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Research Article

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Stable Expression of Manganese Superoxide Dismutase (MnSOD) in Insulinoma Cells Prevents IL-1β–induced Cytotoxicity and Reduces Nitric Oxide Production

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Abstract

The fact that insulin-producing islet β-cells are susceptible to the cytotoxic effects of inflammatory cytokines represents a potential hinderance to the use of such cells for transplantation therapy of insulin-dependent diabetes mellitus (IDDM). In the current study, we show that IL-1β induces destruction of INS-1 insulinoma cells, while having no effect on a second insulinoma cell line RIN1046-38 and its engineered derivatives, and that this difference is correlated with a higher level of expression of manganese superoxide dismutase (MnSOD) in the latter cells. Stable overexpression of MnSOD in INS-1 cells provides complete protection against IL-1β–mediated cytotoxicity, and also results in markedly reduced killing when such cells are exposed to conditioned media from activated human or rat PBMC. Further, overexpression of MnSOD in either RIN- or INS-1–derived lines results in a sharp reduction in IL-1β–induced nitric oxide (NO) production, a finding that correlates with reduced levels of the inducible form of nitric oxide synthase (iNOS). Treatment of INS-1 cells with L-NMMA, an inhibitor of iNOS, provides the same degree of protection against IL-1β or supernatants from LPS-activated rat PBMC as MnSOD overexpression, supporting the idea that MnSOD protects INS-1 cells by interfering with the normal IL-1β–mediated increase in iNOS. Because NO and its derivatives have been implicated as critical mediators of β-cell destruction in IDDM, we conclude that well regulated insulinoma cell lines engineered for MnSOD overexpression may be an attractive alternative to isolated islets as vehicles for insulin replacement in autoimmune diabetes. (*J. Clin. Invest.* 1998. 101:1811–1820.) Key words: insulin-dependent diabetes mellitus • islet β-cells • molecular engineering • immunoprotection • cytokines

Introduction

A large body of evidence has accumulated in support of the idea that inflammatory cytokines cause destruction of insulin-producing β cells of the islets of Langherhans in individuals with insulin-dependent diabetes mellitus (IDDM,[1] reviewed in 1–4). Cytokines have also been reported to be cytotoxic to insulinoma cell lines, but almost all the work in this area has focused on the RINm5F cell line, which lacks glucose-stimulated insulin secretion and has a very low insulin content (5). Our group and others have demonstrated that techniques of molecular engineering can be used to develop new insulinoma cell lines with enhanced functional attributes (reviewed in 6). Such lines could eventually serve as surrogates for isolated islets in transplantation therapy of IDDM. An important component of such an approach will be to choose and develop cell lines that can be effectively protected from immune destruction when transplanted into individuals with the disease.

It has been proposed that cytokine-induced destruction of islet β cells is mediated in part by generation of toxic oxygen radicals (7, 8). Islet cells may be particularly susceptible to this mechanism of destruction due to unusually low levels of expression of enzymes involved in metabolism of reactive oxygen species, including superoxide dismutase, catalase, and various peroxidases (8–10). However, the relative importance of oxygen radicals in islet cell destruction remains unclear, as one group reported that treatment with chemical oxygen radical scavengers provided protection against cytokine killing (11), whereas others using external application of superoxide dismutase or catalase reported no protective effect (12, 13).

Two forms of superoxide dismutase are expressed in mammalian cells, an enzyme that is constitutively expressed and dependent upon copper and zinc ions for activity (Cu/Zn SOD), and a mitochondrial form whose expression is inducible and dependent upon manganese (MnSOD) (14–16). Expression of MnSOD is induced in a variety of cell types, including islet β cells, in response to cytokines such as IL-1β and TNF (17–20). Levels of the inducible form of nitric oxide synthase (iNOS) are also increased by cytokines, particularly IL-1β, and this event and the consequent production of nitric oxide (NO) have been linked to β cell destruction (1–4). Indeed, inhibitors of iNOS effectively block both the short-term metabolic and long-term cytotoxic effects of IL-1β on islet cells (21, 22).

An unresolved and important issue in this area is whether the induction of MnSOD in islets in response to cytokines represents a protective mechanism against free radical toxicity or is, instead, contributory to β cell destruction. In human kidney 293 cells, MnSOD overexpression results in protection against TNF-mediated cytotoxicity (19). However, given the demonstrated sensitivity of islet β cells to NO and its by-products such as peroyxnitrite (1–4, 23), it remains essential to establish that MnSOD does not exacerbate cytokine-induced killing by

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Hans-Ewald Hohmeier and Anice Thigpen contributed equally to this work.

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1. *Abbreviations used in this paper:* IDDM, insulin-dependent diabetes mellitus; iNOS, inducible nitric oxide synthase; MnSOD, manganese superoxide dismutase; NO, nitric oxide; RT, reverse transcription.

potentiating NO production. It is possible, for example, that induction of MnSOD could cause accumulation of NO by removal of the superoxide ion that would otherwise be free to react with NO to form peroxynitrite, as suggested by Eizirik and co-workers (20). Thus, induction of MnSOD could either serve to lower the levels of peroxynitrite or other toxic radicals (protective effect) or increase NO (potentially cytotoxic).

To investigate these issues further, we have studied the cytotoxic effect of cytokines on two types of insulinoma cell lines, and have evaluated the effect of stable expression of MnSOD in such cells. Our results clearly demonstrate that overexpression of this oxygen radical scavenging enzyme protects against IL-1β–mediated destruction of INS-1 rat insulinoma cells, and also uncovers a novel link between MnSOD expression and NO production. Strategies such as that taken here may be useful for blocking or attenuating immunological destruction of engineered cell lines currently under development as islet surrogates for transplantation therapy of IDDM.

Methods

Cell lines and reagents. Studies were conducted with RIN1046-38 cells (24) and several cell lines derived from these cells by stable transfection of the human insulin gene (βG I/17), the human insulin gene and the glucokinase gene (βG 40/110), or the human insulin gene, the GLUT-2 gene, and the glucokinase gene (βG 49/206), as previously described (25, 26). RIN cell lines were cultured in medium 199 supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The rat insulinoma cell line INS-1 (27) was obtained from Claes Wollheim and Philippe Halban (University of Geneva, Geneva, Switzerland) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM Hepes, 2 mM L-glutamine, 1 mM Na-pyruvate, and 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (28). All cells were grown in 10-cm tissue culture dishes at 37°C and 5% CO_2 in a humidified atmosphere and passaged every 4 d by light trypsinization. Recombinant rat IL-1β and recombinant human TNF-α were obtained from Endogen (Cambridge, MA). Recombinant human and rat γ-IFN were obtained from GIBCO BRL (Gaithersburg, MD). A monoclonal antibody for neutralizing γ-IFN was purchased from GenZyme Corp. (Boston, MA). MnSOD immunoblot analyses were performed with a rabbit anti–human MnSOD antiserum that cross-reacts with rat MnSOD obtained from L. Oberley (University of Iowa, Iowa City, IA) (29). iNOS immunoblot analyses were performed with an affinity-purified antibody purchased from Transduction Laboratories (Lexington, KY).

