Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7350–7354, July 1994
Immunology

Identification of immunosuppressant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2

(cyclosporin A/FK-506/rapamycin/WEHI-231 cells/BAL-17 cells)

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Communicated by A. A. Moscona, April 12, 1994

ABSTRACT **Cyclosporin A, FK-506, and rapamycin are immunosuppressants often used as pharmacological probes to study lymphocyte activation and physiological cell death (PCD). Because cyclosporin A and FK-506 are known to prevent PCD in T-cell hybridomas and thymocytes, we used these reagents, as well as rapamycin, to determine whether they alter the pathway leading to apoptosis in murine WEHI-231 cells following surface IgM cross-linking. We observed that the immunosuppressants themselves induced PCD in WEHI-231 cells, but only in sublines susceptible to anti-IgM-mediated apoptosis. PCD was preceded by growth arrest and characterized by the DNA fragmentation pattern typical of apoptosis. In B-cell lines resistant to anti-immunoglobulin- and immunosuppressant-induced PCD, cyclosporin A, FK-506, and rapamycin caused growth arrest. PCD was also induced by inhibitors of protein synthesis in WEHI-231 cells but not in the mature B-cell line BAL-17. Immunosuppressant-induced and protein synthesis inhibitor-induced PCD, but not growth arrest, could be prevented by the overexpression of *bcl-x*$_L$, while transfection with *bcl-2* did not affect PCD or cell cycle arrest. These results suggest that *bcl-2* and *bcl-x*$_L$ may control partially independent systems to inhibit PCD in lymphoid cells and that PCD in B and T cells may be differentially regulated.**

Cyclosporin A (CsA), FK-506 (FK), and rapamycin (Rap) are naturally occurring antifungal agents known to be powerful immunosuppressants (1). These immunosuppressants bind to immunophilins, a family of proteins that have peptidylprolyl *cis–trans*-isomerase activity (1–3). The effects of these drugs are not due to their inhibition of isomerase activity but to the interaction of immunosuppressant–immunophilin complexes with specific effector molecules (4–12). CsA binds the immunophilin cyclophilin and inhibits the calcium/calmodulin-dependent protein phosphatase calcineurin (5, 6). Both FK and Rap interact with the immunophilin FK binding protein (FKBP) (4, 7). The complex of FK and FKBP also binds calcineurin (4–6), but the Rap–FKBP complex inhibits the activity of the $p70^{S6\ kinase}$ (4, 8–10), $p34^{cdc2}$ (11, 12), and $p33^{cdk2}$ (12).

Although CsA, FK, and Rap have been extensively used to study T-cell activation, little is known about the effects of these immunosuppressants on B cells (1). In T cells, CsA and FK inhibit antigen-dependent signaling events required for interleukin 2 gene transcription (1, 4, 7), and Rap blocks biochemical events necessary for interleukin 2-dependent progression from G_1 to S phase in the cell cycle (4, 7). In addition, CsA and FK, but not Rap, were able to prevent physiological cell death (PCD) in thymocytes and T-cell hybridomas induced by either anti-T-cell receptor antibodies or the combination of phorbol 12-myristate 13-acetate and ionomycin (4, 13–16). Experiments by Wicker *et al.* (17) demonstrate that CsA, FK, and Rap inhibit proliferation of B cells stimulated with anti-immunoglobulin reagents, but only Rap blocked proliferation of lipopolysaccharide-activated B cells. In contrast to the effects of immunosuppressants on T cells, the Rap-sensitive events in B cells were completed earlier in G_1 than the events inhibited by CsA and FK (17). In addition, inhibition of anti-IgM-activated B cells with CsA and FK, but not with Rap, resulted in PCD (17). To further study PCD in B lymphocytes, we chose the murine B-cell lymphoma WEHI-231 as a model because it readily undergoes anti-IgM-induced PCD (18–21). After cross-linking of surface IgM, growth of WEHI-231 cells is arrested in the G_0/G_1 phase of the cell cycle (21–23), and death occurs 24–48 hr later. Because of these features, WEHI-231 cells are recognized as a model for immature B cells. In contrast, BAL-17 cells do not undergo PCD after surface IgM engagement and therefore are considered mature B cells. In this report, we describe the ability of the immunosuppressants CsA, FK, and Rap to regulate the cell cycle and PCD in WEHI-231 and BAL-17 cells.

