Discrete and Complementary Mechanisms of Protection of β-Cells Against Cytokine-Induced and Oxidative Damage Achieved by bcl-2 Overexpression and a Cytokine Selection Strategy

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We have been investigating the potential utility of engineered cell lines as surrogates for primary islet cells in treatment of type 1 diabetes. To this end, two strategies that have emerged for procuring cell lines with resistance to immune-mediated damage are *1*) selection of cytokine-resistant cell lines by growth of INS-1 insulinoma cells in iteratively increasing concentrations of interleukin (IL)-1β + γ-interferon (IFN-γ), and *2*) stable overexpression of the anti-apoptotic gene bcl-2 in INS-1 cells. Herein, we show that bcl-2–overexpressing cells are resistant to the cytotoxic effects of reactive oxygen and nitrogen species (ROS/RNS), but are only modestly protected against high concentrations of IL-1β + INF-γ, whereas the converse is true in cytokine selected cells. We also found that the combination of bcl-2 expression and cytokine selection confers a broader spectrum of resistance than either procedure alone, such that the resultant cells are highly resistant to cytokines and ROS/RNS, with no impairment in glucose-stimulated insulin secretion. INS-1–derived cells with combined bcl-2 expression and cytokine selection are also more resistant to damage induced by coculture with mitogen-activated peripheral blood mononuclear cells. Surprisingly, application of the cytokine selection procedure to bcl-2–overexpressing cells does not result in impairment of nuclear factor-κB translocation, iNOS expression, and NO production, as clearly occurs upon application of the selection procedure to cells without bcl-2 overexpression. Further investigation of the diverse pathways involved in the development of cytokine and ROS/RNS resistance may define simplified and specific strategies for preservation of β-cell mass. *Diabetes* 52:1423–1432, 2003

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GSIS, glucose-stimulated insulin secretion; IFN-γ, γ-interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MnSOD, manganese superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; L-NMMA, N^G-monomethyl-L-arginine; PBMC, peripheral blood mononuclear cell; PMA, phorbol-12-myristate-13-acetate; PMA/I, PMA + 1 μg/ml ionomycin; PMA/I + LPS, 10 ng/ml PMA + 1 μg/ml ionomycin + 10 μg/ml LPS; RNS, reactive nitrogen species; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-*N*-acetylpenicillamine; STAT, signal transducer and activator of transcription; STZ, streptozotocin; TNF-α, tumor necrosis factor-α.

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Type 1 diabetes is caused by the autoimmune destruction of pancreatic islet β-cells. Destruction of β-cells appears to result from direct contact with infiltrating T-cells and macrophages as well as from exposure to inflammatory cytokines such as γ-interferon (IFN-γ), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and reactive oxygen/nitrogen species (ROS/RNS) that such cells produce. The success of a recent human islet transplantation trial, involving administration of a mixture of relatively mild, nonsteroidal immunosuppressive drugs, has focused fresh attention on cell-based insulin replacement strategies for treatment of type 1 diabetes (1). However, limited tissue supply and concerns about life-long administration of immunosuppressive agents may limit the applicability of this approach. We and others have therefore been investigating the potential utility of engineered cell lines as surrogates for primary islet cells in the treatment of type 1 diabetes (2–6). We envision that such cells would be delivered in the context of a macroencapsulation device that would prevent contact of cellular elements of the immune system and the engrafted tissue (3). However, immunoisolation barriers will not protect cells from the damage caused by soluble inflammatory mediators such as cytokines and ROS/RNS. Thus, a major goal of our group has been the development of cell lines that are resistant to both cytokine- and oxygen radical–induced damage.

We have recently described a selection strategy for the procurement of cytokine-resistant cell lines (7). Briefly, this involved culture of the rat insulinoma cell line INS-1 in the presence of iteratively increasing concentrations of a mixture of inflammatory cytokines, IL-1β + IFN-γ. The selected cell lines exhibit stable resistance to IL-1β–induced cytotoxicity and transient, reversible resistance to IFN-γ (dependent on the presence or absence of this cytokine in the tissue culture medium) (7).

The purpose of the current study was to compare the protective effects of our cytokine selection strategy with those provided by stable overexpression of the anti-apoptotic gene, bcl-2. We showed that bcl-2–expressing

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cells are highly resistant to ROS/RNS, but only modestly resistant to the cytotoxic effects of high concentrations of IL-1β + IFN-γ, whereas the opposite was found to be true in cells that have been subjected to cytokine selection. Application of the cytokine selection strategy to bcl-2−overexpressing cells conferred a broader spectrum of resistance than either procedure alone, with no loss of glucose-stimulated insulin secretion. Finally, we showed that cytokine resistance is achieved by completely different mechanisms in selected cells dependent on the presence or absence of bcl-2 expression. Exploitation of these new model systems could lead to identification of specific and simplified approaches for enhancing β-cell survival.

RESEARCH DESIGN AND METHODS

Cells and reagents. The rat insulinoma cell line INS-1 (8) was cultured in RPMI 1640 medium containing 10% FCS, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l Na-pyruvate, 50 μmol/l β-mercaptoethanol, 100 units/ml of penicillin, and 100 μg/ml streptomycin (Sigma Chemical, St, Louis, MO) at 37°C and 5% CO_2. Recombinant rat IL-1β was obtained from Pierce Endogen (Cambridge, MA). Recombinant rat IFN-γ was obtained from GIBCO BRL (Gaithersburg, MD). Streptozotocin (STZ), S-nitroso-*N*-acetylpenicillamine (SNAP), and H_2O_2 were obtained from Sigma. 3-morpholinosydnonimine (SIN-1) was obtained from Calbiochem (San Diego, CA). All reagents used for ROS/RNS experiments were freshly solubilized and applied to cells immediately. The INS-1−derived cell line 833/15 and the cytokine selection procedure used to derive it have been described previously (7). These cells were cultured continuously in the presence of 10 ng/ml IL-1β + 100 units/ml IFN-γ before the seeding of cells for experiments.

Stable expression of bcl-2 in INS-1 cells. To obtain the full-length cDNA encoding rat bcl-2 (National Center for Biotechnology Information accession number L14680) (9), oligonucleotides that flanked the start and stop codons of the corresponding mRNA were used in RT-PCRs, using total RNA extracted from INS-1 cells as template. The oligonucleotide sequences used for this amplification were 5′GTACCTGCAGCTTCTTTCCCCGGAAGG 3′ (208–234) and 5′GCAGGTCTGCTGACCTCACT 3′ (960–967), which span the predicted coding sequence from nucleotides 235–945. The resultant cDNA was subcloned into pGEM-T Easy Vector System (Promega, Madison WI), and sequenced. After digestion with *Eco*RI, bcl-2 inserts were subcloned into the vector pCMV8/IRES/neo (10). INS-1 cell lines were stably transfected with empty vector or the bcl-2 expression vector, using a previously described protocol (10).

Selection of cytokine-resistant cell lines. INS-1−derived cell lines were rendered resistant to cytokine-induced cytotoxicity by a selection strategy previously described in detail (7). Experiments were performed on cells that were recovered after the entire selection protocol (8–10 weeks). All of these newly selected cell lines were continually cultured in the presence of 10 ng/ml IL-1β and 100 units/ml rat IFN-γ before the described experiments.