MTT and neutral red viability staining. Cell viability was measured by the previously described MTT assay, with some modifications (30, 31). Cells were seeded at 50,000 cells/well in flat-bottom 96-well tissue culture dishes (Corning Glass Works, Corning, NY). After 24 h, the medium was discarded and 150 μl of the appropriate medium (INS-1 or RIN medium) containing the cytokines was added to the wells. The test medium was left on the cells for the time periods indicated in the figure legends. Medium was discarded and replaced with 75 μg/ml C,*N* diphenyl-*N*′-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dissolved in 115 μl INS-1 or RIN cell growth medium. Plates were incubated at 37°C for 2 h. The resulting formazan crystals were solubilized in 115 μl 0.04 N HCl in isopropanol. The optical density was read at 575 and 650 nm using a SpectraMax 340 (Molecular Devices, Sunnyvale, CA) plate reader. The reduction in optical density induced by cytokine treatment was used as a measure of viability, normalized to cells incubated in the absence of cytokines, which were considered 100% viable. Results with MTT staining were confirmed with neutral red staining (32).

RNA isolation and analysis. Total RNA was isolated from cells and reverse transcribed (RT) according to the manufacturer's protocol using the Microscreen RNA and first strand cDNA synthesis kit (5′→3′, Inc., Boulder, CO). Specific sequences were amplified from the cDNAs by PCR. 50-μl amplification reactions were performed in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA) using *Taq* DNA polymerase (GIBCO BRL) and the following cycles: 40 rounds of 92°C, 40 s/57°C, 40 s/75°C, 1 min 30 s followed by 75°C for 5 min. The oligonucleotides used for amplification are as follows: type I IL-1βr, 5′AAGCTGACCCAGGATCCACG3′ and 5′TCTGCTCTTCAGATGACTGG (33); copper–zinc superoxide dismutase (Cu/Zn SOD) 5′ATTCACTTCGAGCAGAAGGC3′ and 5′CCAAGTCATCTTGTTTCTCG3′ (34); iNOS 5′CCATCATTGCGTGTGCCTGC3′ and 5′AGCTTCTTCAAAGTGGTAGC3′ (35), and as a control for RNA loading, cyclophilin, 5′GACAGCGAAACTTTCGTGC3′ and 5′TCCAGCCACTCAGTCTTGG3′ (36). After subcloning into pNOTAT7 (5′→3′, Inc.), recombinant plasmids were sequenced to confirm the identity of the insert. Inserts from each of the plasmids were isolated by gel purification using the Prep-A-Gene DNA-purification kit (Bio-Rad Laboratories, Hercules, CA). DNA fragments were radiolabeled with ^{32}P-labeled dCTP by random priming with the redi-prime labeling kit (Amersham Life Science, Arlington Heights, IL), and used as hybridization probes. For Northern blot analysis, 5 μg of total RNA was resolved on methyl mercury/1.5% agarose gels as described (37). Samples were transferred to nylon membrane and hybridized with ^{32}P-labeled cDNA probes using Rapid-Hyb buffer (Amersham Life Science) in a Hybaid Micro 4 Hybridization oven (National Labnet Company). After hybridization, nylon membranes were exposed to film to create autoradiographs. Signals were quantified by densitometry with a UMAX UC840 scanner and normalization to the intensity of the cyclophilin signal.

Stable expression of MnSOD in INS-1 cells. To obtain the full-length cDNA encoding rat MnSOD, oligonucleotides that flanked the start and stop codons of the corresponding mRNA were used in RT-PCR reactions, using RIN1046-38 cell total RNA as template. The oligonucleotide sequences used for this amplification were 5′CGCCTCAGCAATGTTGTGTCG3′ and 5′AGGGCTTCACTTCTTGCAAAC3′ (38). The resultant cDNAs were subcloned into pNOTAT7 and sequenced to confirm insert identities and the fidelity of DNA replication. After digestion with BamHI, MnSOD inserts were subcloned into the pCB7/intron vector (25). In this plasmid, hygromycin resistance is conferred by expression of hygromycin phosphotransferase from the SV40 early promoter. βG I/17 and INS-1 cell lines were stably transfected with SOD expression vectors, using previously described protocols (25). Briefly, 10^7 cells were transfected by electroporation (170 V, 510 μF, 129 ohms in a 2-mm cuvette), and maintained in 300 μg/ml hygromycin (Boehringer Mannheim Biochemicals, Indianapolis, IN) for ~2 wk. Single colonies were isolated and screened for the expression of transgenes by the pyrogallol autoxidation MnSOD enzymatic assay, as previously described (39).

Immunoblot analysis. Cells were lysed in PBS containing 0.1% Triton X-100. Supernatants were prepared by centrifugation at 14,000 *g* in a refrigerated microcentrifuge. Protein concentration in the supernatant fractions was determined by the method of Bradford (40), using the Bio-Rad kit. Protein was suspended in an equal volume of 2× sample buffer (100 mM Tris, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 10% β-mercaptoethanol, pH 6.8), heated for 5 min, and electrophoresed using 10% precast Tris-glycine gels (Bio-Rad Laboratories). Protein was transferred to PVDF membranes (Millipore Corp., Bedford, MA) and blocked with 4% dry milk in TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 8.0). Blots were incubated with anti-MnSOD or anti-iNOS antisera diluted 1:1,000 in TBST + 2% BSA for 1 h at room temperature, and bands were visualized with horseradish peroxidase–conjugated goat anti–rabbit IgG (Amersham Life Sciences) and enhanced chemiluminescence (Amersham Life Sciences). Immunoblots were scanned with a UMAX UC840 scanner.

Measurement of nitrite formation. Nitrite formation in cell supernatants as an indication of NO production was measured as described

(41). The cells were plated in 24-well plates at 0.5×10^6 cells/well 2 d before experimental manipulation. The appropriate tissue culture medium was replaced with supplemented RPMI medium with or without cytokines for 24 h. Supplemented RPMI medium (RPMI + 10% fetal calf serum + antibiotics) was used for both INS-1 and RIN cells in this experiment to have identical arginine concentrations. After the 24-h incubation, media were collected, centrifuged for 5 min at 14,000 *g*, and 100 μl of supernatant was incubated with 100 μl of Griess reagent for 2 h. The optical density was read at 540 nm using a SpectraMax340 (Molecular Devices, Sunnyvale, CA) plate reader. Nitrite concentration was calculated using a sodium-nitrite standard curve prepared in RPMI medium, and data were normalized to total cellular protein (40).

Preparation of activated supernatants from human and rat PBMCs. Heparinized blood was collected from a healthy human volunteer or Wistar rats, and PBMCs were isolated over Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) using standard methods (42). 2×10^6 cells/ml were incubated in RPMI medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Cells were stimulated for 3 d with either 10 μg/ml LPS (*Escherichia coli* 0127:B8, obtained from Sigma Chemical Co.) or a combination of 10 ng/ml PMA plus 0.5 μM ionomycin (Sigma Chemical Co.). After harvesting, conditioned media samples were centrifuged at 2,000 *g* for 15 min to remove cellular components. For MTT assay, supernatants were diluted 1:1 with complete RPMI medium. Before adding diluted supernatants to INS-1 cell lines and clones, an appropriate volume of 50× INS-1 supplement (0.5 M Hepes, 100 mM L-glutamine, 50 mM Na-pyruvate, 2.5 mM β-mercaptoethanol, pH 7.4) was added to make the final solution contain the same supplements as normal INS-1 medium.

