Anti-immunoglobulin-induced Apoptosis in WEHI 231 Cells Involves the Slow Formation of Ceramide from Sphingomyelin and Is Blocked by bcl-xL*

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Prolonged (>24 h) exposure to anti-IgM (an antigen surrogate that induces membrane cross-linking and apoptosis) induced a 3-fold increase in the mass of endogenous ceramide measured by 32P labeling by diacylglycerol kinase and a 4-fold increase in ceramide as measured by metabolic labeling with [3H]palmitate in a B-lymphocyte cell line, WEHI 231. This correlated with the induction of apoptosis. Shorter exposure times to anti-IgM (up to 8 h) failed to elicit apoptosis and did not elicit increased ceramide formation. After 8 h, apoptosis occurs concomitantly with ceramide formation over the next 40 h. Further, we showed that exogenous ceramide mimicked anti-IgM-induced apoptosis and that apoptosis was potentiated in serum-free media. Treatment elicit increased ceramide formation. After 8 h, apoptosis anti-IgM (up to 8 h) failed to elicit apoptosis and did not the induction of apoptosis. We showed recently that the time course and extent of anti-IgM-induced apoptosis in WEHI 231 is directly related to ceramide formation (7) and that resistance to anti-IgM-induced apoptosis in a WEHI 231 subline (RE) was the result of insufficient production of ceramide (8). Other signal transduction pathways in RE were either not activated, and, for example, those involving c-myc, c-fos, egr-1, c-fos, p53, bcl-2, bcl-x, bax, and interleukin-converting enzyme, responded the same in wild type and resistant cells (8).

Ceramides (a family of 2-N-acylsphingosines) appear to be an intracellular signal for the regulation of the cell cycle and a cascade that eventually leads to apoptosis (9–17). Thus a sphingomyelin cycle has been identified in which the action of extracellular signaling agents (for example cytokines such as TNF-α) results in the activation of one or more sphingomyelinas, cleavage of membrane sphingomyelin, formation of ceramide, and the activation of multiple cellular and biochemical targets (8, 11, 12, 14, 15, 18). It has also been proposed that ceramide is a potent and specific suppressor of cell growth and an inducer of apoptosis (19–21). Several reports have claimed that the transient (less than 30 min) activation of sphingomyelinase by cytokines leads to apoptosis during the next 24 h (10, 22, 23). However, other reports indicate no changes in ceramide with TNF-α for several hours (24). There is also some controversy as to whether this sphingomyelinase activation is plasma membrane-bound, cytosolic, or lysosomal, or some combination of these (14, 23, 25, 26), but many models proposed rely on this rapid (5–30 min) switching on and off of sphingomyelinase to activate the apoptotic signal. Contrary to the model, several laboratories have reported that ceramide is not necessary for TNF-α activation of nuclear factor-κB and that the observed effects of ceramide might be nonphysiological (27–30). More means of irradiation, corticosteroids, or cross-linking of their antigen receptors. The apoptotic response of the murine B-lymphoma WEHI 231 cell line to the cross-linking of surface IgM receptors provides a model to study the induction of physiological cell death (apoptosis) in lymphocytes (2–4). In WEHI 231, the response is specifically triggered via surface IgM since cross-linking other surface proteins such as IgD, Fc receptor, or major histocompatibility complex class II have no effect (5). Apoptosis is reversed by both phospholipids and by bacterial lipopolysaccharide (6). Apoptosis can also be reversed by the removal of the antigen prior to 12 h of exposure, emphasizing its slow acting nature compared with necrosis and some types of apoptosis. We showed recently that the time course and extent of anti-IgM-induced apoptosis in WEHI 231 is directly related to ceramide formation (7) and that resistance to anti-IgM-induced apoptosis in a WEHI 231 subline (RE) was the result of insufficient production of ceramide (8). Other signal transduction pathways in RE were either not activated, and, for example, those involving c-myc, c-fos, egr-1, c-fos, p53, bcl-2, bcl-x, bax, and interleukin-converting enzyme, responded the same in wild type and resistant cells (8).
recent studies have shown that activation of JNK is not linked to apoptosis, and nuclear factor-κB actually blocks apoptosis (31–34). Thus, ceramide involvement in apoptosis must work along a different pathway than that of activation of nuclear factor-κB and JNK.