Immunoblot analysis. For analysis of bcl-2 expression and Iκ-Bα protein, cells were seeded in 10-cm plates, washed once with PBS, and lysed with buffer containing 1% Triton X-100, 50 mmol/l HEPES, 150 mmol/l NaCl, 0.2 mg/ml phenylmethylsulfonyl fluoride, 100 mmol/l NaF, 2 mmol/l sodium vanadate, 10 μg/ml aprotinin, 5 μg/ml pepstatin, and 5 μg/ml leupeptin (pH 7.2). Lysates were kept on ice for 20 min and centrifuged at 14,000 rpm in a refrigerated microcentrifuge. Supernatant fractions were collected and protein concentrations were determined (11) using a kit from Bio-Rad (Hercules, CA). Samples were suspended in 2× sample buffer (final concentration 60 mmol/l Tris, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 60 mmol/l 2-mercaptoethanol, 100 mmol/l dithiothreitol; pH 6.8), boiled for 5 min, and electrophoresed using 4–15% precast Tris-glycine gels (Bio-Rad). Protein was transferred to polyvinylidine fluoride membranes (Millipore, Bedford, MA) and blocked with 5% dry milk in Tris-buffered saline with Tween (10 mmol/l Tris, 150 mmol/l NaCl, 0.1% Tween 20; pH 8.0). Blots were incubated with anti−bcl-2 (sc-783; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Iκ-B (sc-371; Santa Cruz Biotechnology) primary antibodies at a 1:200 dilution, according to the manufacturer's protocols. The protein bands were visualized with horseradish peroxidase−conjugated goat anti-rabbit IgG (Amersham, Piscataway, NJ) and enhanced chemiluminescence (Amersham). Immunoblots were scanned with a UMAX UC840 scanner.

MTT viability assay. The C,N diphenyl-N′-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay has been previously described (12–14). Cells were trypsinized, counted, and seeded at 50,000 cells/well in 96-well tissue culture dishes (Corning Glass, Corning, NY) or 500,000 cells/ml in 24-well tissue culture plates (Corning). After 24 h, the medium was discarded and replaced with media containing various additives as specified in the figure legends. After the various incubation periods, media were discarded and replaced by INS-1 cell growth medium containing 75 μg/ml MTT. The resulting formazan crystals were solubilized in an equal volume of 0.04 N HCl in isopropanol. The optical density was read at 575 nm and 650 nm using a SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA). The reduction in optical density caused by cytotoxic effects of cytokines, ROS/RNS, or activated peripheral blood mononuclear cells (PBMCs) were used as a measurement of cell viability, normalized to cells incubated in the absence of these agents, which were considered 100% viable.

Measurements of NO and insulin secretion. Nitrite formation was measured in cell supernatants as an indication of NO production, as previously described (12,15). Glucose-stimulated insulin secretion was measured as previously described (7). Cell lines were cultured in the absence of cytokines in their culture medium for 2 weeks before measurement of insulin secretion.

Use of PBMC for in vitro coculture. Heparinized blood was collected from Wistar rats (Charles River Breeding Laboratories, Wilmington, MA) and rat PBMCs were isolated over Ficoll-Paque Plus (Amersham), as previously described (12). INS-1−derived cells were seeded at 5×10^5 cells/ml in 24-well plates and cultured for 24 h before the initiation of coculture experiments. Costar Transwell inserts (VRW Scientific, Westchester, PA) were placed in the wells and 5×10^5 PBMC/ml were seeded into the top Transwell insert in 100 μl of medium. An equal volume of medium containing mitogens at a 2× concentration was prepared fresh and added into each well to give a final concentration of 10 ng/ml phorbol myristic acid (PMA) + 1 μg/ml ionomycin (PMA/I) or 10 ng/ml PMA + 1 μg/ml ionomycin + 10 μg/ml lipopolysaccharide (PMA/I + LPS) (all mitogens from Sigma). Wells containing INS-1 cells without the PBMC-seeded transwell inserts were also treated with the mitogens as controls. Insulinoma cells and PBMCs were cocultured for 48 h before the MTT viability assay and the measurement of nitrite release into the medium.

NF-κB mobility shift assay. Nuclear extracts were prepared by procedures adapted from those of Dignam et al. (16). Binding reactions (20 μl total) were performed by incubating 4 μg of nuclear extract protein with 0.03 pmol (50,000 cpm) of a ^{32}P-labeled, double-stranded oligonucleotide containing the NF-κB binding site (5′-GTTCGACAGAGGGGACTTTCCGAGAGGCAAC-3) in reaction buffer containing 10 mmol/l Tris-HCl (pH 7.5), 50 mmol/l NaCl, 1 mmol/l EDTA, 5% glycerol, 1 mg/ml BSA, and 100 μg/ml poly (dI-dC) as nonspecific competitor DNA. Nuclear protein extracts were incubated with the reaction buffer on ice for 15 min before incubation with the ^{32}P-labeled probe for 20 min at room temperature. DNA-protein complexes were then separated on native 4% polyacrylamide gels in Tris-glycine-EDTA buffer (0.05 mol/l Tris-HCl, 0.4 mol/l glycine, 2 mmol/l EDTA), dried, and exposed to Kodak film.

RESULTS

Stable expression of bcl-2 in INS-1 cells. INS-1 cells were stably transfected with a vector containing the rat bcl-2 cDNA under control of the CMV promoter (pCMV8/bcl-2/IRES/neo) or with the same vector lacking an insert. Multiple neomycin-resistant clones (828/13, 828/29, 828/33, 828/36, 828/37, and 828/44) were isolated and evaluated for bcl-2 protein expression by immunoblot analysis. Figure 1 shows that the levels of bcl-2 protein in the six INS-1−derived cell lines transfected with pCMV8/bcl-2/IRES/neo (*lanes 2–7*) were much higher than in the INS-1 cells transfected with the empty vector (831/1) (Fig. 1, *lane 1*), which contained a very low level of bcl-2 protein.

Bcl-2 expression is more effective than cytokine selection in conferring protection against oxidative stress. The effects of a variety of agents of oxidative stress were compared in bcl-2−overexpressing clones, empty vector−transfected control cells (831/1), and a line previously derived by our cytokine selection protocol (833/15). As shown in Fig. 2, treatment of 831/1 cells with 150 μmol/l H_2O_2, 200 μmol/l of the NO donor SNAP, 1.5 mmol/l of the peroxynitrite generator SIN-1, or 1.0 mmol/l STZ caused a decline in viability to 7 ± 2, 17 ± 3, 20 ± 2, and 8 ± 1%, respectively, relative to 100% viability in control cells

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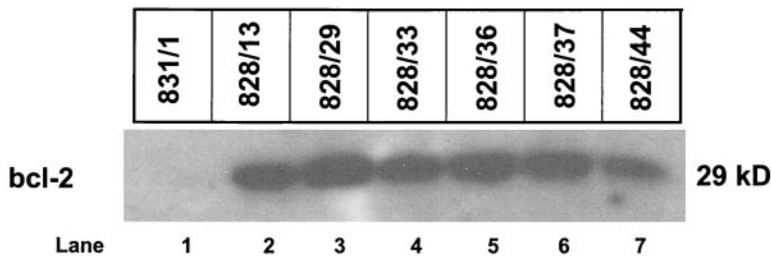

FIG. 1. Bcl-2 protein levels in INS-1–derived cell lines. INS-1 cells were stably transfected with a vector lacking a cDNA insert to yield cell line 831/1 or a vector containing the cDNA encoding rat bcl-2 to yield cell lines 828/13, 828/29, 828/33, 828/36, 828/37, and 828/44. Levels of bcl-2 protein were measured by immunoblot analysis with a rabbit anti–bcl-2 antibody, as described in RESEARCH DESIGN AND METHODS.

incubated in the absence of these agents. After application of the cytokine selection procedure to 831/1 cells, or in studies with a previously selected cell line (833/15), modestly improved viabilities were observed in response to the same agents ($P < 0.05$ vs. unselected 831/1 for all treatments, except H_2O_2). In contrast, overexpression of bcl-2 in the absence of selection raised viabilities to 71–88, 70–76, 66–76, and 85–95% in three independent cell lines exposed to H_2O_2, SNAP, SIN-1, or STZ, respectively. Thus, bcl-2 expression was clearly superior to the cytokine selection procedure for conferring protection against a diverse set of agents that deliver oxidative stress.

