for 72 h with or without $F(ab')_2$ fragments of anti-Ig Abs, in the presence of either the broad range caspase inhibitor Z-VAD-fmk (200 μM) or the equivalent dilution of DMSO (labeled as − on the figure). Equal amounts of whole cell lysates (corresponding to 10^6 cells) were loaded onto each lane of a 8% PAGE gel. After transfer to nitrocellulose membranes, the blots were sequentially incubated with an anti-PARP mAb and HRP-conjugated goat anti-mouse Igs and revealed with a chemiluminescence detection kit. A densitometry scanning of the 85- and 116-kDa bands was performed. The graph in the *lower panel* shows the cleaved/uncleaved PARP ratio calculated from the net intensity of the low and high molecular bands. Representative of three experiments.

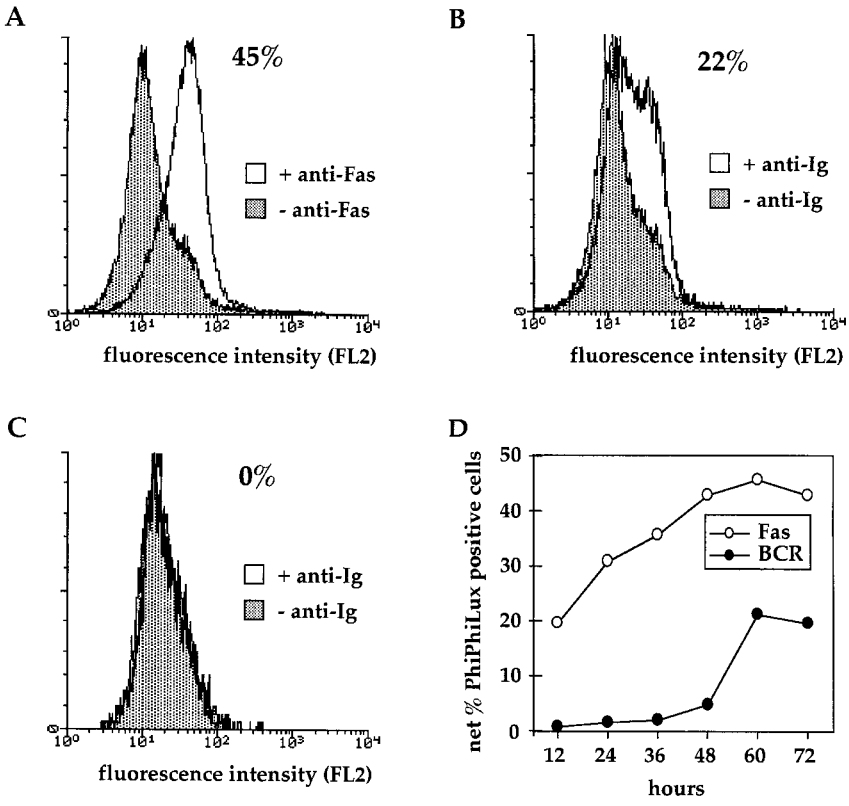

FIGURE 4. BCR ligation on CD40L-activated memory B cells induces activation of a caspase-3-like protease. Memory B blasts recovered after 48 h of stimulation with CD40L were recultured with CD40L and subjected to either anti-Fas or anti-Ig treatment. *A*, B blasts were cultured for 60 h with either the anti-Fas mAb 7C11 or its isotypic control. *B*, B blasts were cultured for 60 h in the presence or absence of $F(ab')_2$ fragments of anti-Ig Abs. *C*, Same culture conditions as in *B*, except that cells were incubated in the presence of 200 μM Z-VAD-fmk. *D*, B blasts were cultured with either Fas mAb 7C11 or its isotypic control (Fas) or in the presence or absence of $F(ab')_2$ fragments of anti-Ig Abs (BCR) for the indicated times. At the end of secondary cultures, cells were incubated with the fluorigenic substrate PhiPhilux-G_2D_2 and analyzed by flow cytometry on the FL2 channel. The net increase in the numbers of fluorescent cells (net percentage of PhiPhiLux positive cells = percentage of positive cells in anti-Fas or anti-Ig-treated samples − percentage of positive cells in control cultures) is indicated. Representative of three experiments.

