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9. A 9-kb Bam HI–Xho I DNA fragment upstream of DQ52 was subcloned as a 2-kb Bam HI–Xho I fragment isolated from phage clone ChSpμ27 [P. Early *et al.*, *Cell* **19**, 981 (1980)] and a 7-kb Bam HI fragment from cosmid clone COS-J17 [C. Wood and S. Tonegawa, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3030 (1983)]. The *V_HT15* gene spanning from a Bam HI site 0.6 kb upstream of the ATG codon to a Bgl II site 60 bp downstream of *J_H1* was isolated from pLLg3R [J. W. Guise, P. L. Lim, D. Yuan, P. W. Tucker, *J. Immunol.* **140**, 3988 (1988)] and ligated to an 0.8-kb Nae I–Pvu II fragment upstream of the heavy chain intron enhancer element. The targeting vector was assembled with the *HSV-tk* gene from pIC19R/MC1-tk [S. L. Mansour, K. R. Thomas, M. R. Capecchi, *Nature* **336**, 348 (1988)] and the *neo*-cassette as modified by Jung *et al.* (FRT-*neo*^{r}-FRT cassette) (*24*). The final construct was linearized with Pvu I at a unique site in the pUC19 vector sequence and used for transfection.
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28. Isotype-specific ELISA was done as described [D. Kitamura *et al.*, *Cell* **69**, 823 (1992)]. Standard antibodies expressing V_HT15Id were BH8 for IgM [J. F. Kearney *et al.*, *Eur. J. Immunol.* **11**, 877 (1981)], S107γ3 for IgG3, and S107γ1 for IgG1. The isotype variants, S107γ3 and S107γ1, were purified from the supernatants of hybridoma cell lines carrying expression vectors for the *V_HT15* gene linked to mouse Cγ3 and Cγ1 genes for mouse, respectively (*32*). The parent cell line used for transfection carried an expression vector for the κ light chain of antibody TEPC15/S107 with V_κ22 and C_κ isolated from plasmid pLLg3R (*9*).
29. Eight-week-old mice homozygous for the *T15i* mutation were immunized with PC-KLH as described (*16*). On day 15 after immunization, spleen cells were prepared and IgM^-IgD^- $B220^+$ cells were purified as described (*23*). We amplified *V_HT15* sequences from complementary DNA with a Cγ primer, 5′-CAGAATTCGGATCCCAGGGGCCAGTGGATAGAC-3′ (*23*), and a *V_HT15* primer, 5′-GGAGGAAGCTTGGTACAGCCTGG-3′ (restriction sites are underlined) [A. Feeney, *J. Exp. Med.* **172**, 1377 (1990)]. Amplified DNAs were cloned and nucleotide sequences determined.
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33. We thank D. Kitamura, R. Kühn, J. Roes, S. Jung, W. Müller, E. Sonoda, and B. Schittek for discussion and advice; Genetic Institute for a LIF-producing cell line; P. W. Tucker, L. Hood, and C. Wood for DNA; J. F. Kearney, M. D. Scharff, and A. Coutinho for antibodies; P. W. Tucker for pLLg3R; C. Uthoff-Hachenberg, R. Zoebelein, and C. Göttlinger for technical assistance; and U. Ringeisen and W. Müller for work on graphs. The animal work was done in accord with official regulations. Supported by the Deutsche Forschungsgemeinschaft through SFB 243, the Federal Ministry for Research and Technology, the Land Nordrheinwestfalen, the Human Frontier Science Programme, and the Fazit Foundation.

26 July 1993; accepted 1 October 1993

Generation of a Mouse Strain That Produces Immunoglobulin κ Chains with Human Constant Regions

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Humanized antibodies are highly efficient as immunotherapeutic reagents and have many advantages over rodent antibodies. A mouse strain was generated by gene targeting to replace the mouse κ light chain constant (C) region gene with the human C_κ gene. Mice homozygous for the replacement mutation (C_κR) produced normal concentrations of serum antibodies, most of which carry chimeric κ light chains, and mounted normal immune responses to hapten-protein conjugates. This technology provides a feasible option for the generation of high-affinity humanized antibodies by means of the powerful somatic hypermutation-selection mechanism.