Bcl-2 expression is less effective than cytokine selection in conferring protection against cytokine-induced cytotoxicity. We next compared bcl-2 expression and our cytokine selection procedure with regard to protection conferred against cytokine-induced cytotoxicity. As shown in Fig. 3, expected cytokine killing profiles (7) were achieved in unselected (831/1) versus cytokine-selected (833/15) cells; that is, low viabilities of 38 ± 3, 57 ± 4, and 19 ± 3% in 831/1 cells versus high viabilities of 92 ± 7, 72 ± 11, and 82 ± 12% in 833/15 cells, when each was exposed for 48 h to 10 ng/ml IL-1, 100 units/ml IFN-γ, or IL-1β + IFN-γ, respectively. Bcl-2 expression conferred an intermediate level of resistance to cytokine-induced damage relative to 831/1 or 833/15 cells (42–44, 70–75, and 41–44% in two independent lines in response to the same set of cytokines); differences compared to 833/15 were significant ($P < 0.04$) for groups treated with IL-1β or IL-1β + IFN-γ, but not those treated with IFN-γ alone. Thus, the cytokine selection procedure was clearly superior to the strategy of bcl-2 overexpression for conferring protection against inflammatory cytokines.

Effects of combining bcl-2 overexpression and cytokine selection. We next determined if the combination of bcl-2 overexpression and cytokine selection could provide protection against both classes of effectors. To test this idea, we applied the cytokine selection procedure to the bcl-2–overexpressing lines 828/33, 828/37 and 828/44, as well as the empty vector control line 831/1. After the selection procedure, we reexamined the levels of bcl-2 expression. As shown in Fig. 4, two bcl-2–negative lines, 833/15 and 831/1, continued to have very low bcl-2 levels after the cytokine selection procedure, just as they did before selection (compare to Fig. 1). Furthermore, analysis of the three stably transfected lines revealed no significant changes in bcl-2 expression before and after the selection procedure (Fig. 4). Thus, the selection procedure did not induce bcl-2 expression in untransfected cells nor did it alter bcl-2 protein levels in previously transfected cells. We would also like to note that although no exact measurements of doubling times were made, no visible differences in cell growth were detectable when comparing the four experimental groups (unselected, bcl-2 untransfected; bcl-2 transfected alone; selected alone; or bcl-2 transfected + cytokine selected).

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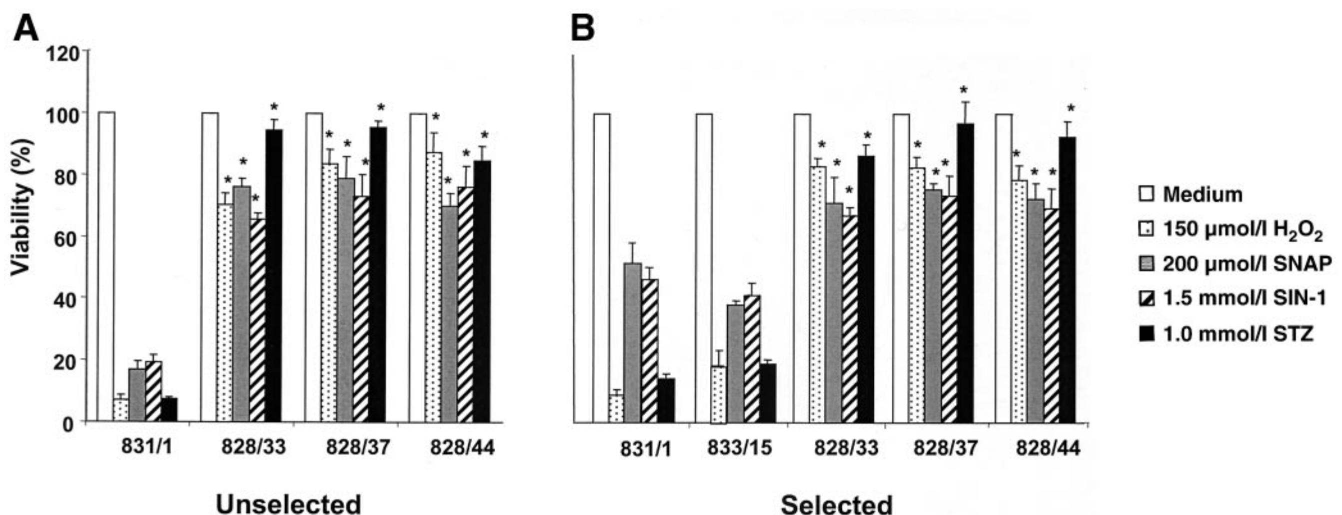

FIG. 2. Viability of INS-1–derived cells treated with agents of oxidative stress. INS-1 cells transfected with an empty vector (831/1) or a vector containing the rat bcl-2 cDNA (828/33, 828/37, 828/44) were studied before cytokine selection (*A*) or after the cytokine selection protocol (*B*), administered as described in RESEARCH DESIGN AND METHODS. The 833/15 line is a previously described cytokine-selected line (7). Selected cells were continually cultured in the presence of cytokines (10 ng/ml IL-1β + 100 units/ml IFN-γ). Unselected and selected cells were seeded in 24-well plates in normal media. After 24 h, cells were incubated with media containing the ROS/RNS species indicated in the figure legend and assayed for viability 18 h later using the MTT assay. Data represent means ± SD for three independent experiments, each performed in triplicate. * $P < 0.05$, for significantly increased viability of bcl-2–transfected cell lines relative to the unselected, empty vector–transfected cell line (831/1) or the selected cell line that lacked bcl-2 overexpression (833/15).