Statistical analysis. Statistical analysis of the data was performed using the two-tailed Student's *t* test, assuming unequal variances.

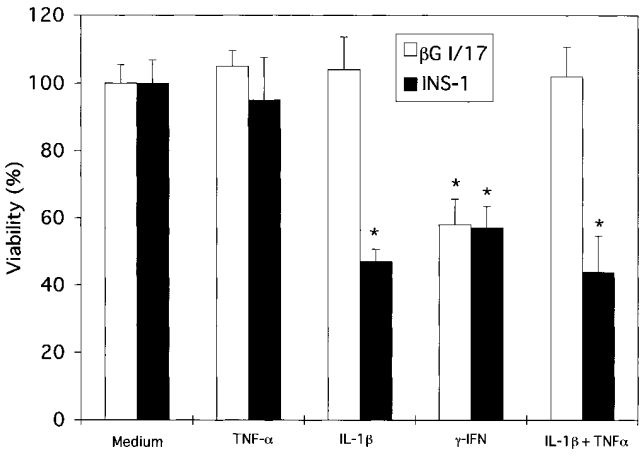

Figure 1. Differential effects of cytokines on RIN-derived and INS-1 insulinoma cells. βG I/17 cells, derived from RIN1046-38 cells by stable overexpression of human insulin (25), and INS-1 cells (27), were incubated in the presence of the indicated cytokines for 48 h. The percentage of cells viable after these treatments was determined by the MTT colorimetric assay as described in Methods, and expressed as a percentage of the viable cells present after treatment with medium lacking any cytokines (*Medium*). The concentrations of cytokines used were: TNF-α, 10 ng/ml; IL-1β, 10 ng/ml; rat γ-IFN, 100 U/ml. Data represent the mean±standard deviation for three independent experiments, each performed in triplicate. *Significantly reduced viability relative to the appropriate medium-incubated control, at a significance level of $P < 0.001$.

Results

Differential sensitivity of various insulinoma cell lines to cytokine-mediated cytotoxicity. Our group has worked with modified RIN1046-38 cells as a potential vehicle for cell-based insulin replacement in IDDM (6, 25, 26, 43). To begin to understand the susceptibility of these cells to cytokine killing we used a clonal line called βG I/17, derived from RIN1046-38 cells by stable overexpression of the human proinsulin cDNA (25, 26). For comparison, we also studied the effects of cytokines on the well established INS-1 insulinoma line (27). Using the MTT viability assay, addition of 10 ng/ml TNF-α had no effect on either cell line, whereas 100 U/ml γ-IFN was equally cytotoxic to both lines (~ 42% of cells killed; Fig. 1). A clear differential effect was noted, however, with 10 ng/ml IL-1β, which killed > 50% of INS-1 cells but was without effect on βG I/17 cells. Further, the combination of IL-1β + TNF-α had a cytotoxic effect on INS-1, but not βG I/17 cells, with a similar percentage of INS-1 cells killed as with IL-1β alone. Although data are presented only for 48 h of incubation with 10 ng/ml IL-1β, the effects of this cytokine on INS-1 cells were observed at concentrations as low as 0.5 ng/ml and within the first 24 h of incubation, whereas in βG I/17 cells, no significant cytotoxic effect was observed, even with 10 ng/ml IL-1β administered for 72 h (data not shown). The resistance to IL-1β cytoxicity exhibited by the βG I/17 line was not a peculiar feature of this transfected clone, as the parental RIN1046-38 cells from which βG I/17 cells were derived and other clonal lines expressing combinations of the GLUT-2, glucokinase, and human insulin genes (βG 49/206 and βG 40/110; 25, 26) were also completely refractory to the effects of IL-1β (data not shown).

The MTT assay provides a measure of cell viability, in that conversion of the MTT substrate to formazan crystals that are detected colorimetrically requires intact and functional mitochondria. Although it was clear that IL-1β or γ-IFN treatment caused an obvious decrease in cell density (see below), further confirmation of the differential effect of IL-1β on RIN and INS-1 cells was also obtained with an alternative viability dye, neutral red, that is sequestered in lysosomes of live cells. Absorbance measurements of extracted neutral red revealed a viability of 103±4% for IL-1β–treated βG I/17 cells, compared to 71±3% viability for IL-1β–treated INS-1 cells ($P < 0.001$; numbers are derived by comparison to the respective cell lines incubated in the absence of cytokines, and represent the mean±SD of eight independent measurements per cell line). Thus, the neutral red assay confirms that IL-1β exerts a cytotoxic effect on INS-1 cells, but not the RIN1046-38–derived cell line. The difference between absolute values obtained with the MTT and neutral red assays (50% viability versus 70%) is consistent with experiences of others who have compared these methods (44, 45).

Stable expression of manganese superoxide dismutase in INS-1 cells. Expression of MnSOD is induced in isolated rat islets in response to IL-1β (20), but it remains unclear whether this event is protective or contributory to the cytotoxic effects of the cytokine. To further evaluate the potential role of MnSOD expression, we measured levels of this protein in βG I/17 and INS-1 cells. As shown in the representative blot in Fig. 2 *A* and validated by densitometric analysis of three independent experiments, βG I/17 cells incubated in the absence of any cytokines contained 2.4±0.8-fold more MnSOD protein than INS-1 cells under the same conditions. Exposure to IL-1β

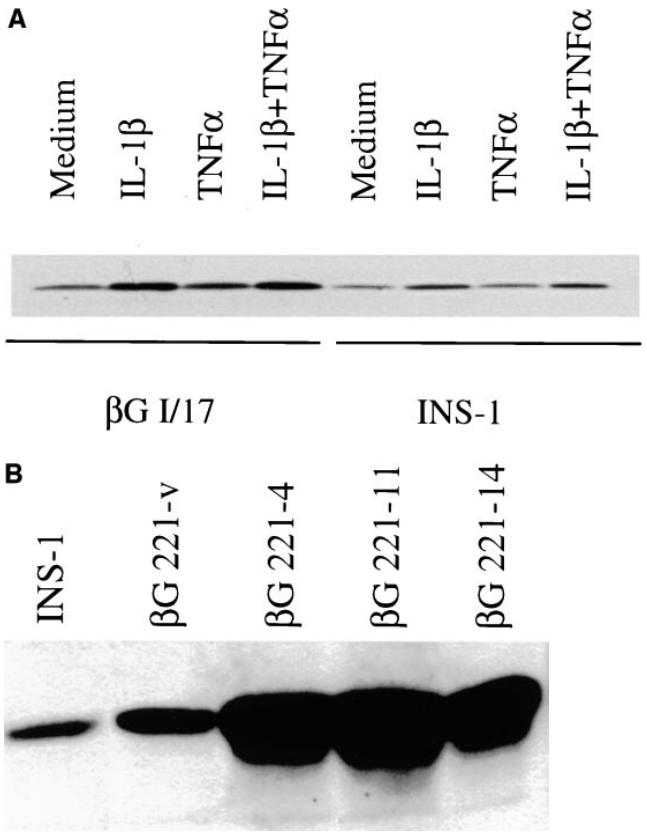

Figure 2. Immunoblot analysis of MnSOD protein levels in RIN-derived and INS-1 cell lines and overexpression of MnSOD in INS-1 cells. (*A*) βG I/17 or INS-1 cells were left untreated, or treated with 10 ng/ml IL-1β, 10 ng/ml TNF-α, or 10 ng/ml IL-1β + 10 ng/ml TNF-α for 16 h. After this treatment, cell extracts were prepared and 25 μg total protein per sample was resolved by SDS-PAGE and immunoblotted with an anti-MnSOD antibody as described in Methods. (*B*) Immunoblot analysis of MnSOD protein levels in native INS-1 cells, INS-1 cells transfected with the pCB7/intron vector lacking the Mn-SOD insert (*βG 221-v*), and three clones stably transfected with pCB7/intron containing the MnSOD cDNA (*βG 221-4*, *βG 221-11*, *βG 221-14*). The blots in each panel are representative of three to five independent experiments (see text for densitometric and statistical analysis).

