CM1 Ligation Induces Apoptosis via Fas-FasL Interaction in Ramos Cells, but via Down-regulation of Bcl-2 and Subsequent Decrease of Mitochondrial Membrane Potential in Raji Cells

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ABSTRACT

Background: CM1 (Centrocyte/-blast Marker I) defined by a mAb developed against concanavalin-A activated PBMC, is expressed specifically on a subpopulation of centroblasts and centrocytes of human germinal center (GC) B cells. Burkitt lymphoma (BL) is a tumor consisting of tumor cells with the characteristics of GC B cell. Previously we reported that CM1 ligation with anti-CM1 mAb induced apoptosis in Ramos (IgM^{high}) and Raji (IgM^{low}) cells. **Methods & Results:** In the present study, we observed that CM1 ligation with anti-CM1 mAb induced Fas ligand and Fas expression in Ramos cells, but not in Raji cells. Furthermore, anti-Fas blocking antibody, ZB4, blocked CM1-mediated apoptosis effectively in Ramos cells, but not in Raji cells. Increased mitochondrial membrane permeabilization, which was measured by $DiOC_6$, was observed only in Raji cells. In contrast to no significant change of Bax known as pro-apoptotic protein, anti-apoptotic protein Bcl-2 was significantly decreased in Raji cells. In addition, we observed that CM1 ligation increased release of mitochondrial cytochrome c and upregulated caspase-9 activity in Raji cells. **Conclusion:** These results suggest that apoptosis induced by CM1-ligation is mediated by Fas-Fas ligand interaction in Ramos cells, whereas apoptosis is mediated by down-regulation of Bcl-2 and subsequent decrease of mitochondrial membrane potential in Raji cells. **(Immune Network 2006;6(2):59-66)**

Key Words: CM1, apoptosis, Ramos (centroblasts), Raji (centrocytes)

Introduction

CM1 molecule is expressed only in germinal center B cells, specifically, in a subpopulation of centroblasts and centrocytes. Immunohistochemical staining has revealed that the CM1 molecule is distributed over the entire GC area. CM1 (centrocyte/-blast marker 1) is defined by a mAb developed against concanavalin-A (con-A) activated peripheral blood mononuclear cells (PBMC)(1).

Burkitt lymphoma (BL) is a tumor with the characteristics of germinal center B cells (2,3). The phenotypic profile of BL cell lines is remarkably similar to that associated with normal B cells found in the GC (4). In the BL cell line, Ramos cells display the centroblast-like surface markers, CD10+, CD38+, CD77+ (5), whereas Raji cells display the centrocyte-like surface markers, characterized as CD10+, CD38+, and CD77−. However, interestingly, both cell lines express CM1 on surface (6).

Apoptosis is a sophisticated mechanism to eliminate cells in a manner that prevents the release of their intracellular contents, which might elicit an unwanted immune response, into the environment (7). The apoptosis of B lymphocytes is essential for

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the elimination of self-reactive B cells and low-affinity B cells during development of the germinal center B cells (8,9). Such a phenomenon in the physiology of germinal center reaction that results in survival of the functional B-cell repertoire and the elimination of abnormal cells, dictates the fate towards B-cell homeostasis or disease (10).

Apoptosis is an active form of cell death controlled by the expression of highly conserved genes, which either activate or suppress the process of cell death (11). The initial phase of triggering apoptosis includes activation of appropriate death receptors by specific ligands, such as tumor necrosis factor (TNF) and Fas ligand (FasL), which are the most extensively studied inducers of apoptosis (12).

Various types of cell death, induced by drugs or mediated by activation of T-cell and B-cell receptors, might employ pathway of triggering apoptosis by up-regulation of FasL expression followed by Fas/FasL dependent signaling (13-16). These types of apoptosis could be effectively prevented by neutralizing anti-Fas IgG. Fas-mediated signals are necessary for the physiologic elimination of self-reactive B cells (17), and CD95 is expressed on B cells in GC where extensive apoptosis occurs. These relationships suggest the possibility that CD95 induction in B cells plays a role in B cell death *in vivo* (18).

One of the major components of cell death machinery is the Bcl-2 family. To date, at least 20 members of the family have been reported in mammals. Bcl-2 family proteins either reside constitutively on the mitochondrial membrane or translocate to mitochondria in response to apoptosis stimuli and regulate the release of pro-apoptotic molecules from mitochondria. Thus, they work as important sensor for cell death signals (19).

