Overexpression of Bcl-2 in Transgenic Mice Decreases Apoptosis and Improves Survival in Sepsis

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Overexpression of Bcl-2 in Transgenic Mice Decreases Apoptosis and Improves Survival in Sepsis


In sepsis there is extensive apoptosis of lymphocytes, which may be beneficial by down-regulating the accompanying inflammation. Alternatively, apoptosis may be detrimental by impairing host defense. We studied whether Bcl-2, a potent antiapoptotic protein, could prevent lymphocyte apoptosis in a clinically relevant model of sepsis. Transgenic mice in which Bcl-2 was overexpressed in T cells had complete protection against sepsis-induced T lymphocyte apoptosis in thymus and spleen. Surprisingly, there was also a decrease in splenic B cell apoptosis in septic Bcl-2 overexpressors compared with septic HeJ and HeOuJ mice. There were marked increases in TNF-α, IL-1β, and IL-10 in thymic tissue in sepsis in the three species of mice, and the increase in TNF-α and IL-10 in HeOuJ mice was greater than that in Bcl-2 mice. Mitotracker, a mitochondrial membrane potential indicator, demonstrated a sepsis-induced loss of membrane potential in T cells in HeJ and HeOuJ mice but not in Bcl-2 mice. Importantly, Bcl-2 overexpressors also had improved survival in sepsis. To investigate the potential impact of loss of lymphocytes on survival in sepsis, Rag-1+/− mice, which are totally deficient in mature T and B cells, were also studied. Rag-1+/− mice had decreased survival compared with immunologically normal mice with sepsis. We conclude that overexpression of Bcl-2 provides protection against cell death in sepsis. Lymphocyte death may be detrimental in sepsis by compromising host defense. The Journal of Immunology, 1999, 162: 4148–4156.

Sepsis is the leading cause of death in the surgical intensive care unit and a major cause of morbidity and mortality in neonatal and medical intensive care units. The Center for Disease Control estimates that each year approximately 500,000 people develop sepsis, and 175,000 die in the United States alone (1). Sepsis is a growing problem; its incidence has tripled from 1972 to 1992 (1). Recently, apoptosis has been identified as an important mechanism of cell death in animal models of sepsis and endotoxiaemia (2–6). In this setting, lymphocytes are the predominant type of cells undergoing apoptotic death. However, parenchymal cells, including intestinal and lung epithelial cells and vascular endothelial cells, also have increased apoptotic cell death in certain models of sepsis (6–8). The potential beneficial or adverse effects of apoptosis on sepsis are unknown. Sepsis is a subset of the systemic inflammatory response syndrome, and apoptosis could be beneficial to the host by eliminating many of the lymphocytes that produce proinflammatory cytokines, such as TNF or IL-1 (7, 9). Alternatively, apoptosis may be detrimental in sepsis by depleting lymphocytes, with resultant compromise of the host defenses against the invading organisms (7, 9). Also, if apoptosis of parenchymal cells is extensive, organ dysfunction may occur. For example, loss of intestinal epithelial cells in sepsis may lead to increased bacterial translocation, thus worsening infection.

The proto-oncogene bcl-2 was discovered by translocation analysis of follicular B lymphomas. The bcl-2 gene family is now known to contain both pro- and antiapoptotic members (10, 11). The protein product Bcl-2 prevents apoptotic cell death from a wide array of noxious stimuli, including hypoxia, serum and growth factor withdrawal, ionizing irradiation, glucocorticoids, and calcium ionophores (11, 12). Because of the known protective ability of Bcl-2 to prevent cell death in a variety of conditions, we investigated its possible role in apoptotic cell death occurring in sepsis. Transgenic mice overexpressing Bcl-2 in T lymphocytes were made septic, and thymus and spleen were examined for the degree of apoptotic cell death. The genetic background strain for the Bcl-2 overexpressors was the C3H HeJ mouse. This strain of mouse is resistant to the lethal effects of endotoxin and recently has been demonstrated to have extensive apoptosis in multiple organs in an intra-abdominal model of sepsis (3, 13). To eliminate the potential confounding effects of endotoxin resistance, an additional strain of nonendotoxin-resistant mice, i.e., C3H HeOuJ mice, very similar in genetic background to C3H HeJ mice, was used as a second control group in all studies.

A second member of the bcl-2 gene family is the proapoptotic Bax. Targeted disruption of Bax blocks cell death after trophic factor deprivation and during development (14, 15). The protein Bax forms heterodimers with Bcl-2, and it is postulated that a preset ratio of Bcl-2/Bax determines the survival or death of cells following an apoptotic stimulus (11, 15). To investigate the potential role of Bax in the apoptotic cell death occurring during sepsis, Bax-deficient mice were examined also.

Cytokines are important mediators of sepsis, and the apoptotic cell death program can be triggered by TNF-α (16). Bcl-2 has been shown to decrease T cell activation and block cytokine production in Jurkat T cells by impairing DNA binding and nuclear translocation of the
nuclear factor of activated T cells (NFAT)\(^1\) (17). Therefore, one potential mechanism for the protective effect of Bcl-2 in sepsis would be to decrease cytokines. To address this question, proinflammatory (TNF-\(\alpha\) and IL-1\(\beta\)) and anti-inflammatory (IL-10) cytokines were measured in thymi and spleens in sham and septic mice.