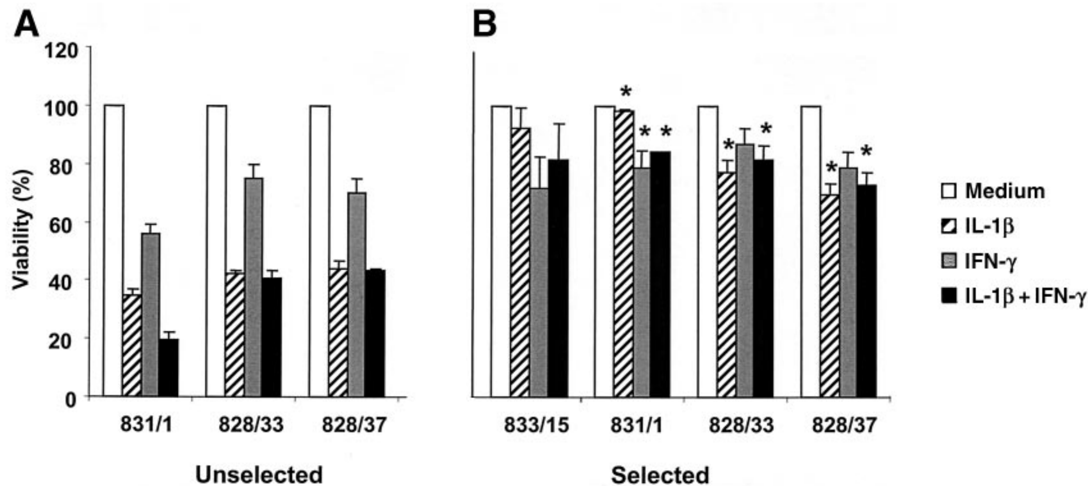

FIG. 3. Viability of INS-1–derived cells treated with cytokines. INS-1 cells transfected with an empty vector (831/1) or a vector containing the rat bcl-2 cDNA (828/33, 828/37) were studied either before cytokine selection (*A*) or after the cytokine selection protocol (*B*), administered as described in RESEARCH DESIGN AND METHODS. The 833/15 line is a previously described cytokine-selected line (7). Selected cells were continually cultured in the presence of cytokines (10 ng/ml IL-1β + 100 units/ml IFN-γ). Unselected and selected cells were seeded in 24-well plates in normal media. After 24 h, cells were incubated with media containing cytokines for 48 h and assayed for viability using the MTT assay, as described. The cytokine concentrations used were 10 ng/ml IL-1β, 100 units/ml IFN-γ, and 10 ng/ml IL-1β + 100 units/ml IFN-γ. Data represent means ± SD for three independent experiments, each performed in triplicate. *$P < 0.01$, for increased viability of the selected cell line relative to the corresponding unselected cell line for each treatment condition.

We next examined the effect of the cytokine selection procedure on three independent cell lines with stable overexpression of bcl-2 (Fig. 2*B*). These lines were 79–83, 71–76, 67–74, and 87–97% viable when treated with 150 μmol/l H_2O_2, 200 μmol/l of the NO donor SNAP, 1.5 mmol/l of the peroxynitrite generator SIN-1, and 1.0 mmol/l STZ, respectively. These levels of viability were not statistically different from those found in the same set of bcl-2–expressing cell lines before cytokine selection, but were clearly improved relative to selected cells (lines 833/15 and 831/1) lacking bcl-2 expression (Fig. 2). Thus, the resistance to oxidative stress conferred by bcl-2 expression was stably retained in cells taken through the selection procedure. However, these selected cells did not gain additional resistance to oxidative stress above the levels obtained by bcl-2 expression alone.

The effect of the combined procedures (bcl-2 expression + cytokine selection) was also evaluated at the level of resistance to cytokine-induced damage (Fig. 3*B*). Viabilities as assessed by the MTT assay were 82 ± 12, 84 ± 1, 82 ± 5, and 73 ± 5% in cell lines 833/15 (previously selected, nonbcl-2 expressing), 833/1 (selected, empty vector-transfected), and 828/33 and 828/37 (selected, bcl-2 expressing), respectively. These values were not statistically different from each other, but were all clearly improved relative to unselected controls. The selection procedure also clearly conferred enhanced resistance to incubation with the individual cytokines IL-1β or IFN-γ relative to unselected cells. However, incubation of cells with IL-1β alone resulted in slightly, but significantly reduced viability in selected, bcl-2–expressing clones (77 ± 3 and 70 ± 5% in 828/33 and 828/37, respectively) than in selected, non–bcl-2–expressing cells (92 ± 7 and 98 ± 1% in 833/15 and 831/1 cells, respectively). Overall, the selection procedure resulted in cell lines with markedly improved resistance to cytokine-induced cytotoxicity, regardless of the presence or absence of stable overexpression of bcl-2.

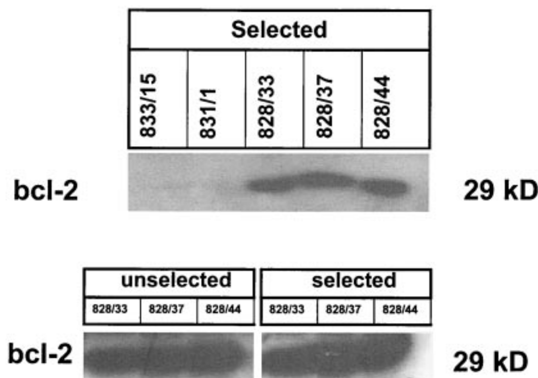

FIG. 4. Bcl-2 protein levels were not altered by the cytokine selection protocol. INS-1 cells transfected with an empty vector (831/1) or a vector containing the rat bcl-2 cDNA (828/33, 828/37, 828/44) were subjected to the cytokine selection protocol as described in RESEARCH DESIGN AND METHODS. The 833/15 line is a previously described cytokine-selected line (7). Selected cells were continually cultured in the presence of cytokines (10 ng/ml IL-1β + 100 units/ml IFN-γ). Levels of bcl-2 protein were measured by immunoblot analysis with a rabbit anti–bcl-2 antibody, as described in RESEARCH DESIGN AND METHODS.

Selected, bcl-2-transfected cells were resistant to the cytotoxic effects of coculture with activated PBMC. The inflammatory response to islet cell transplantation, or as occurs during immunological attack on β-cells in type 1 diabetes, is expected to involve combined attack by cytokines, ROS, and RNS. To model this scenario, we cocultured our various cell lines in the presence of mitogen-activated PBMCs. Such an approach prevents cell-to-cell contact, but allows free transfer of all varieties of soluble toxins produced by PBMC. A mixture of PMA/I and LPS has been shown to stimulate T-cells to produce IL-2, IL-4, IFN-γ, and macrophages to secrete IL-1α/β, IL-6, TNF-α (17,18). ROS, RNS, and peroxynitrite should also be released by macrophages in this system (19–21).

As shown in Fig. 5, coculture of unselected 831/1 cells with PBMC activated with PMA/I for 48 h resulted in 21 ± 2% viability. Bcl-2 transfected cells were modestly pro-