caused a 2.0±0.8-fold increase in MnSOD protein in βG I/17 cells and a 2.4±0.9-fold increase in INS-1 cells, whereas TNF-α alone caused a smaller increase in MnSOD protein in either cell line. Finally, the combination of IL-1β + TNF-α caused a 2.1±0.4-fold and a 2.1±0.6-fold increase in MnSOD protein in INS-1 or βG I/17 cells, respectively. Thus, the MnSOD protein level attained in IL-1β or IL-1β + TNF-α–stimulated INS-1 cells was approximately equal to that in unstimulated βG I/17 cells. These data are consistent with the idea that INS-1 cells contain levels of MnSOD that may be insufficient to protect against damage mediated by IL-1β.

To investigate this issue further, we stably transfected INS-1 cells with a vector in which MnSOD expression is under the control of the CMV promoter (pCB7/intron/MnSOD). After electroporation of INS-1 cells with this plasmid, hygromycin resistant clones that expressed the transgene were initially identified by measurement of superoxide dismutase activity, resulting in the identification of three clones with high levels of activity (βG 221-4, βG 221-11, and βG 221-14). Fig. 2 *B* shows that the level of MnSOD protein in INS-1 cells stably transfected with the vector pCB-7/intron lacking the MnSOD cDNA insert (clone βG 221-v) is similar to that in untransfected cells. In contrast, the three stably transfected clones contained 9.6±4.6, 9.1±5.4, and 9.4±5.0 times as much Mn-SOD protein as found in the βG 221-v control cells, respectively (based on three to five independent determinations; the blot in Fig. 2 *B* is representative). These results provide proof of efficient overexpression of MnSOD in INS-1 cells, allowing hypotheses about the protective effect of this enzyme to be tested. Confidence in these methods is further enhanced by our finding that overexpression of MnSOD in INS-1 cells had no effect on the expression levels of other relevant endogenous mRNAs, including the IL-1β receptor or CuZn SOD. Further, subcellular fractionation of cell extracts into mitochondria-enriched and cytosolic fractions (46) revealed that, like the endogenous enzyme, the majority of overexpressed MnSOD was found in the mitochrondria-enriched samples (data not shown). We also transfected βG I/17 cells by the same methods and obtained a 2.5–3-fold increase in MnSOD protein in two independent clones (βG 224-6, βG 224-10) relative to a clone transfected with the empty vector (βG 224-v; data not shown).

MnSOD overexpression confers protection against IL-1β–mediated cytotoxicity in INS-1 cells. The three representative INS-1 cell lines engineered for stable overexpression of Mn-SOD described above were tested for cytokine-induced cytotoxicity using the MTT assay, and compared with the INS-1 clone that was transfected with the empty vector. As shown in Fig. 3, all three clones engineered for MnSOD expression exhibited complete resistance to killing by IL-1β alone or the combination of IL-1β + TNF-α, whereas only ∼ 50% of the βG 221-v control cells remained viable after these treatments. In contrast, MnSOD expression did not provide any protection against γ-IFN–mediated INS-1 cell destruction at either of the two concentrations of the cytokine tested (20 or 100 U/ml). Confirmation of these results was obtained by photographing the cells and by applying the alternative neutral red viability stain. As shown in Fig. 4, treatment of unengineered INS-1 cells with 10 ng/ml of IL-1β for 48 h clearly reduced the number of viable cells, regardless of which staining technique was used, whereas no change in cell number was noted upon IL-1β treatment of a representative MnSOD-overexpressing INS-1 cell clone (βG 221-14). Absorbance measurements of extracted neutral red revealed a viability of 103±6% for IL-1β–treated βG221-14 cells compared to 70±2% viability for IL-1β–treated and untransfected INS-1 cells (numbers are derived by comparison to the respective cell lines incubated in the absence of cytokines).

MnSOD overexpression decreases NO production and iNOS mRNA and protein levels during IL-1β treatment of RIN or INS-1 cell lines. It is well established that IL-1β–mediated destruction of β cells is accompanied by activation of iNOS expression and production of NO (1–4). To provide further evidence for the importance of NO generation in IL-1β–mediated killing of INS-1 cells, we studied the effect of addition of the iNOS inhibitor L-NNMA. As shown in Fig. 5, L-NMMA provided complete protection against either IL-1β or IL-1β + TNF-α–mediated killing, but could not reverse γ-IFN–mediated cytotoxicity.

To investigate whether the mechanism of protection

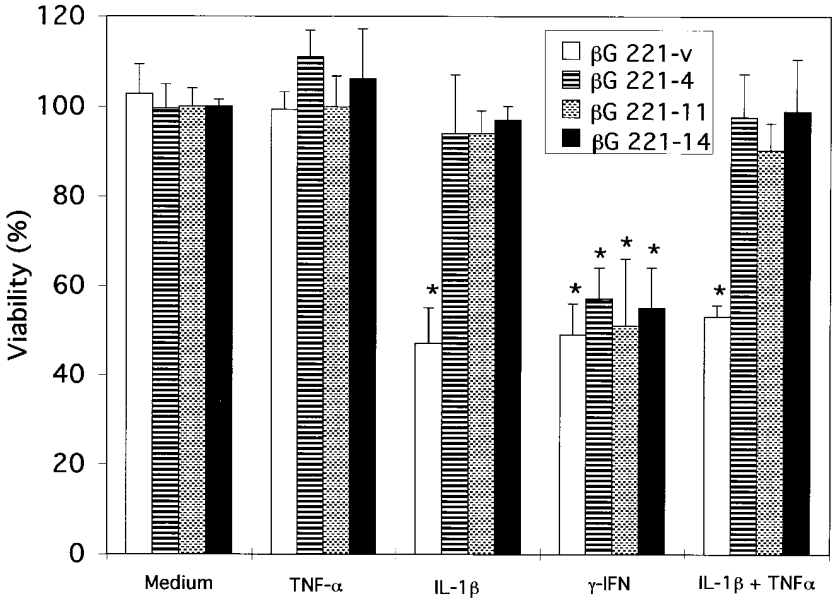

Figure 3. Protection against IL-1β cytotoxicity in INS-1 cells conferred by MnSOD overexpression. INS-1 cells transfected with the pCB7/intron vector lacking the MnSOD insert (*βG 221-v*) and three clones stably transfected with pCB7/intron containing the MnSOD cDNA (*βG 221-4*, *βG 221-11*, *βG 221-14*) were incubated with the indicated cytokines for 48 h. The cytokine concentrations and methods used were the same as defined in the legend to Fig. 1. Data represent the mean±standard deviation for three independent experiments, each performed in triplicate. *Significantly reduced viability relative to the medium-incubated βG 221-v cell line, at a level of significance of $P < 0.001$. Note the absence of IL-1β or IL-1β + TNF-α–mediated cell killing in the three MnSOD-overexpressing lines.