MATERIALS AND METHODS

Cells. WEHI-231 cells, which we named MG and JM for convenience, were obtained from two different sources. The WEHI-231 MG subline (previously shown to be resistant to anti-IgM-induced PCD; refs. 20 and 21) was obtained from Harinder Singh (University of Chicago). The WEHI-231.7 JM subline (susceptible to anti-IgM-mediated PCD; refs. 20 and 21) and BAL-17 were gifts of John Monroe (University of Pennsylvania School of Medicine, Philadelphia). All cells were grown in RPMI 1640 culture medium supplemented with 25 mM Hepes buffer, 2 mM L-glutamine, penicillin/streptomycin mixture, 10% (vol/vol) heat-inactivated fetal calf serum (GIBCO/BRL), and 50 μM 2-mercaptoethanol.

Viability Assay. Cells (2×10^5) were incubated in 24-well plates and treated with CsA (1 μg/ml), FK (10 μg/ml), Rap (10 μg/ml), or an equal volume of EtOH as the vehicle (t = 0). After the indicated amount of time, the cells were collected and resuspended in PBS containing 1% (wt/vol) bovine serum albumin and 0.01% sodium azide. Propidium iodide was added immediately prior to analysis on a FACScan (Becton Dickinson) using LYSIS II software. Cells excluding propidium iodide were scored as viable.

Cell Cycle Analysis. Cells (2×10^5) were incubated in 24-well plates and treated with CsA (1 μg/ml), FK (10 μg/ml), Rap (10 μg/ml), or an equal volume of EtOH as the vehicle. After 24 hr, the cells were collected and resuspended in solution containing propidium iodide (50 μg/ml), 0.3% sodium citrate, and 0.01% Triton X-100; then incubated at

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Abbreviations: PCD, physiological cell death; CsA, cyclosporin A; FK, FK-506; FKBP, FK binding protein; Rap, rapamycin.

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room temperature for 1 hr; and analyzed on the FACScan using LYSIS II software.

DNA Fragmentation Gel Assay. Cells (2×10^5) were cultured in 24-well plates and treated with CsA (1 μg/ml), FK (10 μg/ml), Rap (10 μg/ml), or an equal volume of EtOH as the vehicle. After 24 hr, the cells were collected and lysed in 0.5 ml of 0.6% SDS/10 mM EDTA, pH 7.0. Sodium chloride was added to the lysate to a concentration of 1 M and then mixed by inversion. The mixture was left at 4°C for at least 12 hr and then centrifuged at 14,000 × *g* for 30 min at 4°C. RNase A (50 μg/ml) was added to the supernatant and incubated at 37°C for 60 min. The supernatant was then phenol/chloroform (1:1, vol/vol) extracted, ethanol precipitated in the presence of a carrier, and resuspended in TE (10 mM Tris, pH 8.0/1 mM EDTA). The samples were electrophoresed through a 2% agarose gel containing ethidium bromide at 0.5 μg/ml.

Transfections. WEHI-231.7 JM cells were transfected by electroporation with the pSFFV-Neo plasmid containing human *bcl-2* (24) (JMBCL2 cells), human *bcl-x*$_L$ (25) (JMB-CLX cells), or no insert as a control (JMNEO cells), using a Bio-Rad Gene Pulser with capacitance extender (250 V, 960 μF). Stable transfectants were selected for the acquisition of neomycin resistance by growth in the presence of G418 (1 mg/ml). Expression of bcl-x$_L$ was confirmed by Western blot analysis (see Fig. 5). Expression of bcl-2 was independently confirmed by both Northern blot analysis and by Western blot analysis using the monoclonal antibody 6C8 (26) (data not shown).

Western Blot Analysis. Cells (10^7) were resuspended in 1 ml of lysis buffer (1% Triton X-100/5 mM EDTA/Tris-buffered saline, pH 7.6, plus protease inhibitors). Lysates were cleared by centrifugation at 14,000 × *g* for 15 min at 4°C, then tumbled with anti-bcl-x rabbit serum for 60 min at 4°C, and followed by protein A-agarose beads. The beads were washed four times with lysis buffer and then boiled with reducing SDS sample buffer for 5 min and analyzed by SDS/PAGE on a 15% gel. After electrophoresis, the proteins were transferred to nitrocellulose, and the membrane was blocked with 5% nonfat milk/Tris-buffered saline/0.1% Tween-20. Bcl-x protein was detected by incubating the membrane with the bcl-x antiserum followed by peroxidase-conjugated goat anti-rabbit IgG. The ECL substrate (Amersham) was used as per the manufacturer's protocol.