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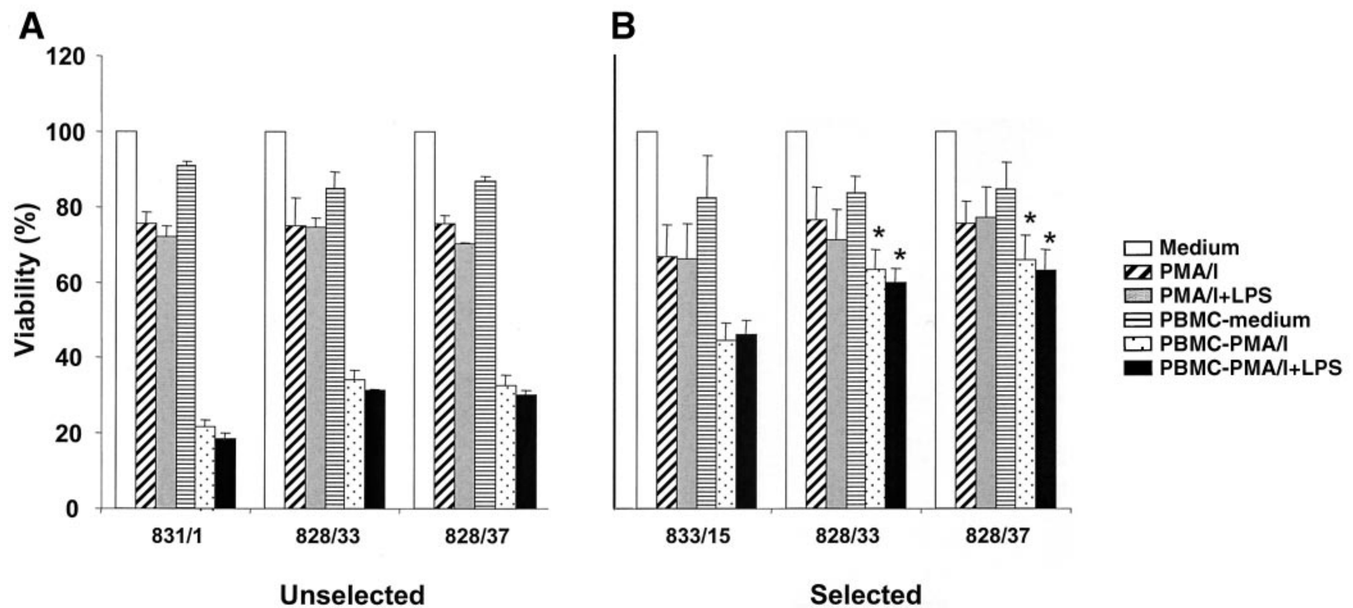

FIG. 5. Viability of INS-1−derived cells cocultured with activated PBMC. INS-1 cells transfected with an empty vector (831/1) or a vector containing the rat bcl-2 cDNA (828/33, 828/37) were studied either before cytokine selection (*A*) or after the cytokine selection protocol (*B*), administered as described in RESEARCH DESIGN AND METHODS. Line 833/15 is a previously described cytokine-selected line (7). Selected cells were continually cultured in the presence of cytokines (10 ng/ml IL-1β + 100 units/ml IFN-γ). Unselected and selected cells were seeded in 24-well plates in normal media. After 24 h, cells were cocultured with rat PBMCs that were either mitogen-activated (PBMC-PMA/I, PBMC-PMA/I + LPS) or left unactivated (PBMC-medium), as described in RESEARCH DESIGN AND METHODS. The concentrations of mitogens used were 10 ng/ml PMA + 1 μg/ml ionomycin (PMA/I), and 10 ng/ml PMA + 1 μg/ml ionomycin + 10 μg/ml LPS (PMA/I + LPS). After 48 h of coculture, INS-1−derived cells were assayed for viability using the MTT assay. The percent viability was calculated relative to cells cultured in the absence of PBMC (medium). Other controls included INS-1−derived cells treated with mitogens only (PMA/I, PMA/I + LPS). Data are means ± SD for three independent experiments, each performed in duplicate. *$P < 0.05$ for increased viability of the selected, bcl-2−transfected cell lines relative to the selected cell line without bcl-2 overexpression (833/15).

tected against the soluble mediators released in the coculture. 828/33 cells were 34 ± 3% viable ($P = 0.039$ vs. 831/1) and 828/37 cells were 33 ± 3% viable ($P = 0.05$ vs. 831/1). The selection procedure alone (833/15) also conferred partial resistance with a viability of 44 ± 5% ($P = 0.001$ vs. 831/1). The greatest level of protection was achieved by the combination of bcl-2 transfection and cytokine selection. Selected 828/33 cells were 63 ± 5% viable ($P < 0.001$ vs. 833/15) and selected 828/37 cells were 66 ± 7% viable ($P < 0.001$ vs. 833/15). Similar results were obtained in cells cocultured with PBMC activated with a different combination of mitogens (PMA/I and LPS) (Fig. 5). Thus, the combination of the two procedures under evaluation (bcl-2 expression + cytokine selection) provided a clear survival advantage relative to either procedure alone for cells tested in an in vitro model of inflammatory attack.

iNOS expression and NO production were not blocked in cytokine-selected, bcl-2−expressing cells. IL-1β is known to stimulate NO production in islet β-cells via induction of iNOS, and the production of NO has been implicated in IL-1β−mediated β-cell destruction (22–25). We therefore examined iNOS expression and NO production in the models currently under study. In agreement with our previous work (7), IL-1β−mediated stimulation of nitrite production was completely blocked in two independent cell lines subjected to the cytokine selection procedure: 833/15 from our previous study (7) and 831/1 selected from the current work (Fig. 6). To our surprise, however, bcl-2−overexpressing cells retained their ability to produce nitrite, even after the cytokine selection process. Thus, selected 828/33 cells produced 19.5 ± 3.7 and 31.1 ± 7.0 μmol/l nitrite after 48 h of IL-1β or IL-1β + IFN-γ treatment, respectively. Similar findings were obtained with selected 828/37 cells (Fig. 6). These nitrite levels were not significantly different from those measured in these same bcl-2−expressing lines before selection (Fig. 6). These results for NO production were well correlated with levels of iNOS protein in the various cell lines as measured by immunoblot analysis (data not shown).

NO production was also measured in the supernatants after 48 h of coculture with activated PBMC (Fig. 7). The source of NO in these cocultures was most likely the INS-1−derived cell lines, as PBMC cultured without INS-1 cells produced negligible amounts of nitrite. Unselected cells (831/1) produced 10.3 ± 0.2 μmol/l nitrite when cocultured with PMA/I-stimulated PBMC and 20.3 ± 0.1 μmol/l nitrite in the presence of PBMC stimulated with PMA/I + LPS. Bcl-2 transfected cells cultured with PMA/I stimulated PBMC produced similar levels of nitrite as the empty vector−transfected cells (Fig. 7). Similar to our observations with cytokine treatment, selection alone (833/15) caused a large decrease in NO production in response to coculture with PBMC stimulated with PMA/I (2.8 ± 0.2 μmol/l nitrite) or PMA/I + LPS (5.5 ± 0.3 μmol/l nitrite). Application of the selection procedure to bcl-2−transfected cells failed to induce this block in PBMC-induced NO production (Fig. 7). Taken together, these data indicate that resistance to cytokine-induced damage conferred by our selection protocol occurred by distinct mechanisms dependent on the presence or absence of bcl-2 overexpression.