We propose that ceramide formation is a physiological event that is sufficient to induce apoptosis over a period of several hours. Thus, apoptosis can be mimicked by the addition of exogenous, soluble C2- or C6-ceramides or bacterial sphingomyelinase and sphingosine derivatives, but cannot be induced by other lipids (apart from sphingosine whose role in apoptosis is also under investigation) or by other lipases (such as phospholipase C). In fact, both diacylglycerol (35) and gangliosides, specifically, GM1 (36, 37), have been reported to have a protective effect indicating that the exogenous ceramide did not induce apoptosis by a nonspecific membrane effect. However, whether ceramide is necessary for any and/or all types of apoptosis and the time course of its action remains to be fully elucidated. In this study, we offer evidence further supporting the requirement of ceramide for apoptosis in the physiological, antigen-induced apoptosis of an immature B-cell line, WEHI 231. We show that ceramide formation is a slow process (which is contrary to many previous studies but in agreement with the Fas-induced killing of SKW6.4 cells (16)) and that bcl-xL protects cells against the ceramide increases. In addition, to emphasize further the critical role of ceramide in apoptosis, we generated cells that were resistant to apoptosis (by prolonged exposure to ceramide inhibitor, N-oleoylethanolamine) and report that they are resistant because they are unable to activate sphingomyelinase in response to physiological signals.

**EXPERIMENTAL PROCEDURES**

**Drugs and Reagents**—C2-ceramide, C2-dihydroceramide, C6-ceramide, C2-dihydrceramide, and N-oleoylethanolamine (OE) were purchased from Matreya Inc., Pleasant Gap, PA.

**Cell Culture and FACS Analysis**—WEHI 231.7 JM cells used for these studies were grown in RPMI 1640 supplemented with Hepes buffer, l-glutamine, antibiotics, 10% fetal calf serum, and 2-mercaptoethanol as described previously (5). WEHI 231.bcl-xL, and WEHI 231.neo transfectants were prepared and maintained as described previously (13). Cell viability was determined by the propidium iodide (PI) exclusion test as described previously (5). The OE-resistant cell lines (OER1, OER2) were obtained by treating wild type WEHI 231 cells with 100 μM oleoylethanolamine for 48 h in serum-free media. The cultures were then left to incubate for 2 weeks at which time two foci of cells were observed. These were grown up separately and identified further as OER1 and OER2. The process was repeated periodically to ensure homogeneous cell populations.

For anti-IgM pulse-chase experiments, 10⁶ WEHI 231 cells were cultured in 24-well plates in the presence of Bet-2 hybridoma supernatant (a 1:40 dilution of the Bet-2 hybridoma secreting anti-IgM antibody as described previously (5)). At the indicated time, the cells were collected, washed, and recultured in the absence of new Bet-2 supernatant to complete a total of 24 h in culture. For continuous anti-IgM exposure studies, cells were exposed to anti-IgM, and all cells were harvested simultaneously. At this time, the cells were harvested and resuspended for viability (PI) and ceramide content studies. For quantitation, cells were run on a FACScan (Becton Dickinson, San Jose, CA) and the results analyzed using Lysys II software as described previously (5). It should be noted that because the physiological inducer of apoptosis, anti-IgM, is derived from a hybridoma supernatant, the exact titers of the antibody may vary over a period of months. This could result in the degree of variation in killing which we observed at later time points. However, in all cases, killing levels were matched by the increase in ceramide formation.

**Diacylglycerol Kinase Assay for Identification and Quantification of Ceramide**—A modification of the method of Preiss et al. (38) was used. Lipids were isolated in a Folch partition as described previously (39), dried, and the ceramide converted to ceramide 1-[32P]phosphate by Escherichia coli diacylglycerol kinase. Labeled lipids (ceramide 1-phosphate and phosphatidic acid) were separated by high performance thin layer chromatography (HPTLC). Following autoradiography, spots corresponding to ceramide 1-phosphate were scraped and counted in a scintillation counter. Quantiﬁcation of ceramide was based on a standard curve of known amounts of ceramide.