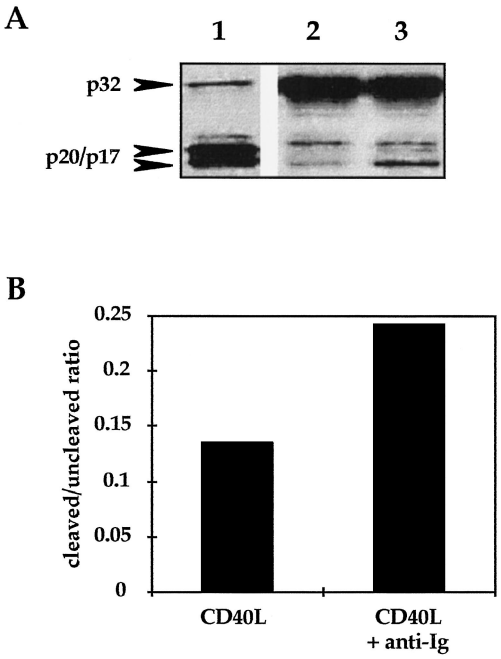

FIGURE 5. BCR ligation on CD40L-activated memory B cells induces cleavage of caspase-3. *A*, Memory B blasts were recultured for 60 h with CD40L with (*lane 3*) or without (*lane 2*) $F(ab')_2$ fragments of anti-Ig Abs. A lysate from Jurkatt cells treated for 12 h with the agonistic anti-Fas mAb 7C11 was used as a positive control (*lane 1*). Equal amounts of whole cell lysates (corresponding to 10^6 cells) were loaded onto each lane of a 15% PAGE gel. The blots were revealed with the anti-caspase-3 rabbit polyclonal Ab and subjected to densitometry analysis, as described in Fig. 3. *B*, The ratios between the 17- and the 32-kDa molecular form of caspase-3 ratios are shown. Representative of three experiments.

apoptotic pathway in both mature human B and T cell lines (13, 24). The capacity of B blasts to cleave the PhiPhilux-G_2D_2 substrate was estimated 60 h after the onset of the secondary cultures. The representative experiment shown in Fig. 4*A* indicates that ligation of Fas on memory B blasts induces an increase in the proportion of cells, producing a significant fluorescence emission upon addition of the substrate (64% in anti-Fas-treated cultures vs 19% in control cultures). Ligation of the BCR (Fig. 4*B*) also induces a rise in the percentage of blasts able to cleave the PhiPhiLux G_2D_2 substrate (42% in anti-Ig-stimulated cultures vs 20% in untreated cultures). Addition of Z-VAD-fmk at the onset of the secondary culture completely suppresses the anti-Ig-induced caspase-3-like activity (Fig. 4*C*). These results provide direct evidence for an activation of a caspase-3-like protease during BCR-induced apoptosis in activated memory B cells. Because other caspases such as caspase-7 could also be responsible for the BCR-induced cleavage of PARP and of the fluorigenic substrate PhiPhiLux G_2D_2, we tested by Western blotting whether anti-Ig Abs promote the release of the active cleavage product of caspase-3. As shown in Fig. 5, in memory B blasts, anti-Ig Abs promote a specific rise in the expression of the 17-kDa active cleavage product of caspase-3. Altogether, these findings indicate that the BCR apoptotic pathway in activated memory B cells involves the late activation of caspase-3 (Fig. 4*D*).