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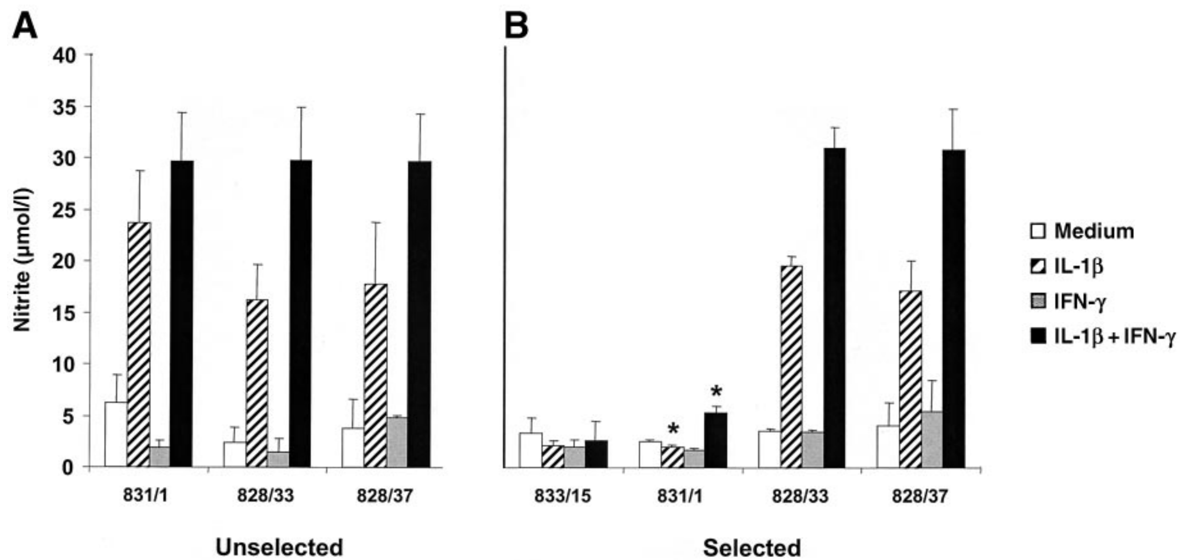

FIG. 6. Cytokine-induced nitric oxide production from INS-1 derived cells before and after cytokine selection. INS-1 cells transfected with an empty vector (831/1) or a vector containing the rat bcl-2 cDNA (828/33, 828/37) were studied either before cytokine selection (*A*) or after the cytokine selection protocol (*B*), administered as described in RESEARCH DESIGN AND METHODS. The 833/15 line is a previously described cytokine selected line (7). Selected cells were continually cultured in the presence of cytokines (10 ng/ml IL-1β + 100 units/ml IFN-γ). The incubation conditions and cytokine concentrations used were identical to those described in Fig. 3. After this treatment, media were collected and assayed for nitrite as a measure of NO production. Data represent means ± SD for three independent experiments, each performed in triplicate. Nitrite levels were not significantly different in bcl-2–transfected cells before (828/33, 828/37 unselected) versus after (828/33, 828/37 selected) the cytokine selection procedure. **P < 0.02 for significantly decreased nitrite production from the empty vector transfected cells after (831/1 selected) compared to before (831/1 unselected) cytokine selection.

Bcl-2 transfected cells selected for cytokine resistance retain IL-1β–mediated NF-κB translocation. IL-1β–mediated stimulation of iNOS expression and NO production is mediated by translocation of the transcription factor NF-κB to the nucleus. We have previously shown an impairment in IL-1β–induced NF-κB translocation in cells subjected to our cytokine selection procedure (7). To evaluate the potential role of these events in the attainment of cytokine resistance in the models currently under study, we performed gel shift assays to measure

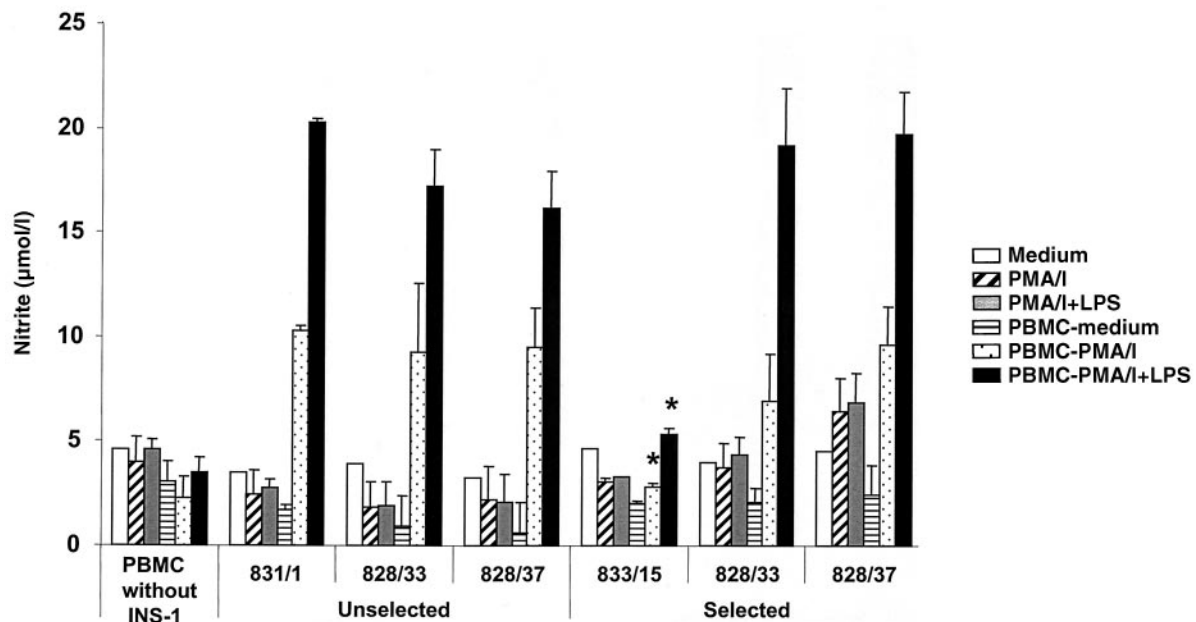

FIG. 7. Nitric oxide production from INS-1–derived cells cocultured with activated PBMC. INS-1 cells transfected with an empty vector (831/1) or a vector containing the rat bcl-2 cDNA (828/33, 828/37) were left unselected or subjected to the cytokine selection protocol, as described in RESEARCH DESIGN AND METHODS. Line 833/15 is a previously described cytokine-selected line (7). Selected cells were continually cultured in the presence of cytokines (10 ng/ml IL-1β + 100 units/ml IFN-γ). Unselected and selected INS-1–derived cell lines were treated as described in the legend for Fig. 5. After this treatment, media were collected and assayed for nitrite as a measure of NO production. As a control, PBMCs were also cultured without INS-1 cells (PBMC without INS-1). Data represent means ± SD for three independent experiments, each performed in triplicate. Nitrite levels were not significantly different in bcl-2–transfected cells before (828/33, 828/37 unselected) versus after (828/33, 828/37 selected) the cytokine selection procedure. P < 0.005 for significantly decreased nitrite production from the selected cells (833/15 selected) compared to the unselected cells (831/1 unselected) for each treatment condition.

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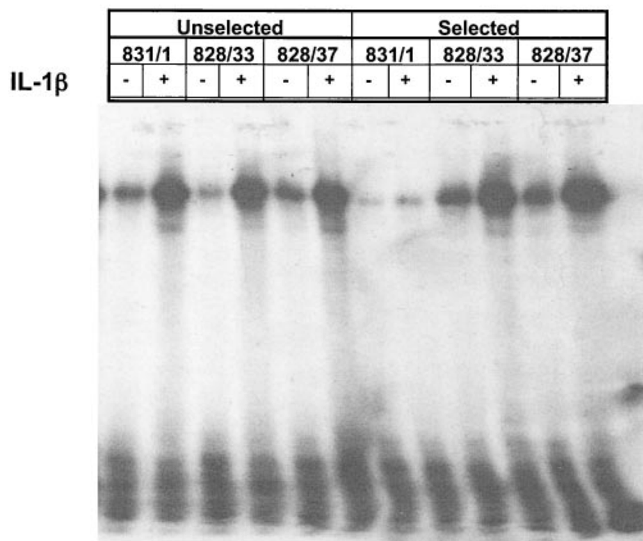

FIG. 8. Cytokine selection failed to suppress nuclear translocation of NF-κB in bcl-2−expressing INS-1 cells. INS-1 cells transfected with an empty vector (831/1) or a vector containing the rat bcl-2 cDNA (828/33, 828/37) were left unselected or subjected to the cytokine selection protocol, as described in RESEARCH DESIGN AND METHODS. Selected cells were continually cultured in the presence of cytokines (10 ng/ml IL-1β + 100 units/ml IFN-γ), whereas unselected cells were cultured in medium lacking cytokines. Both the selected and unselected groups of cells were grown in the absence of cytokines for a period of 24 h before the start of the experiments shown in this figure. Plates of cells were then treated with normal medium (−) or medium containing 10 ng/ml of IL-1 (+) for 1 h. Nuclei were isolated for electrophoretic mobility shift assay analysis as described in RESEARCH DESIGN AND METHODS. Data are representative of two independent experiments.