RESULTS AND DISCUSSION

In preliminary experiments, we determined whether CsA, FK, and Rap could block the surface IgM signal transduction pathway leading to PCD in WEHI-231 cells. Concentrations of CsA up to 100 ng/ml and FK and Rap up to 1 μg/ml did

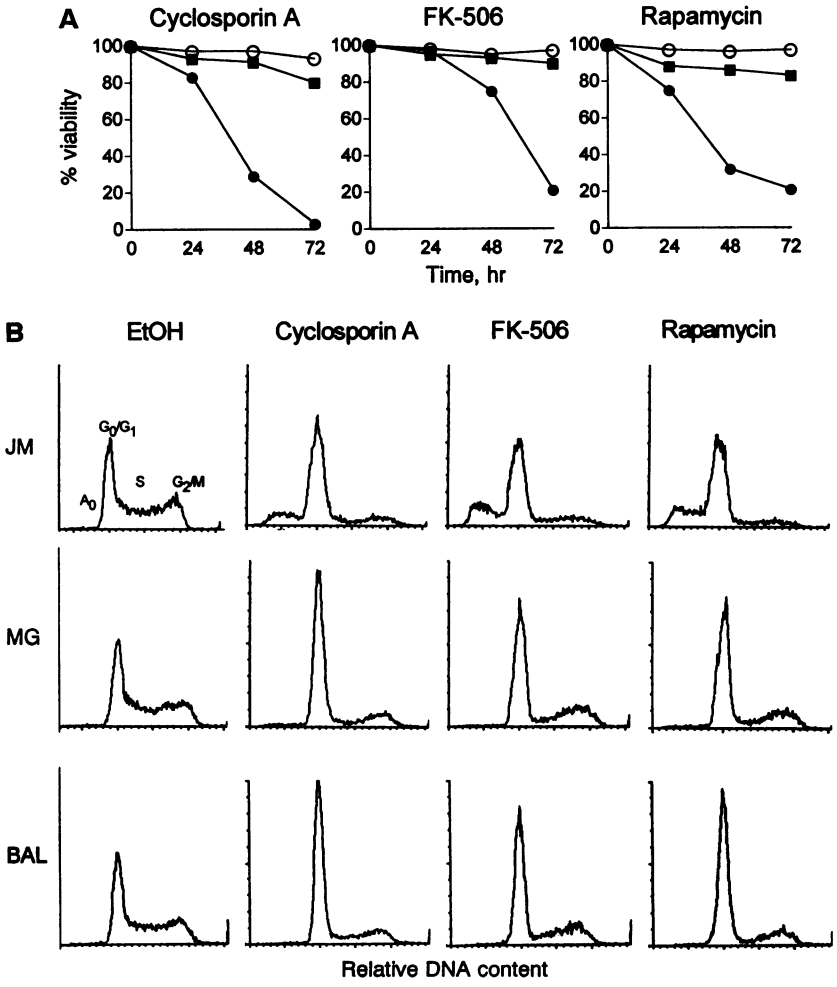

FIG. 1. CsA, FK, and Rap cause growth arrest in WEHI-231 and BAL-17 cells but PCD only in WEHI-231 sublines susceptible to anti-IgM-mediated apoptosis. (*A*) WEHI-231.7 JM (●), BAL-17 (○), and WEHI-231 MG (■) cells (2×10^5) were incubated in 24-well plates and treated with CsA (1 μg/ml), FK (10 μg/ml), Rap (10 μg/ml), or equal volume of EtOH as the vehicle ($t = 0$). After the indicated period of time, viability was determined by propidium iodide exclusion as described in *Materials and Methods*. The data shown here are representative of three independent experiments. (*B*) WEHI-231.7 JM (JM), BAL-17 (BAL), and WEHI-231 MG (MG) cells (2×10^5) were incubated in 24-well plates and treated with CsA (1 μg/ml), FK (10 μg/ml), Rap (10 μg/ml), or an equal volume of EtOH as the vehicle. After 24 hr, cell cycle analysis was performed as described in *Materials and Methods*. The results reported here are representative of eight independent experiments.