The anti-apoptotic members of this family, such as Bcl-2, Bcl-x_L, and Mcl-1, preserve mitochondrial integrity and prevent the release of cytochrome c (20,21). Apoptotic signals, induced by various factors, can be down-regulated by Bcl-2 over-expression and up-regulated by suppression of Bcl-2 expression (22).

The pro-apoptotic protein Bax, which is common to several apoptotic pathways, potentiates death in response to multiple stimuli (23). A decrease in the ratio of Bcl-2:Bax cause changes in the membrane potential of the mitochondria (24).

Mitochondrial changes represent a primary event in apoptotic cell death, since the apoptogenic factor, cytochrome c, is released into the cytoplasm (25-29). Once this translocation occurs, cytochrome c binds to another cytoplasmic factor, Apaf-1, and the formed complex activates the initiator caspase-9 that in turn activates the effector caspase (30,31).

It has been observed that apoptosis is induced by anti-CM1 mAb in tonsillar germinal center B cell and Burkitt lymphoma cell line, such as Ramos and Raji cells, although underlying mechanism remains unclear (1,6).

In the present study, we have observed whether Fas/FasL or pro- and anti-apoptotic members of Bcl-2 family are involved in apoptosis induced by CM1-ligation in Ramos and Raji cells. In addition, we have investigated whether CM1 alters the mitochondrial membrane potential leading to the release of cytochrome c and activation of caspase-9 in Raji cells.

Materials and Methods

Cells and cell culture. The Burkitt lymphoma cell lines Ramos and Raji were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). All cell lines were maintained in continuous log phase growth and cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM of L-glutamine, 10 μg/ml of streptomycin, and 100 U/ml of penicillin. Cells were maintained in a moist atmosphere with 5% CO_2 at 37°C.

Production of anti-CM1 monoclonal antibodies. Balb/c mice were immunized with 1×10^7 con-A activated PBMCs at 2-week intervals for 2 months. Spleens were removed, and 1×10^8 spleen cells were fused with 1×10^7 SP2/0-Ag14 mouse myeloma cells using polyethylene glycol (PEG 4000; Sigma-Aldrich, St. Louis, MO, USA). Hundreds of the resulting hybridoma cell lines were screened by immunohistochemical staining of their supernatants in serial frozen sections of human tonsil. One of these cell lines revealed strong positive staining for some cells in the germinal center, and this was named anti-CM1 mAb. The isotype of the anti-CM1 mAb was IgG_1, as determined by enzyme immunoassay using Screen TypeTM (Boehringer-Mannheim, Germany).

Cell treatments. Anti-CM1 mAb and its isotype control antibody (CD3, mouse IgG_1; BD Biosciene, San Diego, CA, USA) were conjugated with protein-G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hours at room temperature, washed in PBS, and then suspended in 500 μl of PBS. To investigate the apoptosis-inducing effect of CM1, target cells were incubated with anti-CM1 mAb or isotype control conjugated with protein-G plus agarose beads for each time at 37°C.

Analysis of apoptosis by phosphatidylserine exposure and PI uptake. Apoptosis was determined by analyzing phosphatidylserine exposure and membrane integrity by double staining with annexin V-FITC and PI, as previously described (6). The percentages of apoptotic (annexin V+/PI− and annexin V+/PI+)

and necrotic cells (annexin V−/PI+) were determined by counting a fixed number of events in the gate established on Epics Altra supported by the Expo32 program (Beckman Coulter, Fullerton, CA, USA), using the forward and 90° light scatter plots of viable and apoptotic cells.

Analysis of changes in $\Delta\Psi_m$. Changes in $\Delta\Psi_m$ were determined by 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) fluorochrome assay (Molecular Probes Inc., Eugene, OR), which is used to reveal distruption of the mitochondrial transmembrane potential, as described previously (32). In brief, cells (5×10^5) were incubated with $DiOC_6$ (20 nM in PBS) for 15 minutes at 37°C, and then analyzed by flow cytometry (Epics Profile: excitation, 488 nm; emission, 525 nm).