against IL-1β cytotoxicity conferred by MnSOD expression in INS-1 cells is related to iNOS activity, we measured NO production (in the form of nitrite) in control and MnSOD-transfected insulinoma cells. As shown in Fig. 6 *A*, the ability of MnSOD overexpression to protect against IL-1β–mediated cytotoxicity was correlated with its ability to block NO production. Thus, NO production in response to IL-1β treatment was reduced by 79 and 82% in the βG 221-4 and βG 221-11 clones, respectively, relative to βG 221-v control cells. Interestingly, a similar degree of suppression of NO production in response to IL-1β was observed in MnSOD-overexpressing βG I/17 cells relative to control clones transfected with the empty vector (Fig. 6 *B*). However, it should also be noted that the absolute amount of nitrite produced during IL-1β treatment was similar in INS-1 and βG I/17 cells and their engineered derivatives (compare Fig. 6, *A* and *B*), suggesting that the latter cells are not as sensitive to NO as the former, or that other effects of high levels of MnSOD expression such as elimination of toxic

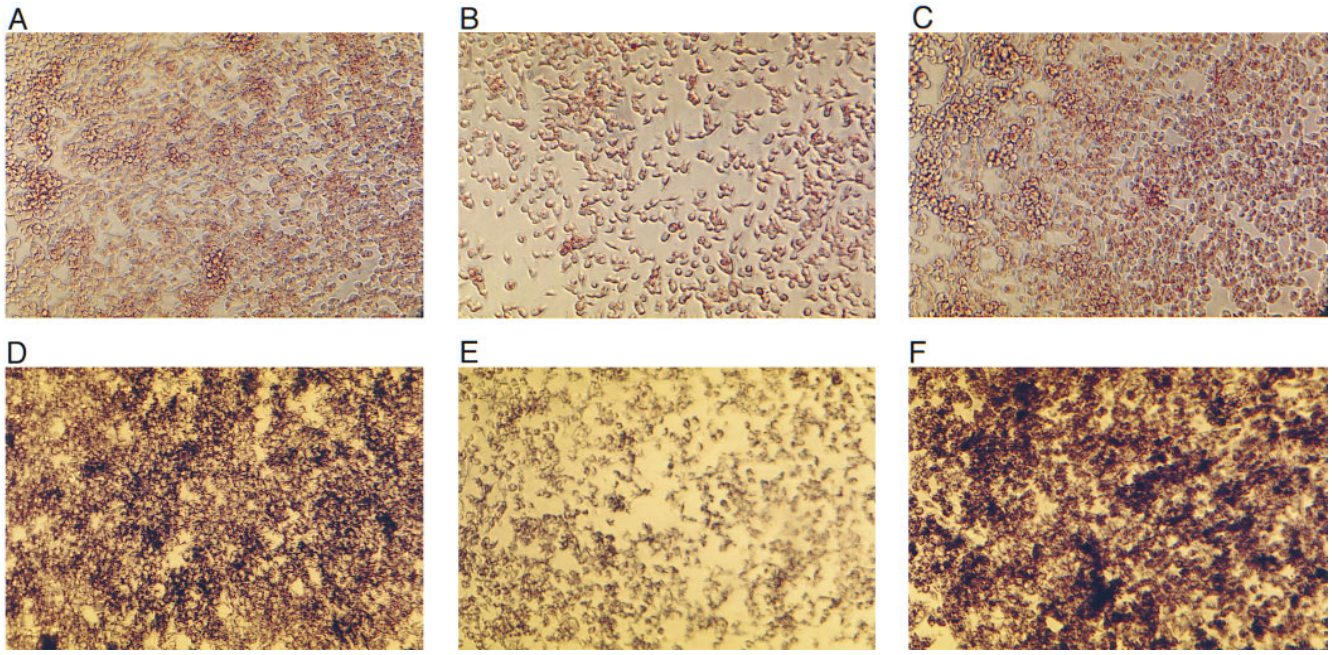

Figure 4. IL-1β effects on INS-1 cell lines. INS-1 cells (*A*, *B*, *D*, and *E*) or βG 221-14 cells with stable overexpression of MnSOD (*C* and *F*) were grown to near confluency and then cultured for 48 h in the absence of cytokines (*A* and *D*) or in the presence of 10 ng/ml IL-1β (*B*, *C*, *E*, and *F*) and stained with neutral red (*A–C*) or MTT (*D–F*). Note that expression of MnSOD (*C* and *F*) prevented the IL-1β–induced cell destruction that is evident in native INS-1 cells (*B* and *E*). *D–F* are representative of the MTT absorbance experiments reported in Fig. 3.

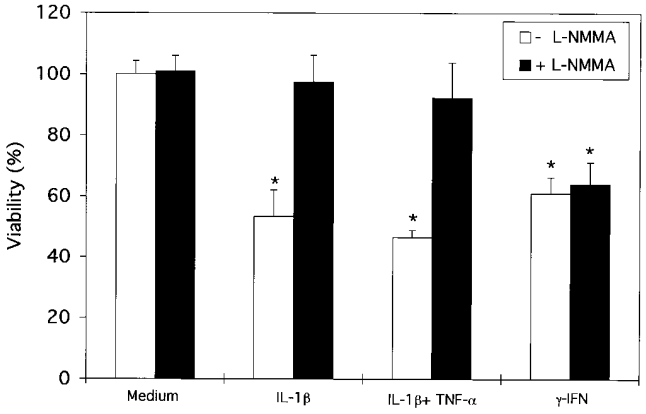

Figure 5. L-NMMA protects against IL-1β–mediated destruction of INS-1 cells. Untransfected INS-1 cells were incubated in the presence of the indicated cytokines and in the absence (*white bars*) or presence (*black bars*) of 2 mM of the iNOS inhibitor L-NMMA for 48 h. The percentage of cells viable after these treatments was determined by the MTT colorimetric assay as described in Methods and expressed as a percentage of the viable cells present after treatment with medium lacking any cytokines (*Medium*). The concentrations of cytokines used were the same as in Fig. 1. Data represent the mean±standard deviation for four independent experiments, each performed in triplicate. *Significantly reduced viability relative to the appropriate medium-incubated control, at a significance level of $P < 0.001$.