In addition to possible effects on cytokines, Bcl-2 may decrease apoptosis by preventing the loss of mitochondrial membrane potential (MMP) (18, 19). A current theory concerning the apoptotic death program maintains that loss of MMP allows leakage of cytochrome \(c\) into the cytosol, which triggers apoptosis. Bcl-2 localizes to the outer mitochondrial membrane and is postulated to block cytochrome \(c\) release by an unknown mechanism. The fluorescent indicator Mitotracker Red and flow cytometry were employed in isolated thymocytes to examine MMP in sepsis and determine potential effects of Bcl-2 on preservation of membrane potential (20, 21). The present experiments were performed primarily to test the hypothesis that Bcl-2 can prevent sepsis-induced lymphocyte apoptosis. Survival studies also were performed to determine whether prevention of lymphocyte apoptosis was beneficial or detrimental to host survival in sepsis.

### Materials and Methods

#### Transgenic mice

Mice that selectively overexpress Bcl-2 in T lymphocytes using the lck-proximal promoter were generated and backcrossed to C3H-HeJ mice for >10 generations as described previously (22). Also as described, all transgenic lines demonstrated Bcl-2 protein in the thymus, spleen, and lymph nodes (both mesenteric and inguinal) (22). Bcl-2 protein was higher in thymus than spleen or lymph nodes, consistent with the percentage of T cells in these organs (22). Mice homozygous for the gene causing Bcl-2 overexpression were mated to C3H HeJ mice, and a tail blood sample was obtained from offspring to verify overexpression of Bcl-2 in T lymphocytes by PCR analysis (23). The genetic background of the Bcl-2 overexpressors is C3H HeJ, a species of mouse that is highly resistant to the lethal effects of endotoxin (24). To avoid potential confounding influence of the endotoxin resistance, a second group of endotoxin-sensitive mice, C3H HeOuJ, also served as a control group. C3H HeOuJ mice have a very similar genetic background to the C3H HeJ mice but are endotoxin sensitive. C3H HeJ and C3H HeOuJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were age and sex matched with the Bcl-2 transgenic mice.

The Bax-deficient mice were generated as described previously (15), and the genotype was verified by PCR analysis (23). Normal littermates, i.e., non-Bax-deficient mice were used as controls. Four Bax-deficient mice and four normal littermates were available for study. Therefore, tissue sample analysis was conducted, but animals were not available for survival studies.

#### Sepsis model: cecal ligation and puncture (CLP)

Mice weighing 17–25 g were housed for at least 3 days before manipulations. The CLP model of sepsis was used to induce intra-abdominal periitonitis as described previously (7, 13). The CLP model was selected because it results in a model that reproduces many of the clinical hallmarks of sepsis that occur in patients (25). Previous studies in our laboratory include positive blood cultures for polymicrobial organisms (aerobic and anaerobic bacteria) from CLP mice but not sham-operated mice (7). The presence of live bacterial organisms is an important component of the model because many drugs that inhibit the inflammatory component of the septic response impair the ability of the host to fight pathogens. The details of the CLP mouse model in our laboratory have been described previously (7, 13). In the CLP group, a midline laparotomy was performed using three-color analysis and flow cytometry. Assessment of MMP was quantified using a commercially available, fluorescein-labeled, mitochondrial membrane potential (MMP) (18, 19). A current theory concerning the apoptotic death program maintains that loss of MMP allows leakage of cytochrome \(c\) into the cytosol, which triggers apoptosis. Bcl-2 localizes to the outer mitochondrial membrane and is postulated to block cytochrome \(c\) release by an unknown mechanism. The fluorescent indicator Mitotracker Red and flow cytometry were employed in isolated thymocytes to examine MMP in sepsis and determine potential effects of Bcl-2 on preservation of membrane potential (20, 21). The present experiments were performed primarily to test the hypothesis that Bcl-2 can prevent sepsis-induced lymphocyte apoptosis. Survival studies also were performed to determine whether prevention of lymphocyte apoptosis was beneficial or detrimental to host survival in sepsis.

#### Quantification of apoptosis

Apoptosis was quantified by four independent methods with varying sensitivity and specificity as described previously (7). Sham or CLP mice were killed 18–22 h postsurgery, and thymus and spleen were removed rapidly. A portion of the thymus and spleen was frozen in liquid nitrogen and stored at −80°C for later use in DNA agarose gel electrophoresis and cytokine determination. A second portion of the tissue samples was placed in 10% neutral buffered paraformaldehyde for hematoxylin and eosin and TUNEL staining. A third portion of the thymus and spleens was used for flow cytometry.