**Lipid Synthesis and Catabolism**—To label lipids, WEHI 231 (2 × 10⁶ cells/flask) were cultured for 24 h in media containing 10 μCi of [3H]palmitate in the presence or absence of the indicated reagents (7). Under these conditions, sphingolipids are labeled well, and palmitate is broken down to acetate and recycled such that de novo synthesized cholesterol is also labeled, and lipids were extracted as described previously (39). The upper phase was discarded and the lower phase evaporated to dryness under nitrogen.

Samples were run as described previously (7), on a 10 × 10-cm LHP-K plate (Whatman) in chloroform:methanol:glacial acetic acid:water (85:4.5:5.0:0.5 v/v), which resolves cholesterol from the ceramide doublet. The upper band consisted mostly of a mixture of C22:0, C24:0 and C24:1 fatty acids (40); the lower band consisted primarily of C16:0, C16:1, and C18:0 fatty acids (40). Sphingomyelin was resolved from phosphoglycerides in chloroform:methanol:acetic acid:water (65:25:5:8.5:4.5 v/v). Plates were sprayed with ENHANCE (DuPont NEN) and developed overnight with Kodak X-Omat AR film to identify the bands by comparison with authentic standards. Bands were then scraped for liquid scintillation counting.

The change in ceramide content was normalized based on total protein or in some cases sphingomyelin as an index of sphingomyelin hydrolysis.

**Sphingomyelinase Assay**—Microsomal and cytosolic cellular fractions were prepared from cells collected after 18–24-h exposure to anti-IgM. Cells were washed in Hank’s buffered saline solution and pelleted in microcentrifuge tubes. The cellular pellet was homogenized in TE buffer (20 mM Tris/HCl, pH 7.5, and 1 mM EDTA) containing 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotonin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin using 20 strokes in a Kontes glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 100,000 × g for 1 h. The microsomal fraction was reconstituted with 20 strokes in the Dual homogenizer.

Neutral sphingomyelinase activity was assayed with [methyl-3H]sphingomyelin (40,000 cpm in 1 nmol of bovine brain sphingomyelin (Sigma) in 0.2% Triton X-100, 80 mM MgCl₂, and 0.1 mM Tris/HCl, pH 7.4, solubilized by sonication and vortexing. After incubating for 0.5–1 h, the reaction was stopped by the addition of 0.5 ml of chloroform: methanol (2:1). The samples were pulse-vortexed three times for 30 s and then briefly centrifuged to separate the two phases. The upper phase, containing labeled phosphorylcholine released from sphingomyelin, was transferred to scintillation vials and counted by liquid scintillation counting. Negative controls containing no enzyme or boiled cell lysates were run concomitantly.

Acid sphingomyelinase (lyosomal) activity was measured as above except that 0.1 mM sodium acetate, pH 5.5, containing 5 mM EDTA replaced the Tris/HCl.

**RESULTS**

**Apoptosis Is Associated with Ceramide Formation in WEHI 231 Cells**—WEHI 231 cells undergo time-dependent apoptosis when treated with Bet-2 hybridoma supernatant containing anti-IgM in the typical manner of oligonucleosomal-DNA fragmentation and membrane blebbing (5). This process requires at least an 8–10-h exposure to anti-IgM before these effects can be seen. To determine the exposure time necessary for anti-IgM-induced apoptosis and ceramide formation, WEHI 231 cells were exposed to anti-IgM for 0–48 h, washed, and replated in anti-IgM free media for the remainder of the experiment. At the time cells were harvested, viability was analyzed by FACS and ceramide mass measured by the diacylglycerol (DAG) kinase assay. We observed that significant cell death did not occur in WEHI 231 cells exposed to anti-IgM for 6 h or less (Fig. 1A). Cells began to die in significant numbers between 12 and 24 h, with at least 50–75% of cells dead by 48 h. Concurrently, ceramide levels in WEHI 231 increased from basal levels of around 100 pmol/dg during the first 6 h to around 350 pmol/dg after a 24-h exposure to anti-IgM (Fig. 1B). Interestingly, we observed a similar pattern in ceramide formation measured by metabolic [3H]palmitate labeling. Early time points saw little changes in ceramide labeling, followed by an increase up to 16 h, followed by sharp increases at later time points (data not shown). Fig. 1C shows a typical autoradiogram of the isolated...
[3H]palmitate-labeled ceramide doublet from control cells and its increase in treated cells. Labeling of most other lipids actually declined following this 24-h treatment since the cells begin to die after 10 h of treatment.