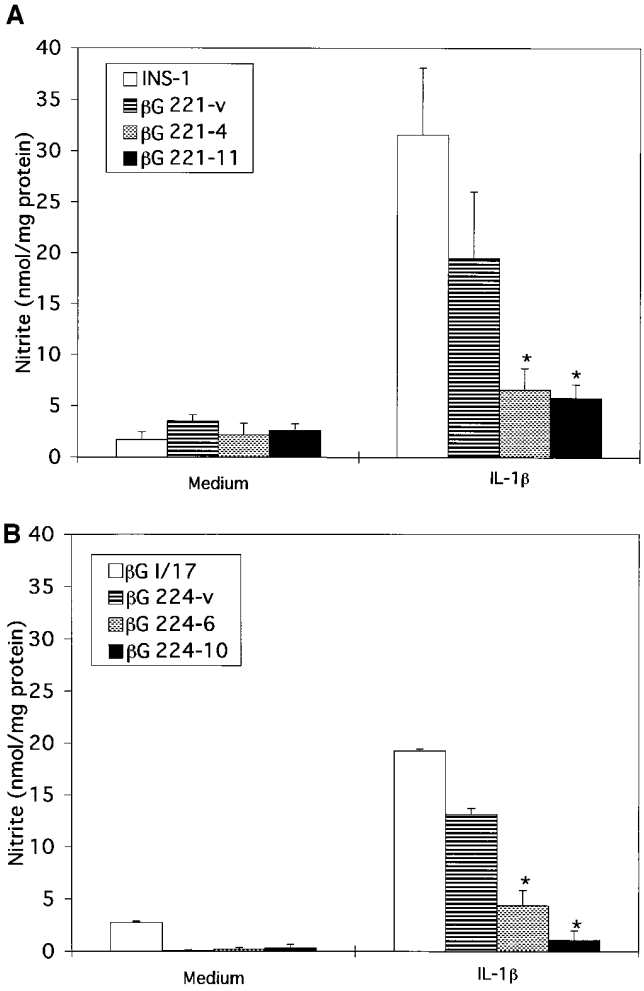

Figure 6. MnSOD overexpression reduces nitrite production from cytokine stimulated INS-1 and RIN-derived cell lines. (*A*) Native INS-1 cells, INS-1 cells transfected with the pCB7/intron vector lacking the MnSOD insert (*βG 221-v*), and two of the clones stably transfected with pCB7/intron vector containing the MnSOD cDNA (*βG 221-4*, *βG 221-11*) were incubated with medium alone or with medium supplemented with 10 ng/ml IL-1β for 24 h. After this treatment, media were collected and assayed for nitrite, as a measure of NO production. Data represent the mean±standard deviation for three independent experiments, each performed in triplicate. *Significantly reduced nitrite production in the MnSOD-overexpressing clones relative to the control βG 221-v cells, at a significance level of $P < 0.001$. (*B*) βG I/17 cells transfected with the empty pCB7/intron vector (clone *βG 224-v*) or with the pCB7/intron vector containing the MnSOD cDNA (*βG 224-6*, *βG 224-10*) were incubated with medium alone or with medium supplemented with 10 ng/ml IL-1β for 24 h. After this treatment, media were collected and assayed for nitrite. Data represent the mean±standard deviation for three independent cell samples per condition. *Significantly reduced nitrite production in the MnSOD-overexpressing clones relative to the control βG 224-v cells, at a significance level of $P < 0.005$.

oxygen radicals are more pertinent to protection against cytokine killing.

To investigate whether MnSOD suppresses IL-1β–mediated NO production by reducing iNOS expression, we measured iNOS mRNA and protein levels in IL-1β–treated control and MnSOD-overexpressing INS-1 cells. As shown in Fig. 7 *A*, IL-1β treatment for 8 h increased iNOS mRNA levels in both groups, but the levels achieved in the MnSOD transfected clones were reduced by 65 (clone βG 221-4) and 66% (βG 221-11) of the levels in the control βG 221-v cells ($n = 3$, $P < 0.001$ for comparison of either MnSOD overexpressing clone with the control). Similarly, as shown in Fig. 7 *B*, all clones exhibited induction of iNOS protein in response to 8 h of IL-1β treatment, but levels achieved in the MnSOD transfected clones were reduced by 90 (clone βG 221-4) and 70% (clone βG 221-11) relative to control βG 222-v cells ($n = 3$, $P < 0.001$ for comparison of either MnSOD-overexpressing clone with the control). These findings strongly suggest that reduced NO production in response to cytokines in MnSOD-overexpressing insulinoma cells is linked to a reduced capacity to increase iNOS gene expression.

MnSOD expression in INS-1 cells confers protection against cytotoxicity induced by supernatants from activated PBMCs. To determine whether MnSOD overexpression provides protection against cytotoxicity during attack by a complex mixture of cytokines and other cytotoxins, we isolated PBMCs from normal rats or humans, stimulated these cells with LPS or PMA + ionomycin, collected the supernatants from the stimulated cells, and incubated control and MnSOD-expressing INS-1 clones with the supernatants. As shown in Fig. 8 *A*, cell killing was reduced from 40% of βG 221-v control cells incubated with LPS-activated rat PBMC supernatants to 10–15% when the βG 221-4 or βG 221-11 clones were tested. Protection was less apparent when PMA-stimulated rat PBMC supernatants were tested, with an improvement from 60% of control cells killed to 40% of MnSOD-expressing cells. This could be explained by the fact that PMA is known to stimulate

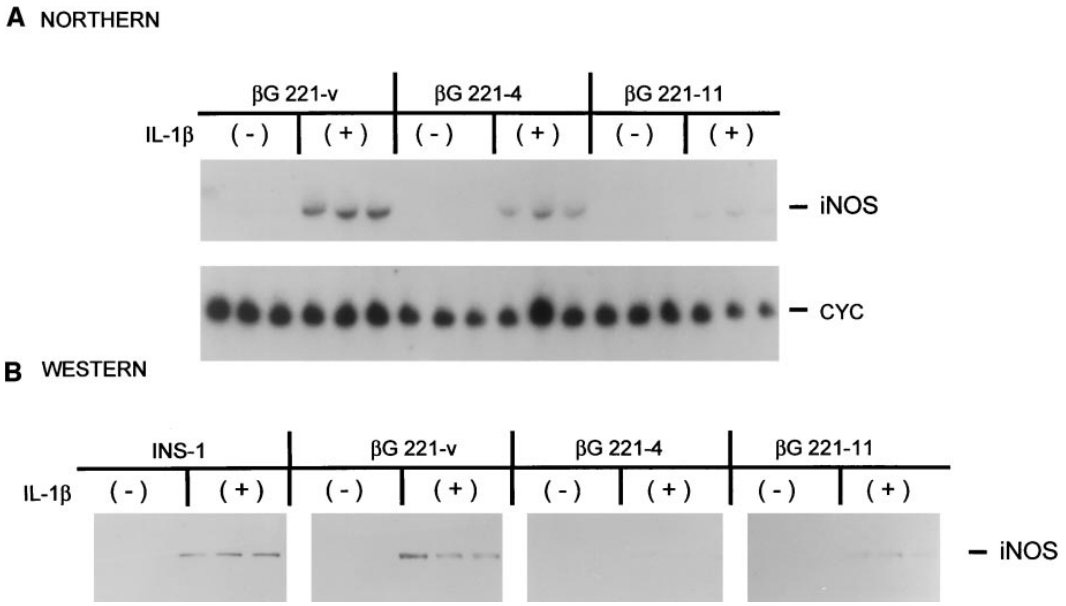

Figure 7. iNOS mRNA and protein levels are reduced in MnSOD-overexpressing INS-1 cells during cytokine stimulation. (*A*) INS-1 cells transfected with the pCB7/intron vector lacking the MnSOD insert (*βG 221-v*), and two of the clones stably transfected with pCB7/intron vector containing the MnSOD cDNA (*βG 221-4*, *βG 221-11*) were incubated with medium alone (−) or with medium supplemented with 10 ng/ml IL-1β (+) for 8 h. Total RNA was prepared and 5 μg per lane was resolved and probed with a radiolabeled iNOS cDNA as described in Methods. (*B*) Cells were treated exactly as described in *A*, except that an additional control group of untransfected INS-1 cells (*INS-1*) was added. After preparation of cell extracts, 30 μg protein/lane was used for immunoblot analysis of iNOS protein as described in Methods. For *A* and *B*, the blots shown are representative of two experiments, each involving samples from three independent groups of cells per condition. Densitometric analysis of the blots is presented in Results.