Various technologies have been developed to overcome problems related to the production of human monoclonal antibodies (mAbs) (*1*), one of which is the generation of chimeric antibodies in which the rodent C regions of heavy (H) and light (L) chains [with or without the framework of the variable (V) region] are replaced by the equivalent domains or sequences of human immunoglobulin (Ig) (*2*). Another strategy attempts to mimic the immune response in vitro through the expression of human Ig V region genes (isolated from human B cell populations or immunodeficient mice engrafted with human lymphoid tissue) in bacteriophages, followed by selection for rare, high-affinity antibodies through antigen binding (*3*). A major drawback to these and similar approaches is the cumbersome work required to generate each specific mAb of appropriate biological function.

An ideal solution to these problems would be the generation of a mouse strain that synthesizes human instead of mouse antibodies. This has been approached by

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the introduction of a mini-locus containing a few human V and C region gene segments in germline configuration into the mouse genome as a transgene (*4*). In such strains, antibodies that carry human H and L chains were indeed produced, but the concentrations produced were low and the repertoire of human V regions was severely limited. Thus, although this approach appears promising in principle, it is not yet at the stage to stand its final test.

We embarked on a less ambitious, but straightforward project whose aim is the generation of a mouse strain that produces high-affinity chimeric antibodies (mouse V and human C regions) to human or other antigens, given that the human C domains allow normal B cell development and function in the mouse. Humanized antibodies of this kind can be expected to be considerably less immunogenic in humans than mouse antibodies, although one would still have to deal with the problem of an immune response to the V region (anti–V region). The latter problem, however, is inherent in any antibody therapy because antibodies to the V region (that is, anti-idiotypic antibodies) can be produced even against autologous V regions, given the extreme diversity of the antibody repertoire (*5*). Here, we report the first step toward this goal, namely the generation of a mouse strain that carries the human instead of the mouse C_κ gene in its genome.

The targeting vector (Fig. 1B) was linearized and transfected into E14-1 embryonic stem (ES) cells (*6*) and cells were selected for resistance to G418. Of 500 ES cell clones screened by polymerase chain reaction (PCR) (Fig. 1), one clone was shown by Southern (DNA) blotting to carry the desired replacement mutation, designated $C_\kappa R$ (Fig. 1D). The human C_κ gene of this clone was amplified by PCR and sequenced to confirm the germline sequence (*7*). Cells of the mutant ES clone were injected into blastocysts of CB.20 mice, and the resulting chimeras transmitted the $C_\kappa R$ mutation into the germ line (Fig. 1D). Homozygous mutant mice were obtained on the (129/Ola × CB.20)F_2 hybrid background.

In the mutant mouse strain, large numbers of B cells that express humanized κ chains on the cell surface are indeed generated (Table 1 and Fig. 2). In the blood of 8-week-old mutants, the concentration of antibodies with humanized L chains was approximately 2 mg/ml, compared to a concentration of 3.5 mg/ml of κ chain–bearing antibodies in control mice of the same age (Fig. 3). The distribution of antibody isotypes was similar in mutant and wild-type animals (Fig. 3). Although in the mutants about 10% of the antibodies carry λ_1

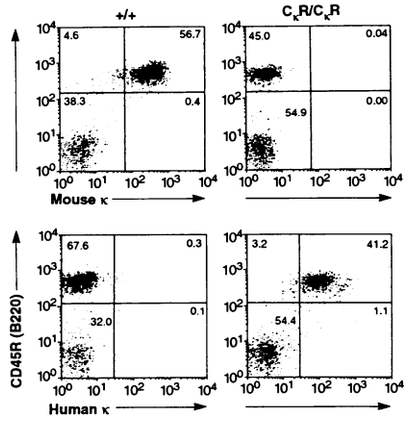

Fig. 2. Flow cytometric analysis of κ L chain expression on peripheral blood lymphocytes of homozygous $C_\kappa R$ mutant mice and control littermates at 8 weeks of age. Single-cell suspensions were prepared from peripheral blood by Ficoll gradient and stained with phycoerythrin (PE)-conjugated mAb to CD45R (B220) and fluorescein isothiocyanate (FITC)–conjugated mAb to mouse or human κ (PharMingen). Data were collected from cells in the lymphocyte gate (*11*). The percentages of cells in a given quadrant are indicated in each quadrant.