In contrast to several other systems undergoing apoptosis, when we looked at ceramide formation after only very short exposures to anti-IgM by either metabolic labeling using [3H]palmitate or quantitation of ceramide mass by the DAG kinase procedure, we observed no sustained or reproducible alterations in the ceramide levels over the first 60 min. Fig. 1D shows a typical experiment where if anything, ceramide levels decreased. Changes such as these were not reproducible from experiment to experiment and did not appear to have any relation to later events. Therefore, these short exposures to anti-IgM were incapable of reducing cell viability, and removing the anti-IgM after 1 h prevented any apoptosis. This underscores the important potential difference between apoptosis-signaling paradigms and classic signal transduction second messenger systems.

In addition we were able to show that two well known paradigms used to block apoptosis induced by anti-IgM, namely the use of phorbol esters and bacterial lipopolysaccharide (6), both blocked the increase in ceramide formation by at least 90% (data not shown).

**Ceramide Is a Biologically Specific Inducer of Apoptosis**—To demonstrate that ceramide could directly mediate apoptosis in WEHI 231 we exogenously added membrane-permeable C2-ceramide and its 4,5-dihydro derivative to cell cultures. The percentage of dead cells after 48 h of culture in the presence of 10, 20, 30 μM ceramide was found to be 3, 40, and 90%, respectively (Fig. 2, right panels), whereas >95% of the cells were viable in cultures containing equivalent concentrations of dihydroceramide (Fig. 2, left panels). In serum-free medium (Fig. 2, lower panels), as little as 1 μM ceramide readily kills these cells. In addition, treating cells in serum-free media required much less ceramide to achieve comparable killing and resulted in 100% killing in less than 6 h. We have shown previously that typical DNA laddering can be observed in cells exposed to 30 μM ceramide (but not dihydroceramide) for 24 h in serum-containing medium (7). We have verified the validity of the PI method of determining apoptosis in cells by using the terminal deoxynucleotidyltransferase assay as well as PI staining to observe the chromatin condensation typical of apoptotic bodies (data not shown). All of our data indicate that we are seeing apoptotic death and not necrotic death. The dihydro derivative of ceramide has no biological activity, whereas ceramide causes death in a dose-dependent manner, agreeing with other studies (41). Other lipids were also found to be ineffective in inducing apoptosis, confirming the biological specificity of ceramide.

**Inhibition of Ceramidase Causes Accumulation of Ceramide**

![Fig. 1. Time course of cell death and ceramide formation in WEHI 231.](http://www.jbc.org/)
and Apoptosis—OE, a synthetic analog of ceramide, was described by Sugita et al. (42, 43) to be a specific inhibitor of ceramidase, the enzyme responsible for the catabolism of ceramide. More recently, Coroneos et al. (44) found OE to be a useful and specific inhibitor of ceramidase in studying ceramidase activation in mesangial cells. We have observed previously that OE is an inducer and potentiator of staurosporine-induced apoptosis in neurons (15) and neurotumor cells (45), presumably because of the inhibition of ceramidase resulting in the accumulation of apoptosis-inducing levels of ceramide. In this study, we treated WEHI 231 with increasing concentrations of OE for 24 h and then analyzed cell viability by PI labeling and ceramide formation by [3H]palmitate labeling. Again, we observed a close correlation. Thus, at high concentrations (50 μM), we observed a decrease in viability (increase in apoptosis) concomitant with an increase in ceramide formation (Fig. 3A). Further, at concentrations that elicit little response on their own (25 μM), coaddition with anti-IgM doubled the efficacy of anti-IgM-induced ceramide formation and death (Fig. 3B). The labeling of sphingomyelin declined by a commensurate amount, suggesting the conversion of sphingomyelin to ceramide (data not shown).