#### Fluorescent TUNEL

At 16–22 h after CLP or sham surgery, mice were killed, and fluorescent TUNEL was performed as described previously (7, 13) with minor modifications. Paraffin-embedded tissue slices were dewaxed and rehydrated. Using an apoptosis detection kit (Boehringer Mannheim, Indianapolis, IN) cells were permeabilized by microwave treatment in citrate buffer and DNA labeled with dUTP fluorescein via action of terminal deoxynucleotidyl transferase, which attaches the dUTP at the 3'-hydroxyl DNA ends (fluorescent TUNEL). Apoptotic nuclei have nicks (strand breaks) in their DNA that are labeled with the fluorescein dUTP. In some cases, a second DNA counterstain, i.e., Hoechst 33342, was used to label nonapoptotic nuclei. Tissue sections were examined at ×400 magnification by fluorescence microscopy, and a minimum of three random fields were evaluated. The percent area of the field that was positively labeled for apoptosis (fluorescent stained) was calculated using an image analysis program (Meta morph, Universal Imaging Corp., West Chester, PA). The percent area was divided by the area of an average cell to obtain the number of TUNEL-positive cells.

#### Light microscopy of hematoxylin- and eosin-stained specimens

Hematoxylin- and eosin-stained specimens were examined by a pathologist (K.E.S.) blinded to sample identity who identified apoptosis based on characteristic cellular morphologic changes, including compacted/condensed nuclei (pyknosis) and/or nuclear fragmentation (karyorrhexis). The specimens were graded at ×400 magnification for the percentage of cells demonstrating apoptotic features. A minimum of three random fields were evaluated in each sample.

#### DNA agarose gel electrophoresis

DNA agarose gel electrophoresis was performed as described previously (7, 13) with minor modifications. Tissue was homogenized in digestion buffer, i.e., DNAzol (Molecular Research, Cincinnati, OH). Following centrifugation, the top layer was transferred to a second tube, and absolute ethanol was added. After a second centrifugation, the precipitate was washed with 95% ethanol, and DNA dissolved in TE buffer (pH 8.0). DNA (7 μg) was pipetted onto a 2% agarose gel containing either ethidium bromide or Sybr Green (Molecular Probes, Eugene, OR), and electrophoresis was performed.

#### Flow cytometry: cell phenotyping and mitochondrial membrane potential

Tissue sections (10–200 mg) from thymi or spleens were gently glass ground to dissociate the cells that were washed twice in PBS with 1% BSA and 0.01% sodium azide. Residual RBC were lysed by hypotonic lysis in ice-cold ammonium chloride. The cells were resuspended in PBS, and the desired fluorescent indicators were added. The degree of apoptotic cell death was quantified using a commercially available, fluorescein-labeled, annexin V product (Apoptosis Detection Kit, R&D Systems, Minneapolis, MN) as previously described (26). Mouse T and B cells were identified using cyochrome-labeled anti-CD3 and phycoerythrin-labeled anti-CD19 Abs, respectively (PharMingen, San Diego, CA). Identification of apoptotic cells using annexin V and cell phenotyping were performed simultaneously using three-color analysis and flow cytometry. Assessment of MMP was performed using Mitotracker Red (catalogue no. M-7513, Molecular Probes) (20, 21). Cells were incubated in 100 nM Mitotracker Red at 37°C for 1 h in the dark, Flow cytometric analysis (50,000 events/sample) was performed on FACSCalibur (Becton Dickinson, San Jose, CA). Cell debris was electronically gated out based on the forward light scatter.

#### Cytokine determination

Tissue concentrations of TNF-\(\alpha\), IL-1\(\beta\), and IL-10 were measured by ELISA using commercially available kits (Genzyme, Cambridge, MA) as

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\(^{1}\) Abbreviations used in this paper: NFAT, nuclear factor of activated T cells; MMP, mitochondrial membrane potential; CLP, cecal ligation and puncture; Rag-1, recombination-activating gene-1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.
The methods for the survival studies in the mouse CLP model have been previously described (27). Approximately 10–15 mg of tissue samples were homogenized in a tissue grinder containing 1 ml of lysis buffer (PBS containing 2 mM PMSF and 1 mg/ml of aprotinin, leupeptin, and pepstatin A) as described by Clark et al. (28). Analysis was performed with 100 μl of standard (diluted in lysis buffer) or 10, 50, or 100 μl of tissue homogenate. Each sample was run in duplicate, and a portion of the sample was analyzed for protein. Data are expressed as picograms per milligrams of protein. For each assay a standard curve was generated and, based on analysis of variance of replicates of the measured absorbance, demonstrated an average coefficient of variance of <10%. Recovery of spiked samples, i.e., containing known amounts of TNF-α, IL-1β, or IL-10, was about 90% (data not presented).

Survival studies in sepsis

Additional groups of mice, C3H HeJ, C3H HeOuJ, and Bcl-2, from over-expressor strains underwent CLP and were included in survival studies. The methods for the survival studies in the mouse CLP model have been described previously (29). Briefly, an investigator (R.S.H.) blinded to the identity of the mice performed CLP in the various groups of mice. Approximately 1 h after CLP, the mice received metronidazole (35 mg/kg; an antibiotic with an anaerobic bacterial spectrum of action) and ceftriaxone (50 mg/kg; an antibiotic with a Gram-negative bacterial spectrum of action). The antibiotics were repeated every 12 h for 48 h and then discontinued. The mice were allowed free access to food and water, and survival was recorded for 6 days.