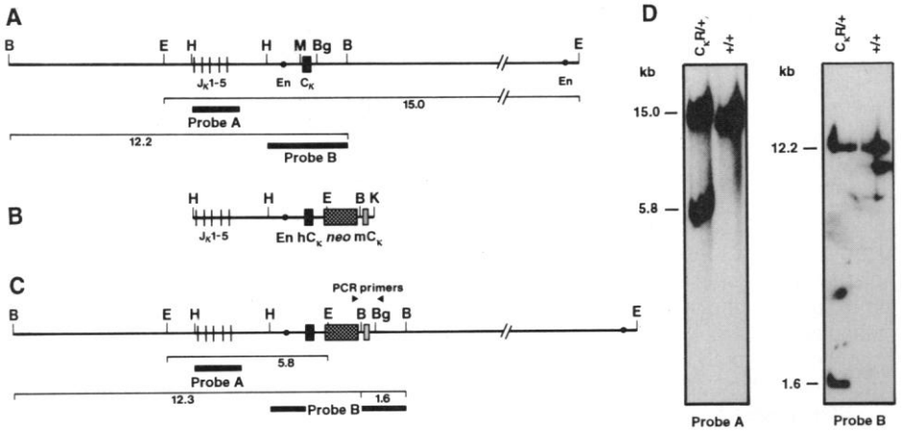

Fig. 1. Strategy for the replacement of the mouse C_κ gene by the human C_κ gene. (**A**) Genomic structure of the mouse C_κ wild-type locus. Exons and enhancers (En) are represented by black bars and closed circles, respectively. B, Bam HI; Bg, Bgl II; E, Eco RI; H, Hind III; K, Kpn I; and M, Mst II. (**B**) The targeting vector contains a 5.5-kb genomic DNA fragment that includes J_κ1–5, an intron enhancer, the mouse C_κ gene (mC$_\kappa$), and its 3′ untranslated region without a polyadenylate [poly(A)] site. A human C_κ gene (hC$_\kappa$) (*14*) and a neomycin resistance gene lacking a poly(A) site (*neo*) were used to replace a short stretch of genomic DNA that contains a splice signal and 12 base pairs of the mouse C_κ gene, rendering the remaining part of mouse C_κ gene (mC$_\kappa$′) nonfunctional. The *neo* gene served as a selection marker for the transfectants and also as a means for enrichment of the homologous recombinants. The Kpn I–linearized vector was introduced by electroporation into E14-1 ES cells. (**C**) Predicted structure of the targeted mouse C_κ locus. Arrowheads indicate the position of the PCR primers. (**D**) Southern blot analysis of the PCR-positive transfectant and the progeny of germline chimeras. Bam HI–digested genomic DNAs from one homologous recombinant candidate and parental ES cells were hybridized to probe B. The transfectant had a 12.2-kb wild-type band in addition to the 12.2-kb and 1.6-kb bands that were derived from the targeted allele (right panel). Tail DNAs from the offspring of germline chimeras were digested with Eco RI and hybridized to probe A. Wild-type animals showed only the 15-kb germline band, whereas the heterozygous mice showed an additional 5.8-kb band, indicative of the $C_\kappa R$ mutation.

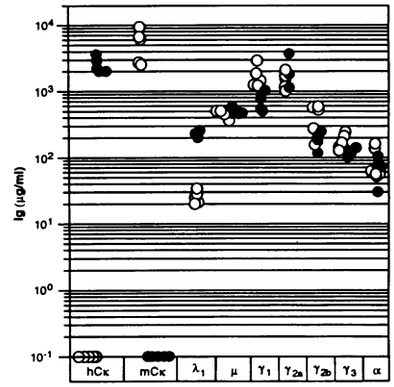

Fig. 3. Serum concentrations of Ig isotypes in homozygous $C_\kappa R$ and control mice. The serum concentrations of the various isotypes in 8-week-old $C_\kappa R$ (●) and normal animals (○) were determined by enzyme-linked immunosorbent assay (ELISA) as described (*6*) (see also the references for the mAbs used in the experiments). The concentrations of mouse and chimeric κ proteins were measured by an ELISA inhibition assay. Plates were coated with mouse κ chain–bearing mAb or human κ proteins from myelomas. Biotinylated mAb to the mouse κ chain or rabbit antibody to the human κ chain (Southern Biotechnology Associates, Birmingham, Alabama), at a concentration tested for maximal sensitivity, was then added into each well in the presence of diluted serum samples. Serum Ig concentrations were calculated with mAbs of the various isotypes as standards. Each symbol represents the value obtained from one mouse.

chains, a large fraction of the IgG antibodies would be associated with (humanized) κ chains because IgG represents the major isotype in the serum. The presence of serum IgG antibodies that carry the humanized L chains indicates that B cells that express the latter can be triggered by environmental antigens to contribute to antibody responses.