Flow cytometric analysis of Fas and Fas-L expression. Cell surface expression of Fas and Fas-L was quantified by flow cytometry. The expression of Fas was analyzed by incubating 10^6 cells/ml with monoclonal anti-Fas-fluorescein isothiocyanate (FITC) antibody (DX2, BD bioscience) or control mouse IgG_1-FITC antibody (BD bioscience) for 30 minutes on ice. FasL was quantified by incubation with monoclonal anti-FasL antibody (NOK-1; BD bioscience) or control mouse IgG_1 antibody (BD bioscience) for 30 minutes on ice, followed by detection using FITC- labeled rabbit anti-mouse IgG (Dako, Ely, United Kingdom) for 30 minutes on ice. Cell so labeled were washed twice and resuspended in PBS. Flow cytometry data was obtained using Epics ALTRA (Beckman Coulter). The Data were analyzed using the Expo32 program (Beckman Coulter).

Western blot analysis. Cells were washed with PBS and lysed in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% Triton X-100 and protease inhibitors. Lysates were centrifuged at 10,000 g for 10 minutes and then supernatants were collected, and the protein concentration of the supernatant was determined using a protein assay system (Bio-Rad, Richmond, CA, USA). The total cell lysates (30 μg of protein) were subjected to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were then transferred electrophoretically to nitrocellulose transfer membrane. After electrotransfer, the membrane was blocked for overnight at 4°C in 5% skim milk. Primary antibodies were incubated for 1 hour at room temperature. Anti-Bcl-2 Ab (mouse monoclonal antibody; Santa Cruz Biotechnology) was used at 1 : 300, and anti-Bax Ab (rabbit polyclonal antibody; Santa Cruz Biotechnology) was used at 1 : 500. Then the membrane was incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature, and specific bands were detected using ECL, according to the manufacturer's protocol (Amersham, Arlington Heights, IL, USA).

Cytochrome c release measurements. Release of cytochrome

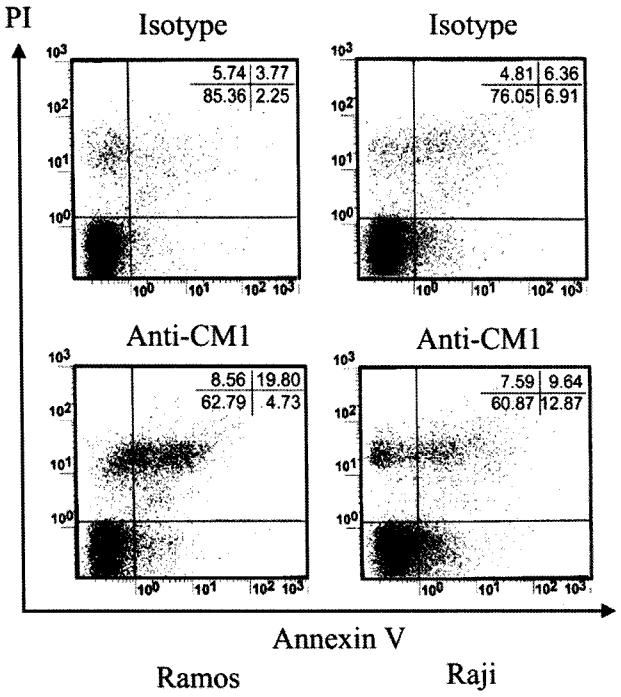


Figure 1. Apoptosis induced by anti-CM1 mAb in BL cells, Ramos and Raji. Target cells, Ramos and Raji, were cultured for 24 hours under the indicated conditions. Cultured cells (5×10^5) were harvested and stained with FITC-labeled Annexin V and PI. Numbers in each quadrant represent percentages. The figure shown is a representative of five different experiments.

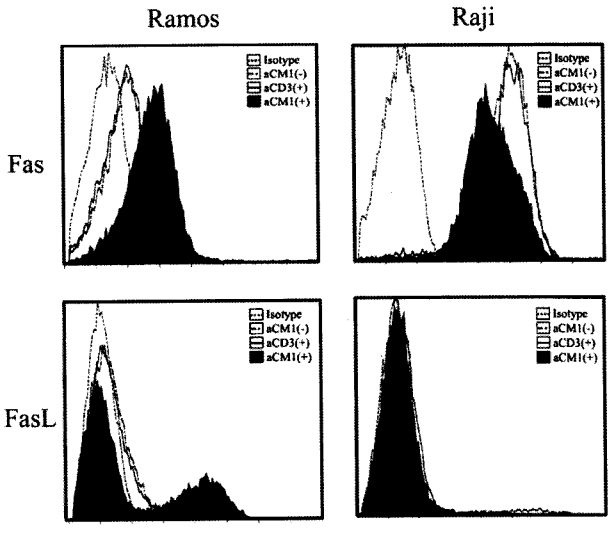


Figure 2. Expression pattern of apoptosis-associated surface molecules. Ramos and Raji cells were placed under each conditions. (- - -) represent isotype controls of staining. (——) represent negative control, untreated with anti- CM1 mAb. (——) represent isotype control, treated cells with anti-CD3 mAb and (■) represents treated cells with anti-CM1 mAb.