Activation of caspases occurs downstream of the mitochondria during BCR-induced apoptosis of activated B cells

The observation that PS exposure but not perturbation of $\Delta\psi$m is blocked by Z-VAD-fmk in anti-Ig-treated blasts suggested that the alteration of the plasma membrane induced by BCR triggering on activated B cells is a caspase-dependent process, while the premitochondrial phase is not. Because we found chemical agents known to block the mitochondrial function to be toxic for normal B cells, we used a cell line model to determine whether caspase activation is consecutive to the BCR-induced mitochondrial alterations. Because Burkitt lymphoma (BL) cell lines have long been recognized to be sensitive to BCR-induced apoptosis (25), experiments were thus undertaken with BL60 cells to address this issue. To test the validity of the Burkitt lymphoma model, we first determined the effect of the broad spectrum caspase inhibitor Z-VAD-fmk on the anti-Ig-induced death of BL60 cells. For this purpose, both $DiOC_6$ staining and annexin V binding were performed on BL60 cells cultured with or without immobilized anti-IgM Abs, in the presence or absence of Z-VAD-fmk. As shown in Table I, BCR-induced death of BL60 cells is rapid and massive, because the rates of specific apoptosis were 85 and 77% on the basis of $DiOC_6$ staining and annexin V binding, respectively, after 24 h of culture with anti-IgM Abs. The caspase inhibitor Z-VAD-fmk marginally affected the disruption of the mitochondrial potential (80% vs 85% specific apoptosis in control cultures), but dramatically reduced PS externalization (26% vs 77% specific apoptosis in control cultures) induced by anti-IgM Abs. This finding indicates that the mitochondrial and membrane alterations induced by anti-Ig Abs follow the same scheme in primary activated memory B blasts and in BL60 cells, that is the loss of $\Delta\psi$m is caspase independent, while alterations of the plasma membrane integrity require activation of caspases. We next investigated whether caspase activation during BCR-induced death is under the control of the mitochondria or regulated by a separate pathway independent from the mitochondria. To discriminate between these two possibilities, we decided to block the dATP-dependent association of cytochrome *c*, Apaf-1 (the mammalian homologue of CED-4), and procaspase-9. This multiprotein complex promotes processing of caspase-9, which in turn cleaves and activates caspase-3 (26). ATP levels were thus artificially reduced in the BL60 cell line by culturing the cells in a glucose-free medium in the presence of 2.5 μM oligomycin, as previously described (27, 28). This procedure prevents production of dATP from both glycolysis and oxidative phosphorylation. The anti-Ig-mediated PS exposure was first estimated on BL60 cells incubated or not in glucose-free medium, supplemented or not with oligomycin. As shown in Fig. 6*A*, neither glucose deprivation nor the addition of oligomycin significantly impaired the BCR-induced apoptosis of BL60 cells, when done separately. By contrast, a strong reduction

Table I. *Effect of Z-VAD-fmk on the BCR-induced disruption of the mitochondrial function and plasma membrane integrity in BL60 cells*[a]

Z-VAD	Anti-IgM Abs	Loss of $\Delta\psi$m (% apoptotic cells)	PS Exposure (% apoptotic cells)
−	−	39.4 ± 1.3	31 ± 2.3
−	+	91.2 ± 0 (85.4)	84.3 ± 0.4 (77.2)
+	−	41.3 ± 0.9	27.5 ± 0.6
+	+	88.5 ± 1.7 (80.4)	46.5 ± 0.6 (26.2)

[a] BL60 cells were cultured for 24 h with or without immobilized anti-IgM Abs, in the presence of the broad-range caspase inhibitor Z-VAD-fmk (200 μM) or the equivalent dilution of DMSO. Both PS exposure and $\Delta\psi$m were monitored at the end of the culture using annexin V binding and $DiOC_6$ staining, respectively. Data are expressed as means ± SD percent apoptotic cells ($DiOC_6^{low}$ or annexin$^+$) from duplicate determinations. The numbers in parentheses indicate the percent-specific apoptosis calculated from the percent apoptotic cells values estimated in the two apoptosis assays. The data are representative of three experiments.