However, it is also clear that the production of κ chain–expressing B cells is less efficient in mutant than in wild-type mice, because homozygous mutants have only half as many B cells in the spleen as the controls, 17% of which express λ chains compared to 5% in the wild type (Table 1). A dramatic effect is seen in heterozygous mutant mice in which B cells that express humanized κ chains are similar in frequency to B cells that bear λ chains (6%), and 85% of the splenic population expresses κ chains from the wild-type locus. This preference may reflect a more efficient pairing of mouse κ domains as compared to the pairing of human κ domains with mouse H chains. However, it is also possible that the $C_\kappa R$ mutation negatively affects the frequency of gene rearrangements in cis, such that in the process of sequential V_κ-J_κ rearrangements during B cell development the wild-type locus usually wins in the competition, just as it usually wins in the competition with the λ locus (8).

To investigate whether the $C_\kappa R$ strain would have its natural antibody repertoire at its disposal and be capable of generating antigen-specific antibodies upon immunization with different antigens, we immunized the $C_\kappa R$ mice with phosphorylcholine (PC) coupled to keyhole limpet hemocyanin (KLH), 2-phenyl-5-oxazolone (phOX)–chicken serum albumin (CSA), and chicken γ-globulin (CG) (Fig. 4). Serum antibodies against these immunogens were measured at the time of immunization (day 0) and on days 7 and 14 after immunization (Fig. 4). It is clear from the results that the response of the mutants to any of these antigens is equivalent to that of wild-type mice, both in terms of the concentration of κ chain–bearing antibodies and the production of different isotypes tested.

A fundamental feature of the antibody response is affinity maturation through somatic hypermutation of the gene segments encoding the antigen binding site and subsequent selection of those B cells that express antibodies of increased affinity (9). Because the hypermutation mechanism could be impaired in the mutant mice because of the presence of the neomycin resistance gene (*neo*^{r}) in the C_κ locus, we examined the mutation frequency in rearranged V_κ genes expressed by B cells responding to immunization with phOX-CSA. Fourteen days after immunization, these cells are known to be contained in a B cell subset that can be brightly stained by phycoerythrin-labeled peanut agglutinin (PNA^{hi}) (10). They are also known to dominantly express a particular V_κ-J_κ rearrangement ($V_\kappa OX1$-$J_\kappa 5$) (11). Accordingly, splenic B cells PNA^{hi} were isolated by cell sorting 2 weeks after immunization, and $V_\kappa OX1$-$J_\kappa 5$ joints were amplified by PCR, cloned, and sequenced (Fig. 5). The data revealed that 66% of the sequences had mutations in the $V_\kappa OX1$-$J_\kappa 5$ region of the chimeric κ chain. Several sequences carried the key mutations in codons 34 and 37, known to increase the affinity of phOX-binding antibodies by approximately 10-fold (12). The average frequency of mutations (two mutations per sequence) in the chimeric L chains is similar to that observed by others (12, 13).

Taken together, the $C_\kappa R$ mouse line described here produces B lymphocytes that synthesize antibodies containing humanized κ chains in amounts that are comparable to those amounts of mouse κ chain–bearing

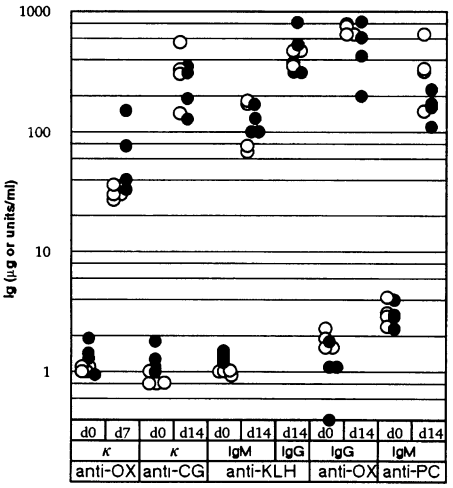

Fig. 4. Serum concentrations of antigen-binding antibodies in $C_\kappa R$ (●) and control (○) mice after immunization with various antigens. Eight-week-old mice received intraperitoneal injections of 100 μg of alum-precipitated 4-hydroxy-3-nitrophenylacetyl-CG, PC-KLH, or phOX-CSA, mixed with 10^9 *Bordetella pertussis* organisms. On days 7 and 14 of the response, sera were collected and the concentrations of antigen-specific antibodies determined by ELISA (15). Days are indicated in the first row beneath the data, and the various antibodies used are indicated in the bottom row.