c from mitochondria to cytosol was measured by Western blot as previously described (33). Cell (1×10^6) were harvested, washed once with ice-cold phosphate-buffered saline and resuspened in 200 μl of ice-cold digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 190 μg/ml digitonin and protease inhibitors). Lysates were centrifuged at 10,000 g at 4°C for 5 min to obtain the supernatants (cytosolic extracts free of mitochondria). Supernatants (50 μg) were electrophoresed on a 15% polyacrylamide gel and then analyzed by western blot using anti-cytochrome c antibody at 1 : 500 (mouse monoclonal antibody; BD Bioscience) and ECL, as described above.

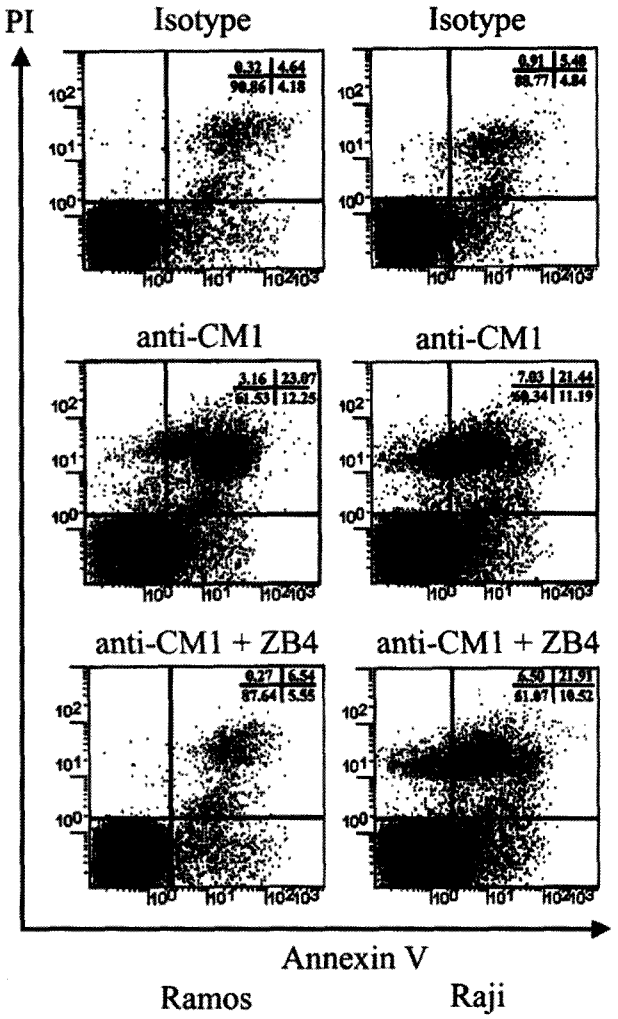


Figure 3. Apoptosis induced by anti-CM1 mAb and effect of ZB4, antagonistic anti-Fas mAb on it in BL cells, Ramos and Raji. Target cells, Ramos and Raji, were cultured for 24 hours under the indicated conditions. Cultured cells (5×10^5) were harvested and stained with FITC-labeled Annexin V and PI. Numbers in each quadrant represent percentages. The figure shown is a representative of five different experiments.

Measurement of caspase-9 activity. The quantitative measurement of caspase-9 activity was performed with a caspase-9 fluorometric assay kit using specific substrates, LEHD-AFC. Briefly, cells were cultured with anti-CM1 mAb for a given period. Then, cytosolic protein was extracted and diluted to 100 μg in a volume of 50 μl and incubated with corresponding substrate, LEHD-AFC at 37°C for 2 hours. We measured the plate on a fluorescent microplate reader using filters that allowed light excitation at 400 nm and collected emitted light at 505 nm.