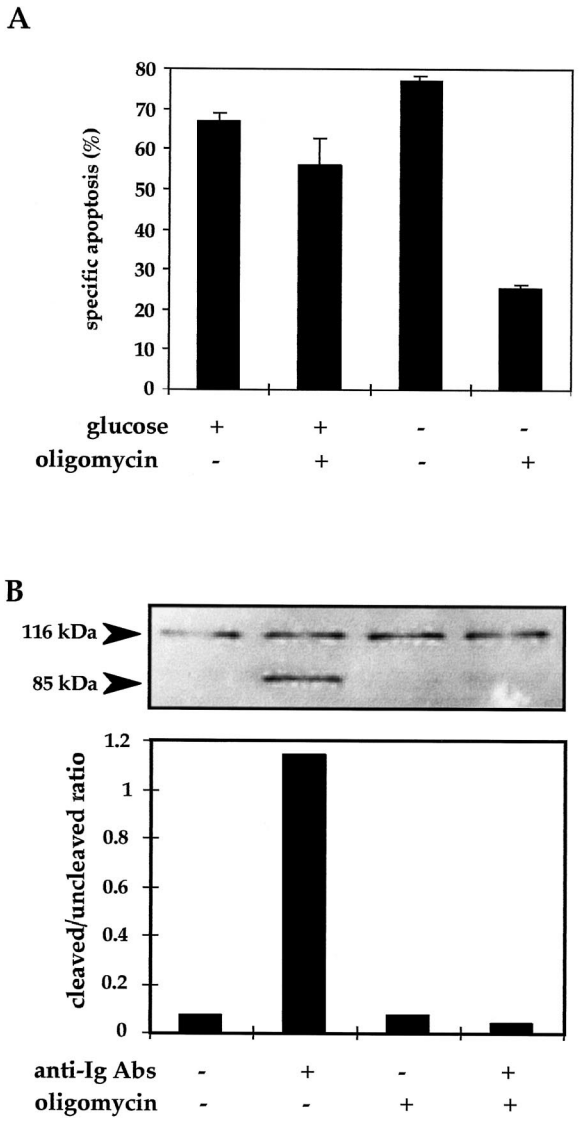

FIGURE 6. dATP depletion prevents the BCR-induced PS exposure and PARP cleavage in BL60 cells. *A*, BL60 cells were cultured for 24 h with or without immobilized anti-IgM Abs in either glucose-containing or glucose-free medium supplemented or not with oligomycin (2.5 μM). Apoptotic cells were estimated by monitoring PS externalization, as revealed by the binding of annexin V. Data are presented as means ± SD of the percent specific apoptosis calculated from duplicate determinations. *B*, BL60 cells were cultured in glucose-free medium for 6 h with or without immobilized anti-IgM Abs, in the presence or absence of oligomycin (2.5 μM). The blots were realized and subjected to densitometry analysis, as described in Fig. 3. The cleaved/uncleaved PARP ratios are shown. Representative of three experiments.

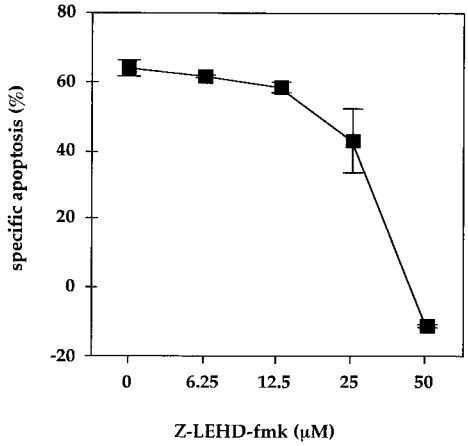

FIGURE 7. The caspase-9 inhibitor Z-LEHD-fmk reverses the proapoptotic effect of anti-Ig Abs on CD40L-induced memory B blasts. Memory B cell blasts were recultured for 72 h with CD40L, with or without $F(ab')_2$ fragments of anti-Ig Abs, in the presence or absence of serial dilutions of the peptide Z-LEHD-fmk or the equivalent dilutions of DMSO. Apoptotic cells were estimated by monitoring PS externalization, as revealed by the binding of annexin V. Data are presented as means ± SD of the percent specific apoptosis calculated from duplicate determinations. Representative of three independent experiments.