To further evaluate the impact of lymphocyte apoptosis in sepsis, an additional sepsis survival study was conducted in Rag-1−/− mice. Rag-1−/− mice are homozygous for the Rag-1−/− Tm1 Mom mutation and produce no mature T or B cells (30). Rag-1−/− mice and their matched normal controls (C57B6/J×129) were obtained from The Jackson Laboratory. Both groups of mice underwent CLP, and survival was recorded as previously stated.

This study was conducted in accordance with the National Institutes of Health guidelines on the use of laboratory animals and with approval of the Washington University animal studies committee.

Statistical analysis

Data are reported as the mean ± SEM. Data were analyzed using a statistical software program RS/1 from BBN Software (Cambridge, MA). Differences in group survival and DNA agarose gels were determined using Fischer’s exact p test. All other data were analyzed using one-way analysis of variance. Significance was accepted at the p ≤ 0.05.

Results

Fluorescent TUNEL

Tissue sections were examined at ×400 magnification. Thymi and spleens from sham-operated Bcl-2, HeJ, and HeOuJ mice had approximately two to five cells positively stained for apoptosis, with many fields entirely negative. Therefore, these tissues were classified as negative for apoptosis.

Thymi from septic HeJ (n = 17), HeOuJ (n = 6), and Bax-deficient (n = 4) mice had many fields in which approximately 10–15% of cells were positively labeled for apoptosis (Fig. 1, A and C, and Fig. 2) and were indistinguishable from each other. It was not possible to count individual nuclei in thymi because many apoptotic cells were confluent or overlapping (Fig. 1, A and C).
Therefore, to estimate the number of TUNEL-positive cells, the TUNEL-positive area first was determined by an image analysis program, Metamorph (Universal Imaging, West Chester, PA). Next, the total TUNEL-positive area was divided by the averaged area of a single cell to determine the estimated number of TUNEL-positive cells. Employing this method, the numbers of TUNEL-positive cells per high powered field (×400) in the various groups were: HeJ, 63.6 ± 16.9; OuJ, 76.5 ± 25.7; and Bax-deficient, 121.2 ± 30.8. Thymi from Bcl-2 mice with sepsis (n = 13) had 9.1 ± 1.6 cells per high powered field positively labeled for apoptosis, with many areas entirely negative for apoptosis (Figs. 1B and 2). Bcl-2 thymi were statistically different from all other groups (p < 0.05).

Spleens from HeJ (n = 13), HeOuJ (n = 6), Bcl-2 overexpressor septic mice (n = 13), and Bax-deficient mice (n = 4) had less apoptotic labeling than did thymi from the same animal. The numbers of TUNEL-positive cells in spleens per HPF from HeJ, HeOuJ, and Bax-deficient mice were 18.6 ± 3.2, 19.8 ± 4.0, and 18.3 ± 3.3, respectively. Spleens from Bcl-2 mice had an average of 8.2 ± 1.0 cells TUNEL-positive/high powered field, and this was statistically different from the values in HeJ and HeOuJ spleens (p < 0.05).

Conventional light microscopy of hematoxylin- and eosin-stained specimens

Histologic examination of specimens was performed by a pathologist (P.E.S.) who was blinded to sample identity. A minimum of three random fields were examined at ×400 magnification, and the percentage of cells demonstrating apoptotic features was recorded. Cells were judged to be apoptotic if they exhibited the characteristic findings of shrunken, compacted nuclei (pyknosis) and/or nuclear fragmentation (karyorrhexis). Thymi and spleens from sham-operated HeJ, HeOuJ, and Bcl-2 overexpressor mice appeared normal, and <1% of cells exhibited apoptotic features (n = 3–5 mice/group). Thymi from septic HeJ (n = 6; Fig. 1D), HeOuJ (n = 6; Fig. 1F), and Bax-deficient (n = 4) mice revealed extensive apoptosis, which was more prominent in cortical than in medullary regions (Fig. 1, D and F). In selected regions, the percentage of cells with apoptotic features was >25% (Fig. 1). There was no readily apparent difference in the degree of apoptosis in thymi in these three strains of mice. Thymi from septic Bcl-2 mice were identical with those from sham-operated mice, with <1% of cells showing evidence of apoptosis (Fig. 1E), and this was statistically different from other groups (p < 0.05).

Spleens from septic HeJ (n = 6), HeOuJ (n = 6), and Bax-deficient (n = 4) mice had less extensive apoptosis than thymi from these mice. Approximately 2–5% of cells had pyknotic or fragmented nuclei. In some regions of the spleen as many as 10% of cells were apoptotic. In contrast to the thymus, spleens from septic Bcl-2 mice (n = 10) were not obviously different from those from the other groups of mice, and approximately 2–5% of cells had characteristic changes of pyknosis and/or karyorrhexis. However, direct comparison of spleens from septic Bcl-2 mice to spleens from HeJ and HeOuJ mice did reveal a difference in the anatomic localization of apoptosis. Apoptosis was present throughout the spleens of septic HeJ and HeOuJ mice, including the area surrounding the periaortiolar lymphoid sheath, which is the T cell-rich area. In contrast, there was a noted lack of apoptosis in the periaortiolar lymphoid sheath in the septic Bcl-2 mice.