Results

CM1 induces Fas/FasL expression in Ramos cell. A previous report showed that CM1 induced apoptosis in Burkitt lymphoma cell line, Ramos and Raji cells (6), As shown in Fig. 1, after incubation with anti-CM1 mAb for 24 hours, the percentage of apoptotic cells was determined by flow cytometry using double staining with FITC-conjugated annexin V and PI.

To evaluate the mechanisms by which CM1 induced apoptosis in Ramos and Raji cell, we determined the signaling pathway of the apoptosis. We first evaluated the change of cell surface expression of Fas and FasL on Ramos and Raji cells. As shown in Fig. 2, following anti-CM1 mAb stimulation, Fas expression was up-regulated in Ramos, but down-regulated in Raji. Also, FasL expression

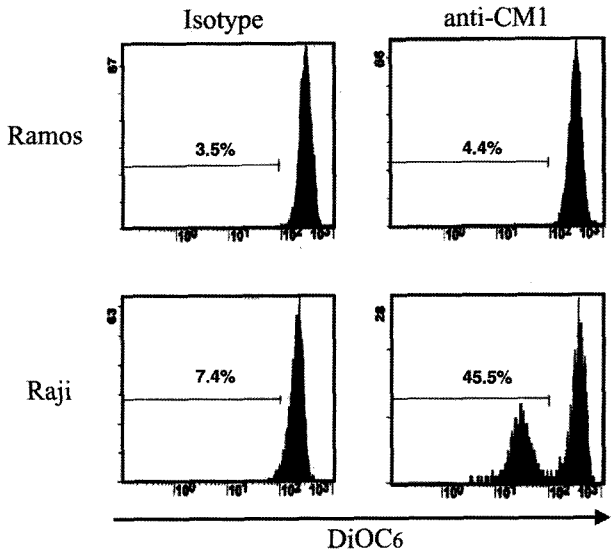
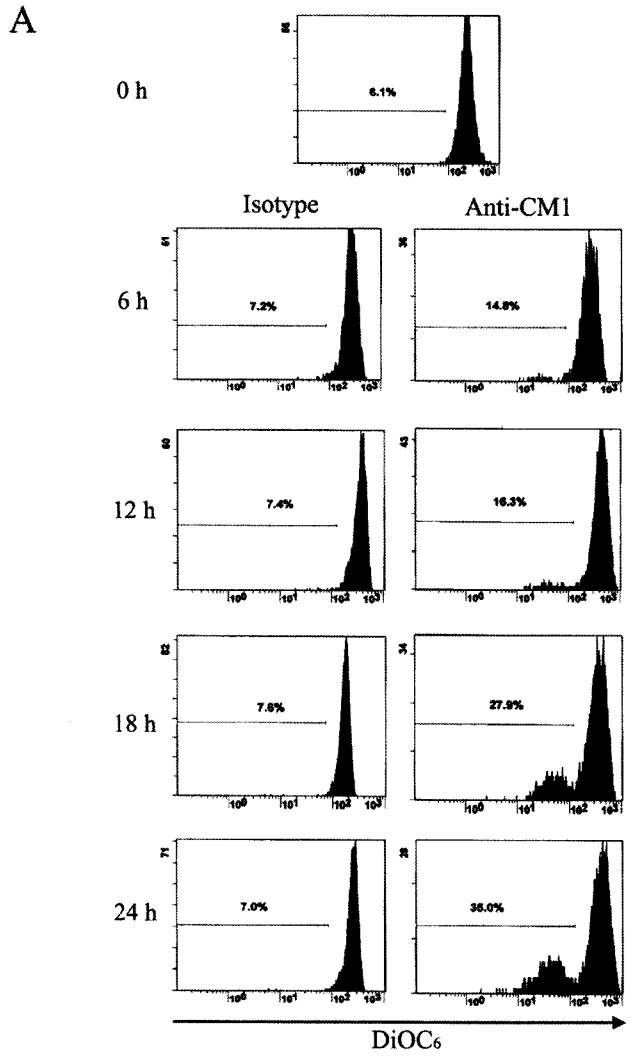


Figure 4. The effect of anti-CM1 mAb on mitochondrial transmembrane potential in Ramos and Raji cells. Ramos and Raji cells were cultured with each condition for 24 hours. For $DiOC_6$ staining, cells (5×10^5) were incubated with $DiOC_6$ (20 nM in PBS) for 15 minutes at 37°C, followed by flow cytometric analysis. Results are representatives of five independent experiments.