not inhibit anti-IgM-induced PCD (A.R.G., unpublished observation) in WEHI-231.7 JM, a subline susceptible to anti-IgM-mediated PCD (20, 21). To our surprise, concentrations of CsA of 1 μg/ml and FK and Rap concentrations of 10 μg/ml induced PCD in the absence of anti-IgM over a 72-hr period (Fig. 1*A*). The observation that CsA, FK, and Rap induced PCD in WEHI-231 is a finding that is in direct opposition to their effects on T cells (4, 13–16). The concentrations of FK and Rap required to cause PCD are significantly higher than those of CsA, probably reflecting the relative levels of cyclophilin and FKBP in WEHI-231.7 JM cells. At the concentrations used, these drugs did not exhibit nonspecific toxicity as both BAL-17, a mature B-cell line resistant to PCD, and WEHI-231 MG, a subline previously shown to be resistant to anti-IgM-mediated apoptosis (20, 21), demonstrated little or no cell death even at 72 hr after treatment (Fig. 1*A*).

To determine if the immunosuppressants exerted detectable effects on B cells resistant to anti-immunoglobulin-mediated PCD, we studied the cell cycle progression after drug treatment. Cell cycle analysis of WEHI-231.7 JM, WEHI-231 MG, and BAL-17 cells showed that their growth was arrested in G_0/G_1 24 hr after treatment with CsA, FK, or Rap (Fig. 1*B*). A close inspection of the data presented in Fig. 1*B* revealed a distinct population of cells (A_0) with decreased DNA staining in the PCD-susceptible WEHI-231.7 JM subline but not in PCD-resistant WEHI-231 MG or BAL-17 cell lines. This population of WEHI-231.7 JM consists of cells that either have lost DNA due to fragmentation or have reduced staining due to condensed chromatin and, in any case, are apoptotic. Our results demonstrate that CsA, FK, and Rap can all intervene in ongoing signaling events required for cell cycle progression in a variety of B-cell lines. The observation that the immunosuppressants induced growth arrest in three B cell lines, but PCD in only one, implies that growth arrest and PCD are two independently controlled events in B-cell lines. Furthermore, the correlation between susceptibility to anti-IgM-mediated and immunosuppressant-mediated PCD suggests an overlap in the signaling pathways leading to PCD.

To verify that the immunosuppressant-induced PCD was apoptosis, we examined cellular DNA by gel electrophoresis. We detected the characteristic ladder caused by internucleosomal DNA fragmentation (4, 13–16, 27, 28) only in WEHI-231.7 JM cells (Fig. 2). This finding confirms that CsA, FK, and Rap cause apoptosis in this cell line. Interestingly, the DNA fragmentation pattern caused by Rap differed from the typical DNA ladders found in anti-immunoglobulin- (20, 21), CsA-, and FK-treated cells (Fig. 2). DNA fragments from Rap-treated cells consisted almost exclusively of large molecular weight fragments, suggesting that Rap inhibits cellular events required for either the activity of the endonuclease(s) or the accessibility of DNA to the endonuclease(s). The differences in DNA fragmentation observed in the FK- and Rap-treated WEHI-231.7 JM cells indicate that the effect of these drugs cannot solely be attributed to the inhibition of the *cis–trans* isomerases.

The data presented here would suggest a model where intact activity of calcineurin and S6 kinase, cdc2, or cdk2 is required to prevent PCD in WEHI-231.7 JM. Apparently, the opposite effects occur in T cells because CsA and FK block positive regulatory events leading to PCD (4, 13–16). The simplest interpretation of our results is that CsA, FK, and Rap interrupt signaling events needed to maintain the negative regulation of PCD in WEHI-231.7 JM. In our view, PCD in WEHI-231 is under constant negative regulation (i.e., WEHI-231 cells are poised to undergo PCD). The findings presented in Fig. 3 are consistent with this hypothesis. Treatment with the protein synthesis inhibitor emetine induced PCD in both the WEHI-231.7 JM and WEHI-231 MG sublines within 8 hr (Fig. 3*A*), suggesting that all the products needed to mediate PCD are preformed but actively inhibited. The presence of the DNA ladder demonstrated that WEHI-231 cells treated with emetine die by apoptosis (Fig. 3*B*). In contrast, BAL-17 cells, which display a mature B-cell phenotype, survived for over 16 hr after the inhibition of protein synthesis, providing additional evidence for the differential regulation of PCD in mature and immature B cells. In agreement with our hypothesis, Illera *et al.* (29) have demonstrated that splenic B cells are also programmed to die, and PCD is accelerated by the inhibition of protein synthesis. Although WEHI-231 cells are particularly sensitive to apoptosis, it is important to note that other metabolic inhibitors do not induce PCD in this cell line. For example, the protease inhibitors leupeptin (100 μM) and E-64d (100 μM) did not cause PCD. Similarly, staurosporine (0.4 μM), a protein kinase C inhibitor, also did not induce apoptosis in WEHI-231 cells. These findings, in conjunction with others (ref. 21;