DNA agarose gel electrophoresis

DNA agarose gels from thymi and spleens of sham-operated mice had no evidence of DNA fragmentation, i.e., ladder formation by the electrophoresis procedure (n = 3–5 mice/group). Thymi from septic HeJ (n = 10), HeOuJ (n = 6), and Bax-deficient (n = 4) mice had 80, 67, and 100% degrees of positive ladder formation, a characteristic feature of apoptosis. In contrast, none of the thymi from Bcl-2 (n = 12) mice had ladder formation, and this was significantly different from all other groups (p < 0.05). An example of HeJ and Bcl-2 is shown in Fig. 3. The spleens from the HeJ (n = 12), HeOuJ (n = 6), and Bcl-2 (n = 11) mice had 33, 50, and

FIGURE 2. Fluorescent TUNEL. Representative black and white images from thymus tissue sections of septic mice were labeled using the fluorescent TUNEL method (to identify apoptotic nuclei) and were counterstained with Hoechst 33342, which stains all nuclei (apoptotic and non-apoptotic). Note the large number of apoptotic nuclei in HeJ, HeOuJ, and Bax thymus but minimal apoptosis in Bcl-2 thymus. Images obtained using a laser scanning confocal microscope at ×400. The TUNEL and corresponding Hoechst images were obtained from the identical microscopic field of view using two different excitation frequencies and emission filters.
In thymi, sepsis caused a marked increase in both the proinflammatory cytokines TNF-α (about three- to sixfold) and IL-1β (two- to ninefold) as well as the anti-inflammatory cytokine IL-10 (1.3- to 2-fold) in all three species of mice (p < 0.05; Table III). The increases in TNF-α and IL-10 in sepsis were greater in HeOuJ thymi than in Bcl-2 thymi (p < 0.05), but were not different from those in HeJ thymi. The increase in cytokine concentrations in spleens was not as great as that which occurred in the thymi. In spleens, there was a 74% increase in TNF-α in septic vs sham HeJ mice (p < 0.05) and a 116% increase in IL-10 in septic vs sham HeOuJ mice (p < 0.05). Cytokine concentrations did not increase in septic vs sham spleens of Bcl-2 mice (Table III).

Survival study

The numbers of mice in the various group were as follows: 15 HeJ, 20 HeOuJ, and 9 Bcl-2 homozygous expressors, and 13 Bcl-2 heterozygous expressors. The Bcl-2 homozygous and heterozygous expressors had very similar survival curves; therefore, their data were combined. There were no deaths in any group at 24 h (Fig. 5). At 48 and 72 h, some of the HeJ and HeOuJ mice, but no Bcl-2 mice, had died. At 72 h there was a statistical difference between Bcl-2 and both groups (p < 0.05). At the end of the 6-day period, there were 26, 55, and 73% survivals in HeJ, HeOuJ, and Bcl-2 mice, respectively. There was a statistically significant improvement in long term, i.e., 6-day survival in Bcl-2 mice compared with HeJ mice (p < 0.05) but not compared with HeOuJ mice. There was also an improved survival in HeOuJ mice vs HeJ mice at 6 days (p < 0.05).

Nineteen Rag-1<sup>−/−</sup> mice and 24 matched, immunologically competent, control mice underwent CLP. At the end of 6 days, there was a 5.3% survival in Rag-1<sup>−/−</sup> mice vs a 45.8% survival in matched controls, and this difference was significant (p < 0.05; Fig. 6).

Discussion

Perhaps the most important finding of the present study was the remarkable ability of Bcl-2 to protect against the lymphocyte cell death occurring in the highly complex and clinically relevant model of sepsis. There was complete protection against apoptosis in thymocytes and splenic T cells during sepsis, a finding that corresponds to the T cell-restricted native overexpressor phenotype in these animals. The ability of Bcl-2 overexpression to prevent lymphocyte cell death was surprising because of previous studies in our laboratory demonstrating a lack of efficacy of TNF-α receptor blockade (13) and transgenic models including Fas receptor knockout and perforin knockout mice (our unpublished observations). Indeed, the ability of Bcl-2 to prevent cell death in sepsis