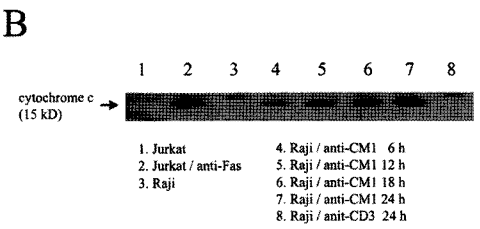


Figure 5. Time kinetics of mitochondrial membrane potentials and cytochrome c release after ligation of CM1 with anti-CM1 mAb in Raji cells. (A) Raji cells were cultured with anti-CM1 for the periods indicated. For $DiOC_6$ staining, cells (5×10^5) were incubated with $DiOC_6$ (20 nM in PBS) for 15 minutes at 37°C, followed by flow cytometric analysis. (B) Cells were cultured with each condition for the periods indicated, and were suspended in digitonin buffer as described in Materials and Methods. Protein extract (30 μg) were prepared and separated on 15% polyacrylamide gel and analyzed by western blotting with anti-cytochrome c Ab.

was up-regulated in Ramos, but not changed in Raji cells stimulated by anti-CM1 mAb.

The effect of antagonistic anti-Fas Ab (ZB4) on anti-CM1 mAb induced apoptosis in Ramos cells. To study whether the Fas receptor-ligand interaction is involved in the CM1-ligation induced apoptosis, Burkitt lymphoma cell lines, Ramos and Raji cells were treated with the antagonistic anti-Fas Ab (ZB4), before the addition of anti-CM1 mAb. ZB4 inhibited the CM1-ligation induced apoptosis in Ramos cells but it exerted no effect on the CM1-ligation induced apoptosis in Raji cells (Fig. 3). Thus, the Fas receptor-ligand system is involved in the CM1-ligation induced apoptosis in Ramos cells but not in Raji cells. These results suggest that CM1-ligation induced apoptosis is associated with the Fas-mediated pathway in Ramos cells. Whereas, CM1-ligation induced apoptosis may be related with another pathway in Raji cells.

CM1 induces apoptosis in Raji cells via decrease of $\Delta\Psi_m$ and subsequent release of cytochrome c to the cytosol. To

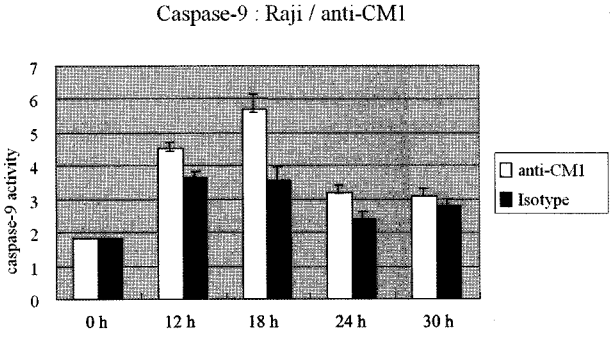


Figure 6. Activation of caspase-9 during anti-CM1-mAb induced apoptosis in Raji cells. Cells were cultured with anti-CM1 mAb for the period indicated, cells extracts were prepared, and activation of caspase-9 was determined with a caspase-9 fluorometric assay kit using specific substrates, LEHD-AFC. Reading the plates was performed on a fluorescent microplate reader using filters that allow light excitation at 400 nm and can collect emitted light at 505 nm. Results are representative of three independent experiments.