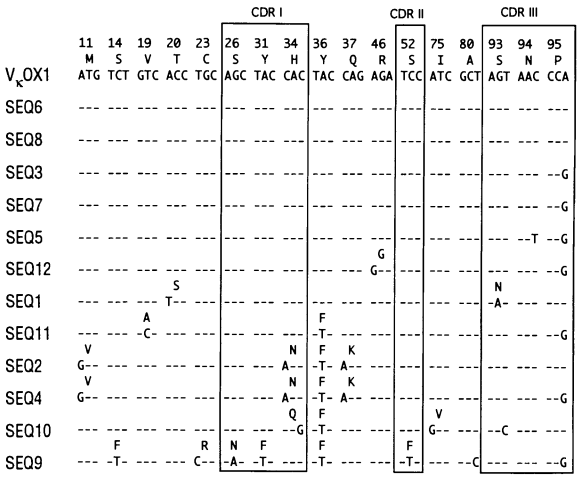

Fig. 5. Somatic hypermutation of $V_\kappa OX1$-$J_\kappa 5$ rearrangements on the $C_\kappa R$ chromosome. On day 14 of the phOX-CSA response, splenocytes from a $C_\kappa R$ mutant mouse were isolated and stained with FITC-conjugated RA3-2B6 and PE-conjugated PNA, followed by sorting of the PNA^{hi} B cell population. The purity of sorted cells was 91%. Complementary DNA sequences of $V_\kappa OX1$-$J_\kappa 5$ were obtained (16) and compared to those of the germline gene (SEQ*n*) (17). All triplets in which mutations were found are shown. Amino acid exchanges are given. The A/G variation in codon 95 is due to different rearrangements, the A being derived from the $V_\kappa OX1$ gene, the G from the $J_\kappa 5$ gene. Complementary-determining regions (CDRs) are indicated. Numbering is as described (18). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Table 1. Representation of different lymphocyte populations in the spleens of mutant and control mice. Single-cell suspensions were prepared from the spleens of individual mice at the age of 8 weeks. Cell numbers were determined in a hemocytometer. Cells were stained with FITC-conjugated antibodies to the mouse λ chain, to mouse κ, to human κ, or to CD3, respectively, in combination with PE-coupled antibodies to CD45 (B220) (15). The flow cytometric analysis was performed on a FACScan (Becton Dickinson). ND, not determined.

Mice	10^7 Nucleated cells (*n*)	10^7 T cells (*n*)	10^7 B cells (*n*)	λ-Producing B cells (%)	Mouse κ–producing B cells (%)	Human κ–producing B cells (%)
+/+	25.2 ± 2.5	7.5 ± 0.9	17.7 ± 1.5	4.9 ± 0.4	ND	<1
+/$C_\kappa R$	ND	ND	ND	5.6 ± 0.5	85.4 ± 1.9	6.1 ± 1.9
$C_\kappa R$/$C_\kappa R$	17.3 ± 1.9	7.7 ± 0.5	9.5 ± 2.4	17.2 ± 2.8	<1	89.1 ± 8.1

antibodies produced by wild-type littermates. When immunized with various T cell–dependent antigens, the mutant and wild-type mice produce equal concentrations of specific antibodies that bear the κ chain. Furthermore, antigen-specific B cells homozygous for the $C_\kappa R$ mutation undergo affinity maturation through somatic hypermutation to the same extent as documented for the wild-type C_κ gene. These mutant mice are now waiting to be crossed with animals bearing an analogous replacement mutation in the H chain locus.