γ-IFN production from peripheral cells, whereas LPS primarily stimulates IL-1β production (47, 48). Consistent with this model, antibody neutralization of γ-IFN in supernatants prepared from PMA-treated rat PBMC reduced killing of MnSOD-expressing INS-1 cells from 40 to 20% (Fig. 8 *A*). Further, incubation of the control βG 221-v clone with stimulated human PBMC supernatants killed ∼ 70% of the cells in 48 h, whereas supernatants from unstimulated PBMC had no effect (data not shown). The activated supernatants from human cells were much less cytotoxic when applied to either the βG 221-4 or βG 221-11 MnSOD-expressing INS-1 clones, such that LPS-activated PBMC supernatants killed 25–30% of the cells, whereas supernatants from PMA-stimulated cells killed ∼ 35% (data not shown). The enhanced capacity of MnSOD expression to confer protection against PMA-activated supernatants from human cells relative to rat cells is consistent with our finding that human γ-IFN is not cytotoxic when added to either INS-1 or RIN cells (data not shown), whereas rat γ-IFN has clear effects (Fig. 1).

The data in Fig. 8 *A* suggest that MnSOD expression provides protection against supernatants from activated PBMC via blockade of IL-1β–mediated effects. To test whether the protection afforded by MnSOD overexpression is related to its capacity to block iNOS and nitrite formation, we evaluated the effect of the iNOS inhibitor L-NMMA on cells treated with supernatants from LPS-activated rat PBMC. As shown in Fig. 8 *B*, L-NMMA or MnSOD overexpression provide the same degree of protection against supernatants from LPS-activated rat PBMC, providing further support for the idea that MnSOD protects INS-1 cells by attenuating iNOS induction in response to IL-1β.

Discussion

IDDM is a disease in which β cells of the islets of Langherhans are destroyed by autoimmune mechanisms (49, 50). Because self-administration of insulin by the patient is clearly less effective for controlling blood glucose concentrations than a normally functioning pancreas, islet transplantation has been investigated extensively as an alternative method for insulin replacement in IDDM. Important unsolved issues that remain with this approach include the difficulty and cost associated with obtaining sufficient numbers of islets for broad application to the growing number of IDDM patients, and the challenge of protecting the transplanted tissue from immunological destruction. For this reason, our group and others have been actively applying techniques of molecular engineering for the development of new insulinoma cell lines that secrete large amounts of insulin in response to appropriate physiological cues, with the idea that such cell lines could serve as surrogates for isolated islets in transplantation therapy of IDDM (reviewed in 6, 51). The current investigation was undertaken to determine if cell lines with attractive secretory properties can be engineered for protection against cytokine-mediated destruction. This endeavor may be relevant even when considering transplantation in the context of protective perm-selective membranes, because for some materials, graft rejection can occur despite the fact that direct contact between cellular elements of the immune response and the transplanted tissue is prevented (52).

As a start point in this process, we have evaluated the cytotoxic effects of cytokines on two types of insulinoma cell lines demonstrated to have robust fuel-stimulated insulin secretion.