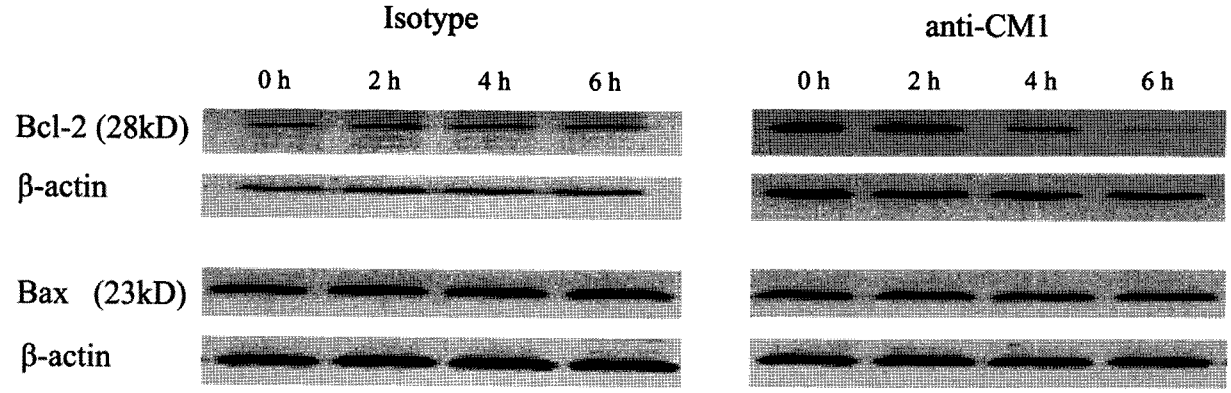


Figure 7. Effects of anti-CM1 mAb on the expression of Bcl-2 and Bax in Raji cells. Effects of anti-CM1 mAb on Bcl-2 and Bax were detected by western blot analysis in Raji cells. Cells were cultured with anti-CM1 mAb for 0, 2, 4, and 6 hours. Cell lysates were analyzed by 15% SDS-PAGE and probed with primary antibodies as described in materials and methods. Results are representative of three independent experiments.

explore the impact on mitochondria, we determined the mitochondrial transmembrane potentials. CM1-mediated signaling in Ramos cells did not induce a reduction in the level of mitochondrial transmembrane potentials as determined by $DiOC_6$ staining. However, anti-CM1 mAb treatment induced significant reduction of the mitochondrial transmembrane poteintial in Raji cells. (Fig. 4)

To analyze the involvement of cytochrome c release in CM1-ligation induced apoptosis in Raji cells, cytosolic fractions were obtained and analyzed for the presence of cytochrome c by the western blot. As shown in Fig. 5B, ligation of CM1 induced the appearance of cytochrome c in the cytosolic fractions of Raji cells.

Cytosolic cytochrome c binds to Apaf-1 and induces activation of caspase-9 (34). To demonstrate that this pathway was activated in CM1-ligation induced apoptosis, caspase-9 was analyzed by caspase-9 fluorometric assay kit in whole cell extracts. Ligation of CM1 induced activation of caspase-9 in the Raji cells, as shown by the increased activity of caspase-9 (Fig. 6).

Anti-apoptotic molecule, Bcl-2 expression significantly down-regulated but Bax expression did not reveal any changes in Raji cells after ligation of CM1. Down-regulation of anti-apoptotic protein, such as Bcl-2 and Bcl-x_L could result in activation of appropriate apoptotic factors, thus supporting initiation of cell death (22). Alternatively, apoptosis might prevent survival signals by enhancing the activity of pro-apoptotic proteins, such as Bax (11). To evaluate ability of CM1 to affect the level of Bcl-2 and Bax proteins, we tested changes in the expression of these proteins in Raji cells undergoing CM1-ligation induced apoptosis.

As shown in Fig. 7, a decrease in the expression of anti-apoptotic molecules, Bcl-2 was observed in Raji cells after addition of anti-CM1 mAb. However, no significant change in the expression of pro-apoptotic molecule, Bax was observed after stimulation of Raji cells with anti-CM1 mAb.

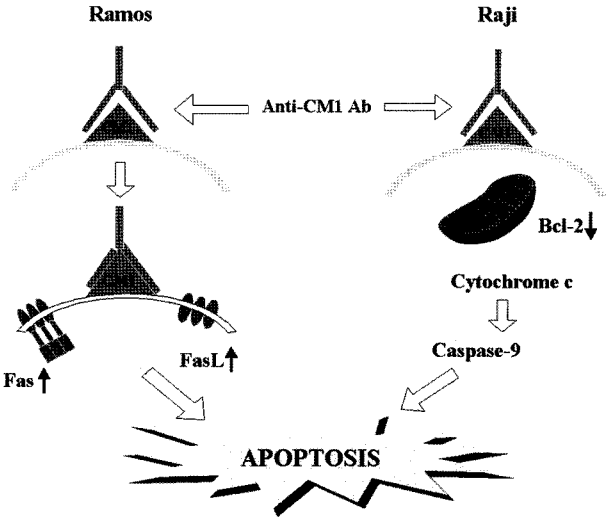


Figure 8. Suggested signaling pathways in CM1-mediated apoptosis of Ramos and Raji cell lines, respectively. In Raji cells, ligation of CM1 induces relative increase in the amounts of Bax via down-regulation of Bcl-2, followed by decrease of $\Delta\Psi_m$, which leads to the release of cytochrome c from mitochondria and triggers caspase-9 activation. In contrast, on stimulation of CM1 with anti-CM1 mAb in Ramos cell, $\Delta\Psi_m$ is not changed but Fas and FasL expression were increased. And ZB4 inhibited the CM1 -mediated apoptosis, suggesting that anti-CM1 mAb induced apoptosis is typically mediated via Fas-FasL interaction.