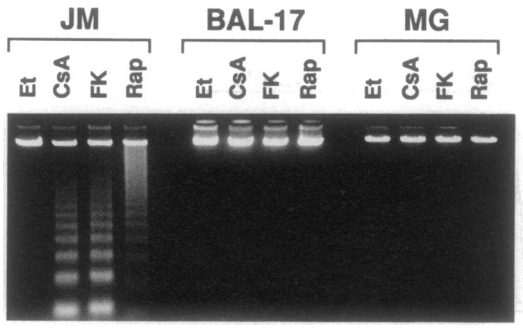

FIG. 2. DNA fragmentation, a characteristic of apoptosis, is induced by CsA, FK, and Rap in WEHI-231.7 JM cells (JM) but not in BAL-17 or WEHI-231 MG cells (MG). Cells (2×10^5) were cultured in 24-well plates and treated with CsA (1 μg/ml), FK (10 μg/ml), Rap (10 μg/ml), or an equal volume of ethanol (Et) as the vehicle. After 24 hr, the cells were collected, and the DNA was isolated and analyzed on a 2% agarose gel as described in *Materials and Methods*.

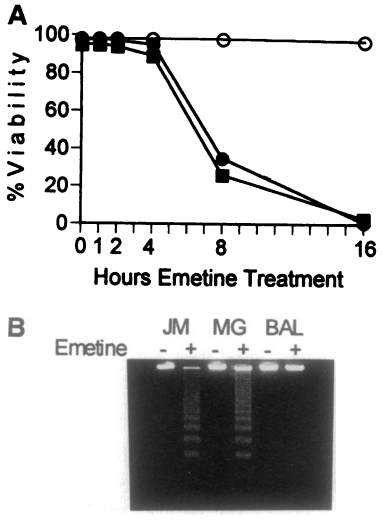

FIG. 3. Inhibition of protein synthesis induces PCD in all sublines of WEHI-231 but not BAL-17 cells. (*A*) WEHI-231.7 JM (●), BAL-17 (○), and WEHI-231 MG (■) cells (2×10^5) were incubated with emetine (1 μM). At the indicated time points, viability was determined by propidium iodide exclusion as described in *Materials and Methods*. (*B*) WEHI-231.7 JM (JM), WEHI-231 MG (MG), and BAL-17 (BAL) cells (2×10^5) were treated with emetine (1 μM). After 24 hr, the cells were collected and the DNA was isolated and analyzed on a 2% agarose gel as described in *Materials and Methods*.

J.Q. and A.R.G. unpublished observations), suggest that there is a considerable degree of selectivity in the effects of metabolic inhibitors.

Because *bcl-2* and *bcl-x_L* have been reported to play critical roles in inhibiting the apoptotic pathway in lymphocytes (25, 26, 30–34), we tested the ability of these genes to prevent PCD induced by CsA, FK, and Rap in the WEHI-231.7 JM subline. Drug-induced PCD was prevented in WEHI-231.7 JM cells by transfection with *bcl-x_L* but not by transfection with *bcl-2* (Fig. 4*A*). The inability of *bcl-2* overexpression to inhibit PCD in WEHI-231 cells is consistent with previous reports demonstrating the lack of correlation between *bcl-2* expression in WEHI-231 cells and resistance to anti-immunoglobulin-induced apoptosis (21, 24). Unlike PCD, growth arrest was not affected by the overexpression of either *bcl-2* or *bcl-x_L* (Fig. 4*B*), an observation that supports the conclusion that cell cycle arrest and PCD are independently regulated in B-cell lines (21).