of the anti-Ig-induced PS externalization was observed when cultures were made in glucose-free medium in the presence of oligomycin (22% vs 65% specific apoptosis in control cultures). We next investigated whether dATP depletion and subsequent blockade of the proapoptotic function of the mitochondria have an impact on the activation of caspase-3 consecutive to the engagement of the BCR. PARP cleavage was therefore examined in BL60 cells cultured in glucose-free medium, with or without anti-IgM Abs, in the presence or absence of oligomycin. As expected, in the absence of oligomycin, ligation of the BCR on BL60 induces the appearance of the 85-kDa cleavage product of PARP. Addition of oligomycin completely prevented the anti-Ig-induced degradation of PARP, thus indicating that caspase-3 activation during BCR-mediated apoptosis occurs downstream of the mitochondria. Because these data were evocative of a possible role for the cytochrome *c*/Apaf-1/procaspase-9 complex in the processing of effector caspases-3 induced by anti-Ig Abs, we next investigated whether caspase-9 intervenes in the apoptotic process elicited by the BCR in CD40L-activated memory B cells. For this purpose, memory B blasts were recultured with or without anti-Ig Abs, in the presence or absence of serial dilutions of the caspase-9 inhibitory peptide Z-LEHD-fmk. Specific apoptosis was estimated after 72 h of culture by evaluation of PS exposure. As shown in Fig. 7, the caspase-9 inhibitory peptide Z-LEHD-fmk blocked the proapoptotic effect exerted by anti-Ig Abs on CD40L-activated memory B cells, in a dose-dependent manner, reaching complete suppression at a 50 μM concentration. To investigate further the caspases involved in the BCR death pathway, we next compared the impact of the caspase-8 inhibitory peptide Z-IETD-fmk on the death of activated memory B cells promoted either by anti-Fas or anti-Ig Abs. The results of a representative experiment illustrated by Fig. 8 show that the broad range caspase inhibitor Z-VAD-fmk strongly reduces apoptosis caused by BCR or Fas engagement. In contrast, while Z-IETD-fmk brought about the same level of inhibition of apoptosis than Z-VAD-fmk in anti-Fas-treated cells, it only marginally affected the BCR-induced PS exposure. Z-IETD-fmk was still unable to inhibit BCR-induced death even when its concentration was increased up to 200 μM (data not shown). This supports the conclusion that caspase-8 activation is not primarily involved in the BCR-induced caspases cascade, leading to death in CD40L-induced memory blasts. Altogether, these findings are compatible with the notion that triggering the BCR on activated B lymphocytes induces a mitochondrial damage, which in turn causes the activation of caspases-9 and -3.

Discussion

This study aimed at exploring the molecular mechanisms involved in the sensitization of human memory B cells to BCR-induced apoptosis and in the execution of this death pathway. The apoptotic

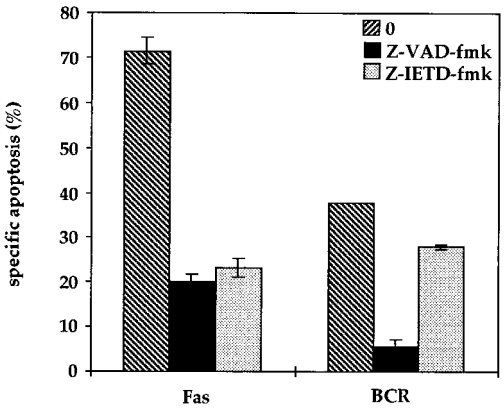

FIGURE 8. The Fas but not the BCR death pathway is sensitive to the caspase-8 inhibitor Z-IETD-fmk. Memory B cell blasts were recultured for 72 h with CD40L, with or without $F(ab')_2$ fragments of anti-Ig Abs (BCR), or with either the anti-Fas mAb 7C11 or its isotypic control (Fas) in the presence or absence of either Z-VAD-fmk or Z-IETD-fmk (100 μM). Apoptotic cells were estimated by monitoring PS externalization, as revealed by the binding of annexin V. Data are presented as means ± SD of the percent specific apoptosis calculated from duplicate determinations. Representative of three independent experiments.

response triggered through the Ag receptor has long been considered as an exclusive feature of immature B cells (29, 30). Nevertheless, two lines of evidence indicate that engagement of the BCR on mature B lymphocytes can also promote their death under certain circumstances. First, mature B cell neoplasms such as group I Burkitt lymphomas undergo apoptosis after BCR cross-linking (25). Second, we have recently demonstrated that BCR ligation enhances the survival of resting memory B cells, but induces their death when they have been previously activated through CD40 or the BCR (18). This observation strongly suggested that activation stimuli can connect the BCR to a death pathway in the mature B cell lineage. The BCR-induced death of mature B cells and AICD of mature T cells are thus strikingly similar inasmuch as both processes are initiated by triggering of the Ag receptor on activated lymphocytes (3, 4). This led us to propose that AICD may contribute to the regulation of both cellular and humoral immune responses.