OE-resistant WEHI 231 Cells Are Also Resistant to Anti-IgM—The evidence presented above indicates that there is a strong correlation between ceramide accumulation and apoptosis, but it could be argued that ceramide accumulation coincides with cell death rather than causing it. To document a direct link between ceramide production and physiological cell death we generated OE-resistant variants. OE-resistant cells were created by successive treatment of WEHI 231 cells with OE and growing up the resistant population (see “Experimental Procedures”). Cells were run on a FACScan, and the results were analyzed using Lysis II software. Viable cells are those with < 10 arbitrary units of red fluorescence. The left bottom panel includes analyses of an ethanol-treated (dark shade) as well as dihydroceramide-treated cultures to indicate that in the absence of serum (broken line), both ethanol and dihydroceramide eventually cause a low level of nonspecific cell death. Ethanol caused little cell death in the presence of serum.

FIG. 2. Exogenously added C2-ceramide kills WEHI, but 4,5-dihydro-C2-ceramide does not. WEHI 231 cells were exposed to the indicated concentrations (1, 10, 20, and 30 μM) of C2 and dihydro-C2 (Dh-C2) (left panels) or C2-ceramide (right panels) in cultures containing 10% fetal calf serum (fcs) or serum-free cultures (w/o fcs, bottom panel). To remove serum, WEHI 231 cells were washed extensively (three times in Hanks’ balanced salt solution using new centrifuge tubes for each wash to eliminate carryover of inhibitory serum components) as described under “Experimental Procedures.” Cells were collected after 24 h and, in the case of serum-free, 4 h, then resuspended in PI-containing solution as described under “Experimental Procedures.” Cells were run on a FACScan, and the results were analyzed using Lysis II software. Viable cells are those with < 10 arbitrary units of red fluorescence. The left bottom panel includes analyses of an ethanol-treated (dark shade) as well as dihydroceramide-treated cultures to indicate that in the absence of serum (broken line), both ethanol and dihydroceramide eventually cause a low level of nonspecific cell death. Ethanol caused little cell death in the presence of serum.
Ceramide-induced Apoptosis in B-cells

Fig. 3. OE induces apoptosis and ceramide accumulation in WEHI 231 cells. Panel A, to assess viability, WEHI 231 cells were collected and resuspended in PI-containing solution following exposure to OE for 24 h as described under “Experimental Procedures.” Cells were run on a FACScan, and the results were analyzed using Lysis II software. Viable cells are those with < 10 arbitrary units of red fluorescence. Ceramide mass was determined on parallel cultures of cells exposed to OE for 24 h by 32P labeling with the DAG kinase assay as described under “Experimental Procedures.” Data represent the mean of two separate experiments. Panel B, labeling of ceramide increases relative to sphingomyelin following 24-h exposure to OE, plus and minus anti-IgM. Ceramide quantitation (ceramide:sphingomyelin ratio by [3H]palmitate labeling) was performed on triplicate flasks of WEHI 231 cells cultured for 24 h in the presence of the indicated reagents as described under “Experimental Procedures.” Data represent the mean of three separate experiments ± S.E.

that after a 24-h treatment the resistant cell lines failed to increase their ceramide mass. Fig. 5 shows the ceramide mass measured by DAG kinase 32P labeling in WEHI 231 wild type and OER2 lines. The wild type shows the typical increase in ceramide mass in response to anti-IgM. However, the OER2 line shows no significant increase in ceramide mass when treated with anti-IgM alone or in combination with OE (data not shown).

Anti-IgM activates a Mg2+-dependent Membrane Sphingomyelinase in Wild Type WEHI Cells but Not in OE-resistant Cells—We observed that 18 h of anti-IgM treatment of WEHI 231 cells produced a 50% increase in Mg2+-dependent, membrane-associated neutral sphingomyelinase activity (pH 7.5) without a detectable change in acid sphingomyelinase activity (pH 5.5). The ratio of neutral to acid sphingomyelinase is at least 20:1 in WEHI (Fig. 6A). In comparable experiments using extracts of human skin fibroblasts we found that 85% of the sphingomyelinase activity was lysosomal (pH 5.5) and only 15% Mg2+-dependent and membrane-bound (pH 7.5), a ratio of 1.6. This indicates considerable heterogeneity in the expression of neutral and acid sphingomyelinases in different cells and suggests that the neutral membrane-bound sphingomyelinase is involved in apoptosis in WEHI 231 cells.