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15. Plastic plates were coated with CG, KLH, phOX–bovine serum albumin (BSA), or PC-BSA (10 μg/ml), respectively. Diluted serum samples were added, and bound antibodies were detected by biotinylated antibodies for the determination of mouse κ and human κ chains, IgM, and for total IgG. The relative concentration of OX-specific IgG or PC-specific IgM was determined by comparison to standard monoclonal OX- or PC-specific antibodies of the same isotypes. The relative concentrations of CG- or OX-specific κ bearing–antibodies as well as KLH-binding IgM or IgG are shown in Fig. 4 as arbitrary units defined by taking the value of the serum from a pre-immune normal or mutant animal as 1 unit.
16. Total cellular RNA was prepared from 1.8×10^5 PNA^{hi} splenic B cells by as described [H. Gu, I. Förster, K. Rajewsky, *EMBO J.* **9**, 2133 (1990)]. Complementary DNA was synthesized with the Super Script Reverse Transcriptase Kit (BRL). $V_\kappa OX1\text{-}J_\kappa 5$ joints were then amplified with synthesis primers carrying the cloning sites of Bam HI and Hind III ($V_\kappa OX1\text{-}J_\kappa 5$ leader sequence specific primer: TGCGGATCCTCAGTCATAATATCCAG; $J_\kappa 5$ primer: CGGAATTCTTTCAGCTCCAGCTTGG). PCR was performed for 35 cycles. Each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 74°C. Amplified L chain fragments were cloned into the PTZ19R vector (Pharmacia).
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19. We thank R. Torres and R. Rickert for critical reading of the manuscript; D. Kitamura, R. Kuhn, S. Taki, and W. Muller for advice and discussion; H. G. Zachau for kindly providing the plasmid containing the human C_κ gene; O. Mäkelä, S. Taki, C. Uthoff-Hachenberg, and I. Wolowczuk for antigens and standard antibodies; C. Göttlinger for technical assistance; and U. Ringeisen for the preparation of the figures. The work with animals was conducted in accordance with official regulations. Supported by the Fazit Foundation, the Deutsche Forschungsgemeinschaft through SFB 243, and by the Human Frontier Science Program.

9 August 1993; accepted 1 October 1993

Bcl-2 Inhibition of Neural Death: Decreased Generation of Reactive Oxygen Species

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The proto-oncogene *bcl-2* inhibits apoptotic and necrotic neural cell death. Expression of Bcl-2 in the GT1-7 neural cell line prevented death as a result of glutathione depletion. Intracellular reactive oxygen species and lipid peroxides rose rapidly in control cells depleted of glutathione, whereas cells expressing Bcl-2 displayed a blunted increase and complete survival. Modulation of the increase in reactive oxygen species influenced the degree of cell death. Yeast mutants null for superoxide dismutase were partially rescued by expression of Bcl-2. Thus, Bcl-2 prevents cell death by decreasing the net cellular generation of reactive oxygen species.

Expression of Bcl-2 inhibits apoptosis induced by Ca^{2+} ionophores without altering intracellular free Ca^{2+} (*1*), suggesting that Bcl-2 inhibits the cellular death program at a point distal to the rise in intracellular free Ca^{2+}. Apoptosis induced by serum deprivation is not associated with a change in intracellular free Ca^{2+} before the onset of internucleosomal DNA degradation (*1*). The lack of correlation between intracellular free Ca^{2+} and cell death in these studies, along with the similarity of Bcl-2–expressing cells to control cells with respect to morphology, differentiation, growth rate, oxygen consumption, and adenosine triphosphate concentrations (*1*, *2*), prompted a search for a cellular parameter that would explain the resistance of Bcl-2–expressing cells to death.

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The hypothalamic neural cell line GT1-7 (*3*) displayed a remarkable sensitivity to toxicity from buthionine sulfoximine (BSO), which was abrogated by the expression of Bcl-2. BSO is a specific and essentially irreversible inhibitor of γ-glutamylcysteine synthetase (*4*) and thus decreases the intracellular concentration of reduced glutathione (GSH), a tripeptide involved in protecting the cell from oxidative injury. Although Bcl-2 inhibits apoptosis, the death of GT1-7 cells induced by GSH depletion is necrotic (*5*). This finding argues that Bcl-2 does not inhibit apoptosis per se; rather, Bcl-2 inhibits a cellular process that may result in apoptosis or necrosis.

Treatment of GT1-7 cells with BSO led to cell death within 40 hours, with an LD_{50} (median lethal dose) of ~100 μM (Fig. 1). Stable expression of Bcl-2 by means of a retroviral vector raised the LD_{50} about three orders of magnitude, to greater than 50 mM (Fig. 1). Measurement of intracellular GSH with monochlorobimane (MCB, Molecular Probes) (*6*) confirmed a decrement after exposure to BSO for both control and Bcl-2–expressing cells (Fig. 2A). However, cells expressing Bcl-2 had two to three times the basal concentrations of GSH and correspondingly higher concen-