The data shown in this work are in agreement with this hypothesis because, as previously described for T cell AICD (23), B cells acquire susceptibility to BCR-induced death upon entry into the S phase of the cell cycle. Because proliferating cells are the preferential targets of the negative regulatory control exerted by the BCR, it can thus be speculated that the sensitivity of mature B cell subsets to AICD should be closely correlated with their ability to cycle in response to primary activation stimuli. In return, the negative feedback control exerted by Ag on B cell responses should not cause massive or premature deletion of the activated B cell pool in the course of the humoral response. Both CD40L and IL-4 have been described to protect Burkitt lymphoma and immature B cell lines from BCR-induced killing (31, 32). This suggests that the proapoptotic function of the BCR on activated B cells could be balanced by T cells. Both Ag and T cell influences would thus contribute to maintain homeostasis of the B cell compartment by regulating the size of the activated B cell pool during the expansion phase of the humoral response. However, neither CD40L nor various T cell-derived mediators (including IL-4) were able to counteract the death-promoting effect of surrogate Ag on activated memory B cells (data not shown). Therefore, the nature of the putative factors conferring resistance to AICD on activated memory B cells still remains elusive.

We next explored the molecular effector mechanisms involved in the death pathway connected to the BCR in activated memory B cells. One of the first transductional events associated with triggering of DD-containing receptors is the recruitment and activation of an initiator caspase (caspase-8 or -10) that is responsible for the subsequent cascade of damages that occurs during the apoptotic cell death (10, 11). Two features of the BCR-induced apoptosis described herein argue against the possibility that B cell AICD could involve the interaction between a DD-containing receptor and its ligand, as it is the case for T cell AICD. First, a caspase-8 inhibitory peptide does not counteract the proapoptotic effect of anti-Ig Abs in CD40L-induced memory blasts. Second, transduction of the death signal from the Ag receptor to the mitochondria does not rely on caspase processing. The ability of Z-VAD-fmk to prevent the membrane but not the mitochondrial alterations resulting from BCR ligation on activated memory B cells strongly argues for a partitioning of the BCR-induced death pathway into a caspase-independent phase and a caspase-dependent one, intervening upstream and downstream of the mitochondria, respectively. We extended this observation by showing that BCR-induced mitochondrial dysfunctioning is involved in controlling caspase activation during this process. This assertion is supported by two lines of evidence. First, dATP depletion, which prevents formation of the cytochrome *c*/Apaf-1/procaspase-9 complex, inhibits both the BCR-induced PS exposure and PARP cleavage in BL60 cells. Second, the inhibitory effect exerted by the Z-LEHD-fmk peptide on the BCR death pathway suggests that caspase-9 participates in the death effector cascade during BCR-induced apoptosis of CD40L-activated memory B cells. These results favor the hypothesis that propagation of the death signal from the BCR involves the mitochondria-dependent cleavage of procaspase-9 that in turn activates other downstream apoptosis effectors.