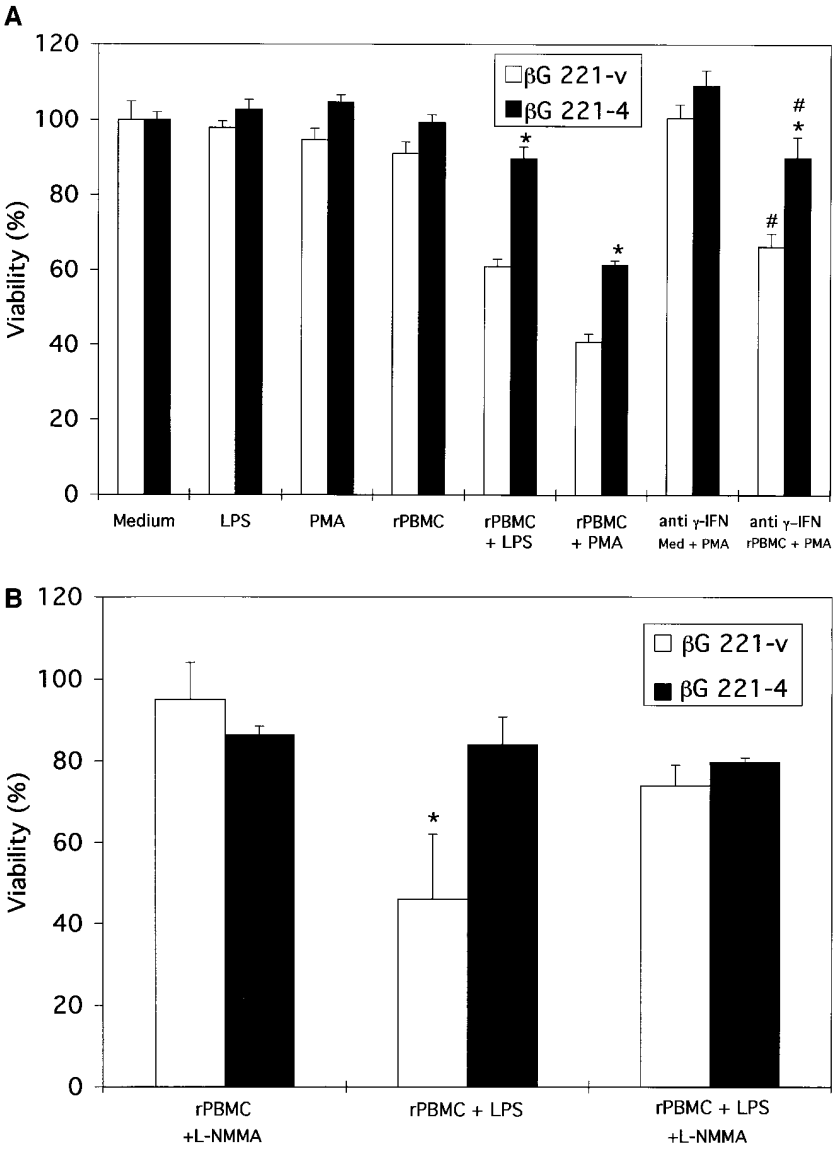

Figure 8. MnSOD overexpression provides protection against killing by activated rat PBMCs. (*A*) PBMCs were prepared from normal Wistar rats and treated with 10 ng/ml PMA + 0.5 μM ionomycin (rPBMC + PMA) or 10 μg/ml LPS (rPBMC + LPS) for 3 d. The conditioned media from these cells were then collected and added to the indicated cell lines for 48 h. Controls included application of medium alone (*Medium*), medium supplemented with LPS (*LPS*), medium supplemented with PMA + ionomycin (*PMA*), or medium from unstimulated rat PBMC (*rPBMC*). The percentage of cells viable after these treatments was determined as described in the legends to Figs. 1 and 3, and expressed as a percentage of viable cells after treatment with medium alone. For experiments involving antibody neutralization of γ-IFN, supernatants were incubated with 10 μg/ml γ-IFN neutralizing antibody for 1 h at room temperature before incubation with cell lines. The neutralizing antibody was added to conditioned media obtained from rat PBMC treated with PMA + ionomycin (anti-γ-IFN + PMA + rPBMC), or as a control, the antibody was added to unconditioned medium supplemented with PMA + ionomycin (*anti-γ-IFN + Med + PMA*). Data represent the mean±standard deviation for three independent cell samples per condition. *Significantly increased viability of the MnSOD-overexpressing line (*βG 221-4*) relative to the empty vector control cell line (*βG 221-v*), at a significance level of $P < 0.01$, whereas # indicates that anti-γ-IFN treatment of rPBMC + PMA supernatants significantly increased viability relative to cells treated with rPBMC + PMA supernatants not immunodepleted of γ-IFN, at $P < 0.02$. (*B*) Supernatants were prepared as described for *A*. Where indicated, the iNOS inhibitor L-NMMA was added to the diluted supernatants before the 48-h incubation with insulinoma cells. Data represent the mean±standard deviation for three to six independent cell samples per condition. *βG 221-v cells treated with supernatants of LPS-treated rat PBMC in the absence of L-NMMA were significantly less viable than cells treated with the same supernatants in the presence of L-NMMA, at a significance level of $P < 0.001$.

The first of these, the INS-1 cell line, was derived by harvesting of isolated colonies of cells with well differentiated properties from a polyclonal population of RINm5F cells (27). The second type of cell line, RIN1046-38, was derived from a radiation-induced insulinoma tumor in rats much like RINm5F, but is a sub-line, which retains a higher insulin content and some glucose-stimulated insulin secretion at low passage numbers (24). Our interest in RIN1046-38 cells derives from our recent finding that stable introduction of combinations of the human insulin, GLUT-2, and/or glucokinase genes confers glucose-stimulated insulin secretion similar in magnitude and dynamics to that observed in normal islets (25, 26).

Surprisingly, RIN1046-38 cells and their engineered derivatives βG I/17, βG 40/110, and βG 49/206 are completely resistant to IL-1β–induced cytotoxicity, even at very high doses of the cytokine. In contrast, IL-1β has clear cytotoxic effects on INS-1 cells, consistent with previous reports of reduced insulin content and secretion caused by IL-1β treatment of these cells (53, 54). The clear differential effect of IL-1β on INS-1 and RIN1046-38 cells provided a unique opportunity to investigate operative mechanisms of IL-1β–mediated cytotoxicity. Our finding of higher basal and IL-1β–induced levels of MnSOD protein in βG I/17 cells compared to INS-1 cells was consistent with the differential cytotoxic effect of the cytokine on the two lines, and provided the impetus for further molecular studies on the protective effects of this radical scavenging enzyme.

That induction of intracellular MnSOD represents a protective mechanism is unequivocally established by our experiments in which INS-1 cells were stably engineered for MnSOD overexpression. Such engineered lines were completely resistant to IL-1β–mediated cytotoxicity and were also well pro-

tected against supernatants from activated human or rat peripheral blood mononuclear cells. These findings suggest that the resistance to IL-1β exhibited by the RIN1046-38–derived lines (i.e., βG I/17) is due to their natural capacity to maintain high levels of expression of MnSOD.

Another novel finding of our study is that the overexpression of MnSOD is linked to the capacity for NO production in insulinoma cells. We observed that MnSOD-overexpressing INS-1 lines had lower levels of iNOS mRNA and protein, when stimulated with IL-1β, than control cells. Consistent with this, IL-1β–stimulated NO production was sharply reduced by MnSOD overexpression in both INS-1 and βG I/17 cells. Further, administration of the iNOS inhibitor L-NMMA to INS-1 cells was equally effective as MnSOD expression at blocking IL-1β–mediated cell destruction. Thus, it appears likely that MnSOD expression exerts its protective effect at least in part via inhibition of iNOS and NO production, although we cannot yet formally rule out that the enzyme may also be working via inhibition of superoxide accumulation or peroxynitrite formation.

The mechanism by which MnSOD leads to reduced levels of iNOS mRNA and protein is unknown. However, a large body of work has made it increasingly clear that reactive oxygen species are potent regulators of signal transduction and transcription in mammalian cells, in some cases, via direct chemical modification of relevant transcription factors or signal transduction elements (55, 56). Regulation of the iNOS gene appears to be highly complex, with some 30 different consensus sequences for transcription factor binding contained in the proximal 1,500 base pairs of the iNOS gene promoter/enhancer region. Further, the 3′ untranslated region of the iNOS mRNA contains a UA rich sequence that is conserved among iNOS transcripts of different species and that is similar to a sequence in the TNF transcript that has been implicated in the control of mRNA stability (4). iNOS and MnSOD are part of a group of "late response genes" that also includes cyclooxygenase II and heat shock protein 70 that are activated by a variety of stressors, including cytokines. These genes are in turn regulated by factors that are induced early in the stress response such as c-fos and c-jun and by translocation of the NF-kB transcription factor from the cytosol to the nucleus (4, 57). Interestingly, both fos/jun activation and binding to the AP-1 *cis*-acting sequence and activation of NF-kB have been shown to be regulated by oxygen radicals (55, 56, 58). Whether constitutive overexpression of MnSOD somehow interrupts the expression or activation of these *trans*-acting factors, or influences the stability of the iNOS mRNA, or both, remains to be determined.

Of interest is our finding that treatment of INS-1 cells with either IL-1β or γ-IFN alone is sufficient to cause cytotoxicity. IL-1β has consistently been shown to be capable of inducing iNOS expression and NO production in a wide variety of insulinoma cell lines and in primary islets, but there is a lack of consensus surrounding the issue of whether it is capable of exerting its cytotoxic effects in the absence of other cytokines (59–62). Similarly, previous studies with rat γ-IFN on isolated rat islets showed the absence of a destructive effect of this cytokine alone, although it was able to potentiate the cytotoxic effect of a sub-optimal dose of IL-1β (63). Controversy also exists with regard to the mechanism of IL-1β–induced cell destruction, as some studies have provided evidence of apoptotic effects of the cytokine, whereas others have failed to do so (4, 59–62). Further studies that provide insight into the mechanism(s) underlying the clear cytotoxic effect of IL-1β on INS-1 cells (Figs. 1, 3, and 4) will be required. Finally, it is of interest that, unlike the case with IL-1β, the destructive effect of γ-IFN is not reversed by L-NMMA or MnSOD expression in INS-1 cells, suggesting that γ-IFN killing occurs by a pathway distinct from that activated by IL-1β. These data suggest that the INS-1 cell system may be ideal for future studies that will be required to address the apparently divergent mechanisms of action of γ-IFN and IL-1β.

We conclude that overexpression of MnSOD renders insulinoma cells less susceptible to immunological damage. Further, to the extent that NO accumulation is central to cytokine-mediated cell killing, the block in NO production conferred by MnSOD overexpression may provide broad protection against this arm of the immune response. The fact that MnSOD-overexpressing INS-1 cell lines are well protected against the cytotoxic effects of supernatants, containing a complex mixture of cytokines and other toxic agents from activated PBMC, provides encouragement for future evaluation of engineered cell lines by transplantation into various animal models of diabetes.

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