NF-κB translocation. As shown in Fig. 8, both bcl-2−expressing and nonexpressing cell lines (831/1, 828/33, and 828/37) exhibited classic and potent IL-1β−induced NF-κB translocation before application of the selection procedure. In agreement with our previous work (7), the empty vector−transfected cell line had markedly impaired NF-κB translocation after cytokine selection (selected 831/1) (Fig. 8). In sharp contrast, bcl-2−expressing cells subjected to the selection process (selected 828/33 and 828/37) retained complete and robust IL-1β−induced NF-κB translocation. These cells also appeared to have elevated NF-κB translocation under basal (non−IL-1β−stimulated)

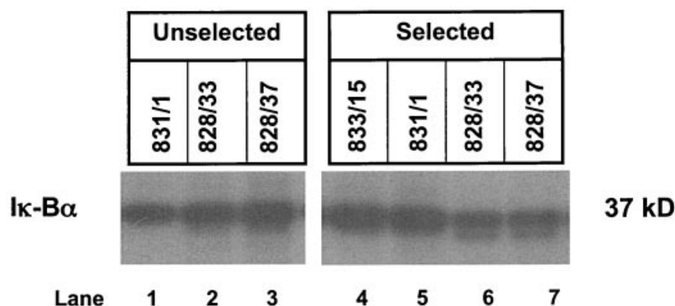

FIG. 9. Immunoblot of Iκ-Bα protein levels in unselected and selected cells. INS-1 cells transfected with an empty vector (831/1) or a vector containing the rat bcl-2 cDNA (828/33, 828/37) were left unselected or subjected to the cytokine selection protocol as described in RESEARCH DESIGN AND METHODS. Line 833/15 is a previously described cytokine-selected line (7). Selected cells were continually cultured in the presence of cytokines (10 ng/ml IL-1β + 100 units/ml IFN-γ). Unselected cells were cultured in medium lacking cytokines. Levels of Iκ-Bα protein were measured by immunoblot analysis with a rabbit anti−Iκ-Bα antibody, as described in RESEARCH DESIGN AND METHODS.

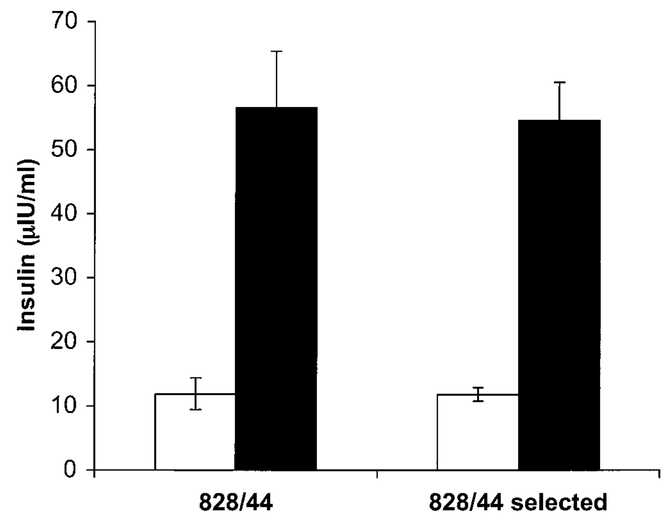

FIG. 10. Glucose-stimulated insulin-secretion was not impaired by bcl-2 expression or the cytokine selection procedure. The bcl-2−overexpressing cell line 828/44 was studied before (828/44) or after (828/44 selected) application of the cytokine selection procedure. Insulin secretion was measured after 2 h of static incubation in 3 mmol/l (□) or 15 mmol/l (■) glucose. Data represent means ± SD for three independent experiments, each of which was performed in quadruplicate.

conditions compared to unselected bcl-2−transfected cells. These findings are consistent with those in Fig. 7 and provide further support for distinct mechanisms of selection-induced cytokine resistance dependent on the background of bcl-2 expression.

Bcl-2 transfected cells selected for cytokine resistance had reduced Iκ-Bα protein levels. Because the selected, bcl-2−transfected cells had persistent NF-κB translocation, we investigated the concentrations of the regulatory protein, Iκ-Bα, which inhibits NF-κB function by retaining it in the cytosolic compartment. Previous studies reported reduced Iκ-Bα in response to adenovirus-mediated bcl-2 expression in neonatal myocytes, apparently mediated by an increased rate of degradation of the inhibitor (26). Nevertheless, the effect of bcl-2 on Iκ-Bα levels could not be predicted a priori in our system, as Iκ-Bα is itself a target gene of NF-κB (27). Figure 9 (*lanes 6–7*) shows that Iκ-Bα protein levels are reduced in bcl-2 transfected cells subjected to the cytokine selection procedure. Non−bcl-2−transfected cells had similar high levels of Iκ-Bα protein regardless of exposure to the selection procedure (*lane 1* vs. *lanes 4–5*), and bcl-2 overexpression alone did not result in decreased Iκ-Bα levels (*lanes 2–3*). Thus, Iκ-Bα levels were decreased only in cells that have experienced the dual manipulation of bcl-2 overexpression and cytokine selection.

Glucose-stimulated insulin secretion was not impaired by bcl-2 expression or the cytokine selection procedure. After stable overexpression of bcl-2, the six clones shown in Fig. 1 were screened for glucose-stimulated insulin secretion (GSIS) at 3 vs. 15 mmol/l glucose. The results revealed a range of phenotypes, spanning from no detectable response to a 4.7-fold response. This compares to the twofold glucose response of the starting polyclonal population of INS-1 cells used in this study (28). The heterogeneity in glucose responsiveness among INS-1−derived subclones is entirely consistent with our previ-