In contrast, it can be seen that anti-IgM was unable to stimulate any sphingomyelinase species in the OE-resistant cell line OER2 (Fig. 6B). It should be noted that although the OE-resistant cells fail to activate sphingomyelinase in response to stimuli, there appears to be around a 30% increase in sphingomyelinase basal activity. We believe this to be unimportant because of earlier experiments demonstrating no increase in resting ceramide levels.

Fumonisin B1 Does Not Block Apoptosis: Sphingomyelin Synthesis Is Not Required for Apoptosis—WEHI 231 cells were equilibrium-labeled with [3H]palmitate in the presence of different concentrations of fumonisin B1. Fumonisin B1 inhibited both sphingomyelin synthesis and ceramide synthesis (Fig. 7A). Concentrations of fumonisin B1 greater than 50 μM were toxic for WEHI 231 cells. Fig. 7A shows an autoradiogram of a typical HPTLC of total lipids labeled for 24 h with [3H]palmitate in the presence of increasing concentrations of fumonisin B1. At 50 μM, fumonisin B1 did not prevent the 50% cell death (apoptosis) induced by 24 h of anti-IgM treatment (Fig. 7B), indicating that de novo synthesis of ceramide was not necessary.

Overexpression of bcl-xL Protects against Apoptosis but Not Ceramide Formation—We have previously generated physiologic cell death-resistant WEHI cells by the overexpression of bcl-xL (but not bcl-2) (13). The protection afforded to WEHI 231 cells by the overexpression of bcl-xL can be seen in Fig. 8A. Further, Fig. 8A shows how cells transfected with neo controls are killed readily by exogenous ceramide but that the overexpression of bcl-xL confers resistance to ceramide-induced apoptosis. Interestingly, bcl-xL-transfected cells synthesize similar levels of ceramide in response to anti-IgM (Fig. 8B). Whether we examine cell ceramide by 32P labeling or [3H]palmitate labeling we failed to observe significant changes in the ability of the cell to increase ceramide mass. From these data we conclude that ceramide acts upstream of bcl-xL. This is reasonable because bcl-xL is believed to block apoptosis at its later stages, with ceramide being important in the earlier signal transduction of the death signal.

DISCUSSION

The death of immature lymphocytes following receptor cross-linking provides the means to purge out self-reactive clones and represents a good example of apoptosis. Under physiologic circumstances, clonal deletion occurs when developing lymphocytes encounter self-antigens, but these conditions can be mimicked in vitro by exposing T- or B-lymphocytes to antibodies that cross-link their clonal receptors. Despite intensive research from many laboratories, it is still not known how lymphocytes biochemically trigger apoptosis. The results presented in this paper, in conjunction with those published elsewhere (7, 8), provide an answer in a clonal transformed cell line, WEHI 231, representative of mouse B-lymphocytes. We have shown previously that exogenous ceramide (but not its dihydro derivative) induces apoptosis in WEHI 231 (7). The demonstration that ceramide induces apoptosis is in agreement with previous observations by others (14–17, 46–48), but we strongly disagree that the reported rapid time courses for ceramide formation have anything to do with apoptosis. Often, if the stimulant is removed (after several hours in many cases) the cells can recover and not undergo apoptosis. We have demonstrated previously that ceramide accumulates in WEHI 231 cells exposed to conditions known to induce apoptosis, such as irradiation, dexamethasone, and anti-immunoglobulin (7). We con-
firm and extend those findings in this paper, showing that agents that increase ceramide induce apoptosis, and failure to produce ceramide coincides with resistance to apoptosis in two distinct cell lines. These observations suggest that ceramide is a mediator of apoptosis.