Table I. Percent apoptotic cells determined by flow cytometry<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>HeJ sham</th>
<th>HeOuJ sham</th>
<th>Bcl-2 sham</th>
<th>HeJ clp</th>
<th>HeOuJ clp</th>
<th>Bcl-2 clp</th>
</tr>
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<tbody>
<tr>
<td>Thymus T cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>6.9 ± 1.2 (4)</td>
<td>6.3 ± 1.2 (4)</td>
<td>5.1 ± 0.8 (5)</td>
<td>15.6 ± 1.2* (6)</td>
<td>50.1 ± 4.2*Δ (6)</td>
<td>6.2 ± 0.9 (10)</td>
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<tr>
<td>Spleen B Cells</td>
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<tr>
<td>7.0 ± 0.5 (4)</td>
<td>10.0 ± 2.0 (4)</td>
<td>7.1 ± 1.2 (5)</td>
<td>17.7 ± 0.8* (6)</td>
<td>35.9 ± 2.6*Δ (6)</td>
<td>10.0 ± 0.8 (10)</td>
<td></td>
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<tr>
<td>Spleen T Cells</td>
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<tr>
<td>9.6 ± 1.2 (4)</td>
<td>11.4 ± 4.4 (4)</td>
<td>8.4 ± 1.4 (5)</td>
<td>33.9 ± 4.8* (6)</td>
<td>29.2 ± 5.2* (6)</td>
<td>9.3 ± 0.8 (10)</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Values expressed as mean ± SEM. The percent of T or B cells that were undergoing apoptosis were determined using annexin V and flow cytometry as described in Materials and Methods. Cecal ligation and puncture (clp) caused an increase in apoptosis in T and B cells in HeJ and HeOuJ mice but not in Bcl-2 mice; *, p < 0.05 clp>sham for matched strains. The change in the percent apoptotic in T or B cells in clp HeOuJ mice was greater than the percent apoptotic in T or B cells of HeJ mice. The number of mice in each group is identified in parenthesis. The flow cytometry study was conducted twice.
is extraordinary given the complex pathophysiologic nature of this disorder and the failure of the previous transgenic models to block apoptosis. An array of interlocking pathways and mediators is recruited in sepsis, and a partial list includes cytokines, complement, leukotrienes, oxygen radicals, and coagulation factors. The inhibitory effect of Bcl-2 on cell death in sepsis implies that it acts in a final common pathway of apoptosis. Studies conducted on isolated cells have shown that Bcl-2 has a wide-ranging capability to block cell death from an array of noxious stimuli, including gamma irradiation, hypoxia, growth factor withdrawal, and oxidative stress (12, 22). The present study is important because it demonstrates that Bcl-2 has a protective effect in a clinically relevant in vivo model.

Although the Bcl-2 transgenic mice used in this study had overexpression of Bcl-2 limited to T cells, there was also an apparent decrease in B cell apoptosis in septic Bcl-2 mice as well. The 42% increase in splenic B cell apoptosis in septic vs sham Bcl-2 mice did not achieve statistical significance and was much less than the marked increases in B cell apoptosis in HeJ (150%) and HeOuJ (259%) mice (p, 0.05). There is no ready explanation for this protective effect in B cells, although histologic examination may provide a clue. As is apparent by both light and fluorescence microscopy, apoptotic cell death in sepsis occurred in islands or clusters (Fig. 1, A, C, D, and F, and Fig. 2). This focal anatomic pattern suggests a potential paracrine effect, and recent studies indicate that such a phenomenon exists in apoptosis. Su et al. demonstrated that paracrine apoptosis occurred in Jurkat T cells and was mediated by shedding of FAS ligand (31). Therefore, if T splenocyte apoptosis is decreased in the spleen, neighboring B cells may also be protected.

**FIGURE 4.** MMP. Suspensions of thymic cells were prepared from sham or septic mice as described in Materials and Methods. The cells were incubated with Mitotracker Red (Molecular Probes), which is a sensitive indicator of MMP. There is a decrease in the fluorescent intensity of Mitotracker Red (x-axis), with loss of MMP. Note the increase in the percentage of thymocytes from septic HeJ mice with decreased MMP (27.3% in HeJ septic vs 3.4% sham). No loss of MMP occurred in Bcl-2 septic mice. The arrow in HeJ septic identifies the subset of T cells with decreased MMP. *, 27.3% of cells. This experiment was performed on two occasions and demonstrated reproducible results.

**Table II.** Percent Thymus T cells with decreased MMP*  

<table>
<thead>
<tr>
<th>Thymus T Cells</th>
<th>HeJ sham</th>
<th>HeOuJ sham</th>
<th>Bcl-2 sham</th>
<th>HeJ clp</th>
<th>HeOuJ clp</th>
<th>Bcl-2 clp</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3 ± 0.4† (4)</td>
<td>7.1 ± 0.6 (4)</td>
<td>5.3 ± 1.6 (4)</td>
<td>28.4 ± 2.2* (6)</td>
<td>18.9 ± 2.6* (6)</td>
<td>5.4 ± 1.5 (6)</td>
<td></td>
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</table>

* The percent of thymic T cells with decreased MMP were determined using flow cytometry and the fluorescent indicator Mitotracker as described in Materials and Methods. There was a loss of MMP with sepsis in T cells from HeJ and HeOuJ but not in Bcl-2 mice. †, clp—sham for matched strain, p < 0.05. Septic Bcl-2 was less than both clp HeJ and HeOuJ, p < 0.05. See Fig. 5 for representative flow data. †, Values are mean ± SEM and expressed as pg/mg protein. The number of mice in each group is identified in parenthesis. This experiment was conducted twice and results of the two studies were combined.
Another important implication of this study relates to the role of apoptosis in the pathophysiology of sepsis. Investigators postulated that apoptosis might be beneficial by causing elimination of lymphocytes, which are a major source of cytokines in sepsis. A decrease in cytokines could reduce the uncontrolled systemic inflammatory component of sepsis that contributes to organ injury (7, 9). Alternatively, apoptosis of lymphocytes may be detrimental in sepsis by compromising the ability of the host to combat infection (7, 9). Overexpression of Bcl-2 prevented T cell death and caused improvement in 3-day survival in Bcl-2 transgenic mice compared with that in both HeJ and HeOuJ strains.