Two independent sets of data establish that caspase-3 is activated during BCR-induced apoptosis in activated memory B cells: 1) the caspase substrates PARP and PhiPhiLux G_2D_2 are cleaved in anti-Ig-treated cells; 2) anti-Ig Abs enhance the release of the active cleavage product of caspase-3 in CD40L-activated memory B cells. We found activation of caspase-3 to be delayed during BCR-induced apoptosis with respect to its kinetics of activation during Fas-mediated killing (data not shown). However, our observation that mitochondrial damages precede caspase activation and PS exposure following BCR ligation suggests that anti-Ig-treated cells are already committed to death before caspase processing occurs. Therefore, the late and moderate activation of caspases during BCR-induced apoptosis should not be regarded as an indication of the poor efficiency of the death signaling pathway triggered by the Ag receptor in B cells. Interestingly, Graves and colleagues (33) recently provided evidence that protein neosynthesis is required for BCR-induced apoptosis to occur in a B lymphoma cell line. We have shown in this work that the postmitochondrial phase of BCR-induced death essentially relies on processing of preformed caspases. It is thus tempting to speculate that de novo synthesis of a protein component is more likely to be involved in transduction of the apoptotic signal from the BCR to the mitochondria. However, because Z-VAD-fmk does not prevent the first cleavage step of caspase-3 (which produces the intermediate p20/p12 inactive heterodimer) during BCR-mediated apoptosis of Ramos cells (13), we cannot formally exclude that proteases other than caspases participate in the BCR-mediated activation of caspase-3 in activated memory B cells. A hypothetical model for the transduction of the apoptotic signal through the BCR, based on our present experimental findings and on the published literature, is depicted in Fig. 9.

In conclusion, this study illustrates that sensitization of memory B cells to BCR-induced apoptosis relies on their cycling status. This study also highlights that the Ag receptor death pathway in

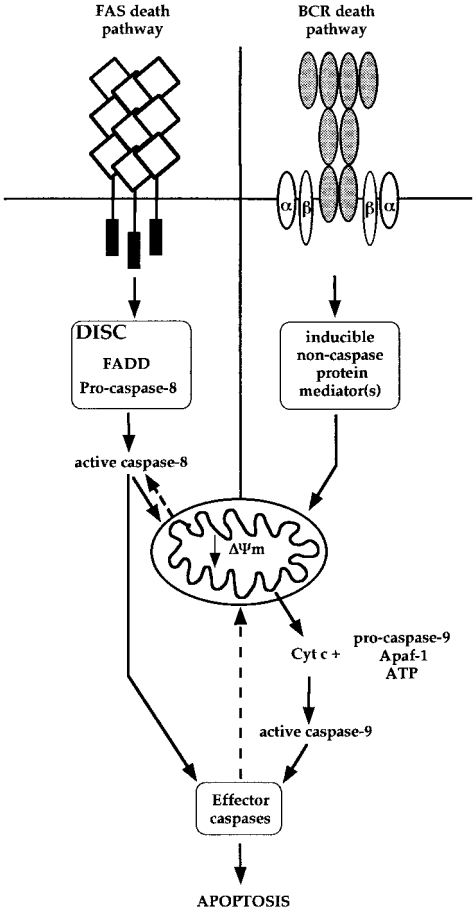

FIGURE 9. Hypothetical model of the apoptotic pathways coupled to Fas and the Ag receptor in mature B cells. Triggering of Fas on activated memory B cells results in the early formation of a DISC and subsequent recruitment and activation of initiator caspases such as caspase-8, through the adaptor molecule FADD. Caspase-8 could also be further activated downstream of the mitochondria (dotted arrow). Caspase-8 activates downstream effector caspases (such as caspase-3) either directly or indirectly through disruption of the mitochondrial function. Engagement of the BCR on CD40L-activated memory B cells primarily induces breakdown of the mitochondrial membrane potential in a caspase-independent fashion, which leads to processing of caspase-9 and subsequent activation of downstream effector caspases. These activated apoptosis effectors may in turn affect the mitochondrial function, thus amplifying the apoptotic process initiated by Fas or the BCR (dotted arrow).

mature B cells consecutively involves a caspase-independent and a caspase-dependent phase taking place upstream and downstream of the mitochondria, respectively. It describes for the first time in activated normal human B cells that connection of the BCR to the caspase effectors of apoptotic cell death is ensured by the mitochondria. Which molecular event causes the rewiring of the Ag receptor to the death-inducing pathway in activated memory B cells? What are the molecules upstream of the mitochondria that cause disruption of its transmembrane potential and how do they alter mitochondrial integrity? Are they constitutively connected to the BCR? These are some of the next questions to be addressed in future studies.

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