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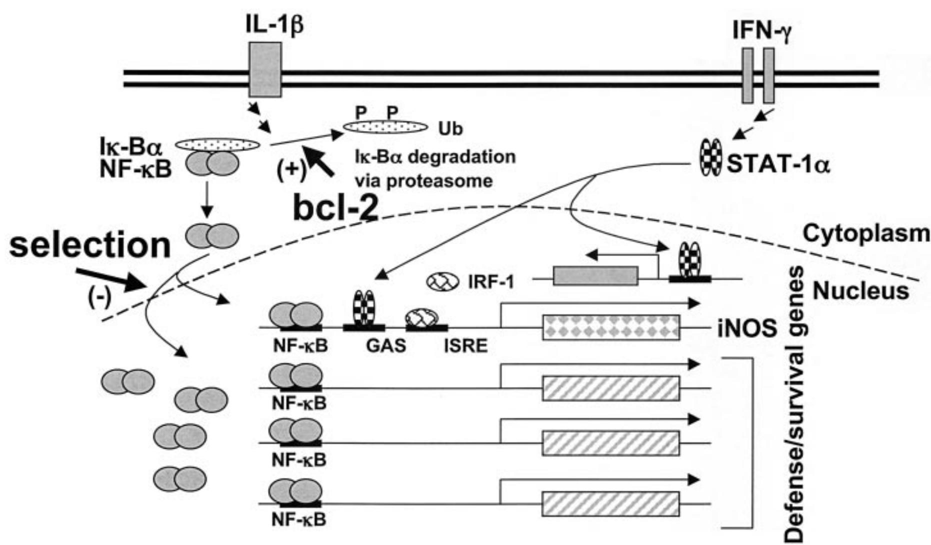

FIG. 11. Model for explaining the differential pathways for acquiring cytokine resistance in selected cell lines dependent on the presence or absence of stable overexpression of bcl-2. In cells lacking bcl-2 expression, the selection procedure impairs NF-κB–mediated activation of the iNOS gene (7 and this study). In bcl-2–expressing cells, the impairment in NF-κB translocation and activation of iNOS does not occur. Instead, these cells must rely on induction of NF-κB–activated defense/survival genes such as MnSOD or Hsp70 to remain viable in the face of high levels of nitric oxide. The failure of the cytokine selection procedure to impair NF-κB activation and iNOS expression in bcl-2 expressing cells may be attributable to bcl-2–mediated phosphorylation of Iκ-Bα, leading to an enhanced rate of degradation of this cytoplasmic NF-κB regulatory molecule, resulting in more NF-κB translocation to the nucleus. GAS, growth arrest specific; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; P, PO; Ub, ubiquitin.

ous studies (7,28). Further analysis of the best responder from the current study, clone 828/44, is shown in Fig. 10. A 4.7-fold response to glucose is evident in cells with bcl-2 overexpression alone and bcl-2–overexpressing cells after cytokine selection. The retention of GSIS after cytokine selection was also extensively documented in our earlier paper describing the selection procedure (7). We conclude that neither bcl-2 overexpression alone nor the combination of bcl-2 overexpression and the cytokine selection procedure results in impairment of GSIS.

DISCUSSION

Although the Edmonton trial has defined a method of human islet transplantation with a very high success rate (1), several potential liabilities and risks associated with life-long immunosuppression as advocated by this procedure can be imagined, particularly in children. Thus, the search for less invasive ways to protect insulin-producing cells from immunological attack must continue. To this end, our group and others have investigated a variety of strategies for conferring resistance to agents of inflammation. This has included expression of manganese superoxide dismutase (MnSOD) (12,29), dominant negative elements of specific cytokine signaling pathways (30–34), and anti-apoptotic genes, most notably bcl-2 (35–37). One group has expressed dominant negative reagents in mouse insulinoma cells in concert with stable overexpression of bcl-2 (30,31,36). However, in this and all other cases, only partial resistance against damage incurred in the presence of a mixture of inflammatory cytokines has been achieved. In an effort to overcome this problem, our laboratory recently devised a cytokine selection strategy, resulting in the isolation of INS-1–derived cell lines with near-complete resistance against the damaging effects of high concentrations of IL-1β + IFN-γ (7). In the current study, we carried our analysis of these selected cell lines further, and discovered that the selection strategy provided only weak protection against oxidative stress, the other "arm" of the inflammatory response. We also showed for the first time that β-cells gain substantial resistance against multiple agents of oxidative stress when engineered for overexpression of bcl-2.

These findings of reciprocal modes of protection in selected versus bcl-2–transfected cells caused us to investigate the potential additive protection that might arise by combining the two strategies. This led to the striking observation that cytokine-selected, bcl-2–overexpressing cells are significantly more viable than cells subjected to either procedure alone, based on studies with an in vitro model of inflammation involving coculture of INS-1–derived cells with mitogen-activated PBMC. To our surprise, however, the selection strategy leads to cytokine resistance via distinct pathways dependent on the presence or absence of a background of bcl-2 expression. In the absence of bcl-2, the selection procedure resulted in suppression of NF-κB translocation, reduced iNOS expression, and a block in NO production, whereas none of these events occurred in selected cells when bcl-2 was overexpressed. The discovery of alternate pathways by which resistance can be gained represents a framework for future discovery of the specific molecular events involved, possibly leading to the development of highly specific and effective strategies for intervening in immune-mediated destruction of transplanted β-cells or cell lines.

Figure 11 depicts a model explaining how divergent pathways can lead to cytokine resistance with or without suppression of NF-κB/iNOS signaling. We believe that cells lacking bcl-2 overexpression must reduce iNOS expression and NO production to attain cytokine resistance. This idea is supported by prior work showing that the iNOS inhibitor N^G-monomethyl-L-arginine is an effective blocker of NO production and IL-1β–mediated cytotoxicity (7,24), although cytokines may also work through other mechanisms, as indicated by retention of partial cytotoxic effects in islets of iNOS knock-out mice (38,39). We further suggest that bcl-2–transfected cells subjected to the selection procedure can become cytokine resistant, even in the absence of suppression of NF-κB translocation and NO production via activation of cytoprotective genes.

To elaborate on this idea further, IL-1β and IFN-γ induce activation of the transcription factors NF-κB and signal transducer/activator of transcription-1, respectively. The rat iNOS promoter contains two NF-κB sites, an IFN-γ–activated site (GAS), and two IFN-stimulated response elements (ISREs) that are required for IL-1β induction and

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IFN-γ potentiation (40). Binding of the transcription factors to these sites stimulates iNOS gene transcription and subsequent NO production, and explains in part the synergistic effects of IL-1β + IFN-γ in causing islet cell death (41,42). NO has direct cytotoxic effects on β-cells, but can also mediate damage by reacting with O_2^- to form peroxynitrite (43,44). In the initial weeks of the selection process, cells that survived developed mechanisms to cope with this cytotoxic environment. In cells not expressing bcl-2 (831/1 and 833/15), the path chosen was to avoid damage by blocking production of NO via impairment of NF-κB translocation. Cells transfected with bcl-2, in contrast, maintained the ability to activate this factor in response to cytokines. This may have been mediated by the BH4 domain of bcl-2, which has been suggested to cause degradation of Iκ-Bα, thereby releasing NF-κB for nuclear translocation (26,45,46). In this way, bcl-2 expression could serve to maintain NF-κB protein levels and activity in the nucleus (47). Because of these actions of bcl-2, these cells are unable to downregulate NF-κB and iNOS, but may become more able to activate protective genes. Consistent with this model, NF-κB has been reported to enhance transcription of both pro-and anti-apoptotic genes (48,49). Examples of the latter include Hsp27, Hsp70, and MnSOD (50). In addition to these NF-κB−activated genes, IL-1β + IFN-γ can also upregulate Hsp40, GADD-153, MGMT (O6-methylguanine-DNA methyltransferase), metallothionein, and Gas-5 growth arrest homolog to provide additional protection against cytokine-mediated destruction (42,51–54). Although further studies will be required to fully develop these ideas, our work has introduced a new model system for the identification of factors that can protect islet cells from immune-mediated damage.

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