Fig. 5 shows that the extent of protection provided by bcl-x_L correlates with the amount of protein expressed by the cells. When susceptibility to CsA was tested in two different *bcl-x_L* transfectants, we noticed that one cell line displayed a highly resistant phenotype while the other was significantly more sensitive (Fig. 5*A*). Upon investigation of bcl-x_L expression, we determined that the more resistant transfectant expressed ≈3 times more protein than the other (Fig. 5*B*). The transfectant expressing low levels of bcl-x_L was still less susceptible to CsA than both the *bcl-2* transfectants and the neo controls. In addition to preventing immunosuppressant-induced PCD, bcl-x_L was also capable of blocking apoptosis induced by emetine, indicating that bcl-x_L acts downstream from the putative inhibitor of the apoptotic machinery (Fig. 5*C*).

In conclusion, CsA, FK, and Rap prevent cell cycle progression and induce PCD in WEHI-231.7 JM cells. PCD can be actively suppressed by overexpression of *bcl-x_L* but not *bcl-2*. To our knowledge, this is the first report showing that *bcl-2* and *bcl-x_L* can differentially regulate apoptosis. The ability of *bcl-x_L* and the inability of *bcl-2* to protect against immunosuppressant-induced PCD have several possible interpretations. One explanation is that these two genes regulate partially independent systems to inhibit PCD in lymphoid cells. Their differential regulation of PCD could be caused by the expression of a bcl-2 inhibitor that does not affect the function of bcl-x_L. For example, bax has been shown to inhibit the ability of bcl-2 to prevent apoptosis in an interleukin 3-dependent cell line (35). We detect expression of bax in WEHI-231.7 JM cells by Northern blot analysis and immunoprecipitation from biosynthetically labeled cells (data not shown). Since resistance to immunosuppressant-induced PCD in WEHI-231 MG and BAL-17 might be due to overexpression of the endogenous *bcl-x_L* gene, we investi-