In the present study broad spectrum antibiotics were employed in the model because it is believed to reflect the conditions that occur in patients (25). Importantly, live bacterial pathogens can be cultured from blood and peritoneal fluid in mice that have undergone CLP (7). Patients in whom the circulating lymphocyte count decreased for a 3-day duration had a greatly increased risk of nosocomial sepsis (32). The particular model of sepsis used to examine therapeutic efficacy is of great importance in study design (25). We selected the CLP model because it is believed to reflect the conditions that occur in patients (25).

Table III. Tissue cytokines

<table>
<thead>
<tr>
<th>Tissue Cytokines</th>
<th>HeJ sham</th>
<th>HeOuJ sham</th>
<th>Bcl-2 sham</th>
<th>HeJ clp</th>
<th>HeOuJ clp</th>
<th>Bcl-2 clp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>7</td>
<td>15</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>TNF-α</td>
<td>218.4 ± 46.3*</td>
<td>268 ± 50.9</td>
<td>256.5 ± 50.3</td>
<td>1010.4 ± 93.9*</td>
<td>1657.4 ± 221.7*†</td>
<td>803.7 ± 78.1*†</td>
</tr>
<tr>
<td>IL-1β</td>
<td>231 ± 7.5</td>
<td>329.2 ± 102</td>
<td>206.2 ± 20.6</td>
<td>742.3 ± 139.1*</td>
<td>3024.1 ± 1036.8*</td>
<td>739.0 ± 234.3*</td>
</tr>
<tr>
<td>IL-10</td>
<td>605.2 ± 110.0</td>
<td>1423.2 ± 362.1</td>
<td>614.5 ± 61.5</td>
<td>1613.2 ± 313.1*</td>
<td>2438.8 ± 419.4*</td>
<td>811.7 ± 179.9*</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>4</td>
<td>7</td>
<td>19</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>TNF-α</td>
<td>171.1 ± 21.7</td>
<td>176 ± 17.2</td>
<td>166.8 ± 22.2</td>
<td>297.8 ± 24.5*</td>
<td>369.3 ± 64.7</td>
<td>279.5 ± 39.2</td>
</tr>
<tr>
<td>IL-1β</td>
<td>52.6 ± 6.5</td>
<td>32.7 ± 4.2</td>
<td>68.7 ± 12.3</td>
<td>78.3 ± 5.6</td>
<td>61.2 ± 15.3</td>
<td>99.3 ± 10.7</td>
</tr>
<tr>
<td>IL-10</td>
<td>200 ± 28.3</td>
<td>110.5 ± 13.7</td>
<td>230.3 ± 63.1</td>
<td>263.2 ± 27.3</td>
<td>238.7 ± 37.3*</td>
<td>250.2 ± 20.4</td>
</tr>
</tbody>
</table>

* Tissue cytokines were measured using a commercially available ELISA kit as described in Materials and Methods. †, TNF-α, IL-1β, and IL-10 were increased in clp vs sham thymi in all three groups of mice for matched strain, p < 0.05. Note that the sepsis-induced increase in cytokines in thymi were much greater than in spleen. ‡, Values are means ± SEM and expressed as pg/mg protein. The number of mice in each group is identified. The tissues were obtained from three separate experiments and then analyzed together at one time.

FIGURE 5. Survival curve: percent survival of mice following CLP. Fifteen HeJ, 20 HeOuJ, and 22 Bcl-2 overexpressors had CLP and received i.p. antibiotics (metronidazole and ceftriaxone) 1 h after surgery as described in Materials and Methods. Survival was recorded for 6 days. Δ, On day 3, Bcl-2 had improved survival compared with both HeOuJ and HeJ (p < 0.05). *, On day 6, Bcl-2 had improved survival compared with HeJ (p < 0.05). †, On day 6, HeOuJ had improved survival compared with HeJ, (p < 0.05). The survival experiment was conducted in one large study.

FIGURE 6. Survival curve: percent survival of mice following CLP. Nineteen Rag-1 mice and 24 matched immunologically competent control mice underwent CLP as described in Materials and Methods. *, At the end of 6 days, there was a 5.3% survival in Rag-1 mice and a 45.8% survival in matched controls, and this difference was significant (p < 0.05). The survival study was conducted in one large study.
The resistance of HeJ mice to endotoxin is due to a mutation in a single gene on chromosome 4 and is related partially to a decreased production of circulating cytokines, e.g., TNF, in response to endotoxin challenge (13). Interestingly, in some models of sepsis, HeJ mice are more susceptible to death than are mice of the endotoxin-sensitive parent strain (24). The findings of extensive lymphocyte apoptosis in thymus and spleen of septic HeJ mice confirm the results of our previous study, indicating that endotoxin is not a sine qua non for apoptosis in sepsis (13).