Does ceramide cause apoptosis, or could it be argued that ceramide accumulation occurs nonspecifically in dying cells, i.e. that it is the result not the cause of apoptosis? Many laboratories have shown that ceramide (both synthetic and natural) is able to induce apoptosis, but this is also true of other drugs such as dexamethasone, etoposide, and staurosporine. Several pieces of evidence strongly suggest the importance of ceramide as a mediator of cell death. First is the biological specificity of ceramide. We have shown here (Fig. 3) and previously (7) that only ceramide has biological activity, whereas the dihydro derivative does not. This has been confirmed in other studies (41, 49). Another major proof is that we can show the activation of a neutral membrane-bound sphingomyelinase and the generation of ceramide from a physiological precursor, sphingomyelin, both under physiological conditions (receptor cross-linking) and in response to drugs that cause apoptosis. This also has been shown in Fas- (16) and TNF-α (22) induced apoptosis.

We believe some of the most compelling evidence for the mediating role ceramide plays comes from our studies with the specific inhibitor of ceramidase, OE. By specifically blocking the catabolism of ceramide, we were able to induce apoptosis at high concentrations of OE (50–100 μM) but more importantly, potentiate the effect of anti-IgM at low concentrations (10–25 μM). Using the ceramidase inhibitor OE to select for low ceramide-producing WEHI 231 cells, we generated a subline that was resistant to anti-IgM. The existence of OE-resistant variants of WEHI 231 provides compelling evidence for a causative role of ceramide in apoptosis because if ceramide were not a mediator of death, selection with OE for low ceramide production would not yield anti-IgM resistance. Moreover, we have shown previously that a naturally anti-IgM resistant WEHI cell line was also deficient in ceramide production (8). Thus we have now described two different apoptosis-resistant WEHI 231 cell lines, and both are characterized by low ceramide production while other signaling pathways remain intact.

What is most notable about these studies is the observation the apoptosis does not occur without a sustained signal and that the agonist must be present for many hours. Thus, apoptosis in WEHI 231 is reversible in its early stages. This contrasts with the typical agonist-induced signaling pathway but is reminiscent of growth factor signaling and withdrawal. Our
data also contrast with previous reports of the role of ceramide in apoptosis in serum-starved cells in response to cytokines such as TNF-α or Fas ligand and other agents (10, 14, 22, 23, 50–52). These studies have described the rapid activation of an acid sphingomyelinase resulting in the transient increase (15 min) of ceramide which then can activate downstream targets such as nuclear factor-κB or JNK. This was interpreted as suggesting a trigger mechanism for subsequent apoptosis, which then takes place over the next 24–48 h. This is not the case with apoptosis in WEHI 231 since removal of anti-IgM or OE at any time up to 10 h will reverse the commitment to apoptosis, and it is only after 10 h of continuous exposure that we see the irreversible commitment to apoptosis. We also found that anti-IgM exposure increased neutral sphingomyelinase activity in WEHI 231 leading to hydrolysis of sphingomyelin and sustained intracellular accumulation of ceramide, but only after 10 h of continuous exposure to anti-IgM. Although both neutral and acidic sphingomyelinases have been implicated by others in the production of ceramide in response to agents that induce apoptosis (10, 14, 22, 23, 50–52), we find that plasma membrane-bound neutral, Mg²⁺-dependent sphingomyelinase is responsible for ceramide production in WEHI 231. This observation is also in agreement with the report by Andrieu et al. (28) that human fibroblasts from acid sphingomyelinase (lysosomal)-deficient (Niemann-Pick) patients showed normal responses to TNF-α. Tepper et al. (16) have shown recently that ceramide production is important in Fas-induced apoptosis. In their study, they too found that a neutral membrane-bound sphingomyelinase was involved, that early alterations in ceramide levels were not important, but that the sustained accumulation of ceramide over several hours was crucial. This, however, was contradicted somewhat by Gulbins et al. (53), who reported rapid changes (2 min) in ceramide levels prior to activation of the Ras signaling pathway. It is conceivable that depending on the cell type and the kind of apoptosis-inducing stimulus used, different sphingomyelinases contribute to ceramide production, which results in different cellular events. What is clear, however, is that anti-IgM-induced apoptosis appears to work through a different sphingomyelinase pathway than that which has been reported for TNF-α.