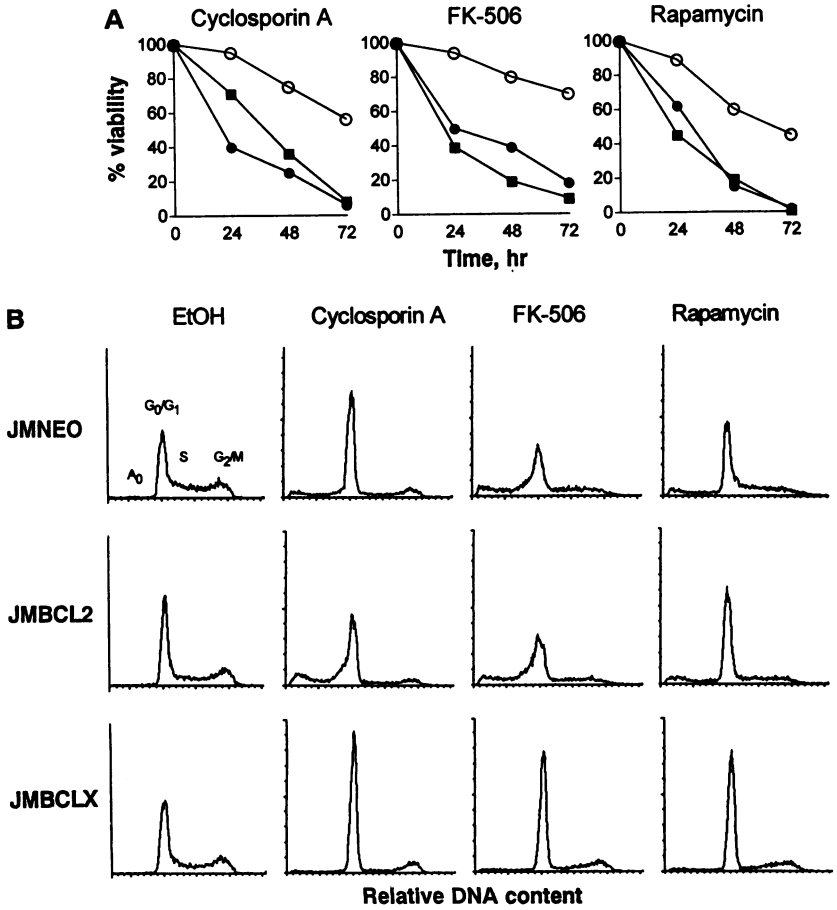

FIG. 4. Immunosuppressant-induced PCD is prevented by bcl-x_L but not by bcl-2. WEHI-231.7 JM cells were transfected by electroporation with the pSFFV-Neo plasmid containing human *bcl-2* (24) (JMBCL2), human *bcl-x_L* (25) (JMBCLX), or no insert as a control (JMNEO). (*A*) JMBCLX (○), JMBCL2 (●), and JMNEO (■) cells were incubated in 24-well plates and treated with CsA (1 μg/ml), FK (10 μg/ml), Rap (10 μg/ml), or an equal volume of EtOH as the vehicle (data not shown). Viability was determined by propidium iodide exclusion as described in *Materials and Methods* at the indicated time points. The data shown here are representative of eight independent experiments. (*B*) JMBCLX, JMBCL2, and JMNEO cells were incubated in 24-well plates and treated with CsA (1 μg/ml), FK (10 μg/ml), Rap (10 μg/ml), or an equal volume of EtOH as the vehicle. After 24 hr, cell cycle analysis was performed as described in *Materials and Methods*. The results reported here are representative of seven independent experiments.

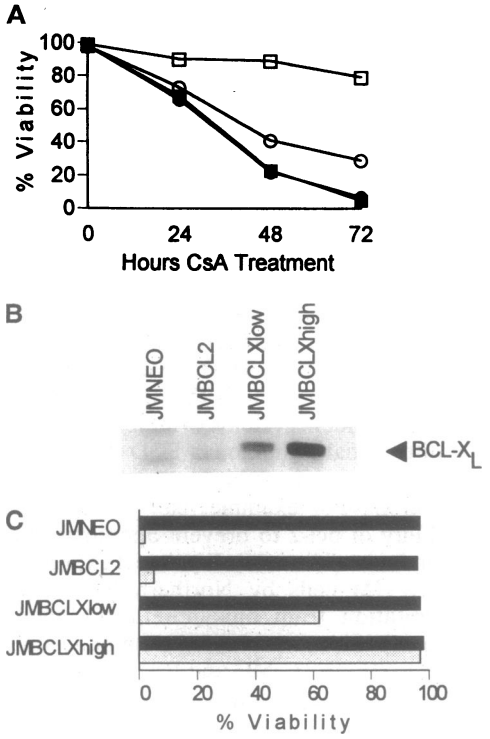

FIG. 5. Resistance to PCD correlates with the amount of bcl-x_L protein expressed. (*A*) JMNEO (■), JMBCL2 (●), JMBCLXlow (○), and JMBCLXhigh (□) cells were incubated in the absence or presence of CsA (1 μg/ml). Viability was determined by propidium iodide exclusion after the indicated period of time. (*B*) Bcl-x_L protein was detected by Western blot analysis as described in *Materials and Methods*. (*C*) JMNEO, JMBCL2, JMBCLXlow, and JMBCLXhigh cells were incubated in the absence (black bars) or presence (stippled bars) of emetine (1 μM). After 16 hr, viability was determined by propidium iodide exclusion.

gated the levels of *bcl-x* in these cell lines. We observed detectable levels of *bcl-x$_L$* mRNA in BAL-17 cells but not in WEHI-231 MG cells (data not shown), which implies that bcl-x_L is sufficient but not required to prevent PCD.

Although the immunosuppressive effects of CsA, FK, and Rap have mainly been attributed to their ability to inhibit T-cell activation (1), our results suggest two additional mechanisms for the immunosuppressive effects of these drugs. First, CsA, FK, and Rap appear to prevent cell cycle progression in B cells, indicating that these pharmacological reagents may be capable of inhibiting the clonal expansion of B cells after exposure to antigen. Second, these drugs can induce apoptosis in an immature B-cell line, implying that interruption of ongoing signal transduction events may cause clonal deletion of developing B cells. Our results suggest that the seemingly paradoxical use of immunosuppressants to treat selected B-cell lymphomas/leukemias and B-cell-associated autoimmune diseases should be explored.

We thank Carol Lynne McShan for her excellent technical assistance, Dr. Suren Sehgal at Wyeth-Ayerst Research for supplying rapamycin and for insightful discussion, and Fujisawa USA, Inc., for supplying FK-506. This work was supported by National Institutes of Health Grants PO1 CA-19266, RO1 AI-19589, T32 GM-07281, and T32 GM-07183. L.H.B. is a fellow of the Leukemia Society of America.

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