Discussion

CM1 was shown to be distributed over the distal part of the dark zone (a part of the centroblasts) and

the entire area of the light zone (the centrocytes). Cross-linking of CM1 on the GC B cells induced apoptosis, suggesting that CM1 might be involved in apoptosis of the germinal center B cells (1).

The phenotypic profile of BL cell lines were remarkably similar to that associated with normal B cells found in the GC (4). We chose two BL cell lines, Ramos and Raji cells as a model to analyze the effects of CM1 on centroblasts and centrocytes, respectively. Ramos expressed CD38+/CD77+, and Raji expressed CD38+/CD77− on their surface.

A previous report showed that CM1 induced enhanced expression of Fas, and that CM1-ligation induced apoptosis could be prevented by the caspase-8-specific inhibitor, z-IETD in Ramos cells (6). In this study, we first demonstrated that ligation of CM1 enhanced expression of both Fas and FasL expression and antagonistic anti-Fas Ab (ZB4) inhibited the CM1-ligation induced apoptosis in Ramos cells. This results suggested that CM1 ligation induces apoptosis in Ramos cells via a typical pathway of Fas/FasL interaction.

However, in apoptosis of Raji cells induced by anti-CM1 mAb, neither up-regulation of Fas/FasL nor inhibitory effect of caspase-8-specific inhibitor was observed, suggesting that some other mechanism, such as a mitochondrial pathway might be involved in CM1-ligation induced apoptosis of Raji cells. Indeed, the reduction of the mitochondrial transmembrane poteintial in CM1-ligation induced apoptosis has been reported in Raji cells (6).

As shown in Fig. 7, a decrease in the expression of anti-apoptotic molecule, Bcl-2 was observed in Raji cells after addition of anti-CM1 mAb. However, no significant change in the expression of pro-apoptotic molecule, Bax was observed after stimulation of Raji cells with anti-CM1 mAb.

Our results demonstrated that cytochrome c release from mitochondria is an event in CM1-ligation induced apoptosis. Once cytochrome c is released, a sequential ordering of caspase activation has been reported (35). Our results demonstrated that CM1 induces activation of caspase-9. Thus, the cytochrome c release by ligation of CM1 could be sufficient to trigger caspase-9 activation. In conclusion, the results presented in this report demonstrate that CM1-ligation induced apoptosis involves release of cytochrome c from mitochondria preceding caspase-9 activation and loss of $\Delta \Psi_m$.

Bcl-2 was first discovered by virtue of its translocation (t(14;18)) and elevated expression in follicular B-cell lymphomas (36). The bcl-2 oncogene was thought to confer oncogenic potential upon cells by inhibiting programmed cell death (37). Bcl-2 was localized to the mitochondrial inner membrane, suggesting that Bcl-2 might play some roles in modulating mitochondrial function. However, Bcl-2 was also found in the endoplasmic reticulum and nuclear envelope (38,39). It has also been reported that IFN-γ induces the apoptosis of WEHI 279 by down-regulation of both Bcl-2 and Bcl-x_L (40).

The direct effects of CM1 on B cells, especially the induction of apoptosis, were observed in GC B cells and BL cells, Ramos and Raji cells (1,6). However, the mechanisms of apoptosis of GC B cells and BL cells by ligation of CM1 were not well understood. In this study, we proposed two mechanisms of CM1-ligation induced apoptosis : Fas/FasL system in Ramos cells and down-regulation of Bcl-2 and cytochrome c release in Raji cells.

Previous reports suggested that ligation of CM1 initiates apoptosis in caspase 8-dependent manner in Ramos cells and in a mitochondria-controlled manner in Raji cells (6). In this study, confirming that Fas and FasL interaction is one of the direct targets of CM1-ligation induced apoptosis of Ramos cells and down-regulation of Bcl-2 is important targets of CM1-ligation induced apoptosis of Raji cells.

These results suggest that CM1 might be involved in providing apoptotic signals to low affinity GC B cells, through the Fas/FasL system or mitochondrial pathway.

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