We do not know precisely what initiates sphingomyelinase activation, but in the two resistant WEHI cell lines (RE and OER2), a failure to activate sphingomyelinase appears to be the reason for failure of anti-IgM to induce apoptosis. Not much is known about the activation of sphingomyelinase, but Jay-
have no evidence that anti-IgM can activate phospholipase A2. A recent report that anti-IgM treatment mediated by 32P labeling with DAG kinase. After 24 h of anti-IgM treatment, there was no block in the activation of the sphingomyelinase and production of ceramide. Data represent the mean of three separate experiments ± S.E.

adve et al. (54) have proposed that arachidonate is the physiological activator. This polyunsaturated fatty acid is typically generated by the action of phospholipase A2. At present we have no evidence that anti-IgM can activate phospholipase A2 or that the OE-deficient cell line is deficient in phospholipase A2. However, there are other candidates for the role of sphingomyelinase activator such as the staurosporine-activated 60-kDa protein kinase (55). In addition, lysosomal (acid) sphingomyelinase is known absolutely to require an activator protein (saposin D, and to a lesser extent saposins A and C) for hydrolysis of sphingomyelin (56), and an analog of this protein could be the target for activation of the neutral sphingomyelinase. We propose that membrane disturbances/alterations associated with the protracted process of surface IgM capping and internalization may generate the late signals that activate this Mg2+-dependent membrane sphingomyelinase.

Exogenous, soluble C2-ceramide has also been claimed to protect cells from growth factor withdrawal (57). However, this protection was short lived with almost all protection being lost in 48 h. This protection comes at very low concentrations (<5 μM), and higher concentrations (50 μM) produced apoptosis. It is possible that this effect may be through stimulation of the low affinity nerve growth factor receptor (p75NGF) (58) and temporally fulfilling the NGF requirement for survival. Protection may also come by the induction of retinoblastoma protein dephosphorylation, resulting in growth arrest (59). This also appears to be a low concentration phenomenon. It is conceivable that the role of ceramide is biphasic, being protective at low concentrations and toxic at high. However, studies in which ceramide analogs are used as an exogenous drug may produce many unanticipated effects on cells and should be interpreted with caution. In our study, we believe our results with exogenous ceramide are substantiated by the data measuring endogenous ceramide.

How does ceramide kill cells? Ceramide has been claimed to directly activate a number of specific cytosolic kinases and even a phosphatase. Recent candidate targets include protein kinase Cζ (60) and the stress-related kinase family (17) as well as Ras (53). The apoptotic message is now directed toward the cell nucleus but can be regulated by the protective bcl-2 and bcl-xL proteins, which are localized to the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope. Their localization to the major sites of oxygen-derived free radical generation suggest that they mainly protect against oxidative messages, but this remains to be proved, and other factors have been proposed. Support for the location of bcl-xL downstream of ceramide comes from our observation that bcl-xL-transfected cells were less readily killed by both anti-IgM and ceramide, whereas OE-resistant cells and the RE cells generated by anti-IgM resistance were killed by ceramide equally as well as wild type cells. Furthermore, the observation that the bcl-xL transfectants were unaffected in their ability to produce ceramide in response to apoptotic stimuli strongly supports the role of ceramide in apoptosis as well as the order of ceramide and bcl-xL. A related study recently found that bcl-2 was capable of blocking ceramide-mediated cell death (61). In that study, they found similar results in that bcl-2 blocked ceramide-induced cell death but did not block the induction of ceramide synthesis in response to apoptotic stimuli. Overcoming the “bcl-xL-block” results in the activation of proteases and the rapid phase of apoptosis. The interleukin-converting enzyme/ced-3 family of proteases is the cell execution enzyme dephosphorylation, resulting in growth arrest (59).

Fig. 8. Panel A, bcl-xL expression protects against anti-IgM and ceramide. WEHI viability was determined by PI and FACS analysis after 24-h treatment with anti-IgM or increasing concentrations of C2-ceramide (25–50 μM). Panel B, bcl-xL transfectants fail to alter ceramide synthesis in response to apoptotic stimuli strongly supports the role of ceramide in apoptosis as well as the order of ceramide and bcl-xL.

REFERENCES
Ceramide-induced Apoptosis in B-cells

Anti-immunoglobulin-induced Apoptosis in WEHI 231 Cells Involves the Slow Formation of Ceramide from Sphingomyelin and Is Blocked by bcl-xL

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