The failure of Bax deficiency to prevent lymphocyte cell death in this sepsis model is important given the purported interrelationship between the antiapoptotic Bcl-2 and the proapoptotic Bax (11). Bax is known to heterodimerize with Bcl-2 and a preset ratio of Bcl-2/Bax is thought to be critical in determining survival in certain, but not all, types of cells following specific apoptotic stimuli (11). B-deficient mice should have demonstrated decreased sepsis-induced apoptosis in thymi and spleens, but no protection was seen. Recently, Knudson and Korsmeyer used a genetic approach with gain-and-loss-of-function models of Bcl-2 and Bax (23). They determined that two characteristic physical features of Bcl-2-deficient mice, i.e., apoptosis and thymic hypoplasia, are largely absent in mice that are deficient in Bax. In contrast, overexpression of Bcl-2 still decreased apoptosis in the absence of Bax. These investigators concluded that while an in vivo competition exists between Bax and Bcl-2, each family member is able to regulate apoptosis independently. The present results in a sepsis-induced apoptosis model further support the concept that Bcl-2 may function independently of Bax to regulate cell death in some circumstances (23).

An intriguing question that follows from the present study is the role of the different lymphocyte subsets in sepsis. Preliminary studies in transgenic mice in which Bcl-2 is overexpressed in B lymphocytes show complete protection against sepsis-induced apoptosis in B cells (our unpublished observations). In addition, these mice also have improved survival in sepsis compared with wild-type controls with sepsis (our unpublished observations). Although the improved survival in Bcl-2 transgenic mice in sepsis is encouraging, caution is necessary in extrapolating results in animal models to humans. Findings in many previous animal studies of sepsis were not duplicated in clinical trials (25). This lack of correlation in animal and human diseases undoubtedly is related to both the lack of an animal model of sepsis, which totally reproduces the human condition, and fundamental differences in the species’ response to sepsis.

The mechanism of the protective effect of Bcl-2 in apoptosis is under intense investigation. One way in which Bcl-2 might be protective in certain cases could be by reducing cytokine production (17). Linette and colleagues demonstrated that Bcl-2 blocked IL-2 production by activated T cells (17). Bcl-2 inhibited IL-2 production by preventing the translocation of NFAT from the cytosol to the nucleus (17). In addition to IL-2, NFAT is involved in production of numerous cytokines that play important roles in sepsis. Although there was a difference in TNF-α in septic HeOuJ vs Bcl-2 mice, it is unlikely that changes in TNF-α are responsible for the ability of Bcl-2 to prevent apoptotic death. First, there were no differences in thymic tissue cytokines in septic Bcl-2 vs septic HeJ mice (Table III) despite the absence of lymphocyte apoptosis in the former and the presence of apoptosis in the latter. Similarly, splenic TNF-α and IL-1β concentrations were not different in septic HeOuJ vs septic Bcl-2 mice despite the decreased apoptosis in the latter relative to the former. Additional evidence against the hypothesis that Bcl-2 protects by decreasing cytokines is provided from our previous study in which administration of a TNF receptor blocker failed to decrease apoptosis in the identical mouse CLP model (13). However, it is possible that Bcl-2 prevented the production of other unmeasured cytokines, e.g., IL-6 or that the effect of Bcl-2 on cytokine production occurred at an earlier time point in the course of disease.

The present findings are consistent with the current hypothesis that Bcl-2 prevents apoptosis by preserving MMP (18, 19). Thymocytes in which Bcl-2 was overexpressed had no loss of MMP with sepsis, whereas a loss in MMP did occur in a subset of cells from septic HeJ and HeOuJ mice (Fig. 4 and Table II). The loss of MMP is one of the common manifestations of the apoptotic process and occurs in diverse types of cells regardless of the stimulus (18, 19). This mitochondrial alteration is thought to mark the “point of no return” and may be a key mechanism (18). It is speculated that the loss of MMP is due to the opening of the mitochondrial permeability pores. Subcellular localization studies demonstrate that Bcl-2 has a patchy distribution at the contact sites between the outer and inner mitochondrial membranes (33). Most studies indicate that Bcl-2 and other antiapoptotic Bcl-2 family members must localize to mitochondria to prevent apoptosis (18). The mechanism by which Bcl-2 prevents loss of MMP is unknown.

In conclusion, transgenic mice overexpressing Bcl-2 in T cells had complete protection against T lymphocyte apoptosis and partial protection of B lymphocytes in sepsis. A subset of T cells in septic HeJ and septic HeOuJ mice had loss of MMP, and this was prevented by Bcl-2 overexpression. The mechanism of the protective effect of Bcl-2 is unlikely to be due to regulation of cytokine production, but may be related to preservation of MMP. The improved survival in Bcl-2 transgenic mice compared with HeJ and HeOuJ mice and the decreased survival in the Rag-1−/− mice considered together suggest that lymphocyte apoptosis is sepsis is detrimental to host survival.

References