Selection of Insulinoma Cell Lines With Resistance to Interleukin-1β– and γ-Interferon–Induced Cytotoxicity

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Engineered insulinoma cell lines may represent an alternative to isolated islets for transplantation therapy of type 1 diabetes. Success of this approach may require development of cell lines that can withstand cytokine-mediated damage. To this end, we have cultured INS-1 insulinoma cells in increasing concentrations of interleukin-1β (IL-1β) + γ-interferon (IFN-γ), with approximate weekly iterations over an 8-week period. Based on the C,N diphenyl-*N′*-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay, the selected cells, termed INS-1_{res}, were 100% viable after 5 days of treatment with 10 ng/ml of IL-1β. These cells were also 78 ± 1.2% viable after 5 days of exposure to the combination of 10 ng/ml IL-1β and 100 U/ml IFN-γ, whereas parental INS-1 cells treated in the same manner were only 0.3 ± 0.03% viable. INS-1_{res} cells were also resistant to treatment with supernatants from activated rat peripheral blood mononuclear cells, whereas only 20% of parental INS-1 cells survived such treatment. The resistance to IL-1β conferred by this procedure was stable, whereas the partial resistance to IFN-γ was transient but reinducible by culture in the presence of cytokines. Stable transfection of INS-1_{res} cells with a plasmid containing the human insulin cDNA and expansion of the transfected colonies in the absence of cytokines produced cell lines that were on average more resistant to IL-1β + IFN-γ (53 ± 11%) than similarly transfected clones derived from parental INS-1 cells (15 ± 7%). Importantly, several INS-1_{res}–derived clones retained the capacity to secrete insulin in response to glucose concentrations over the normal physiological range. With regard to the mechanism by which selection was conferred, we found normal levels of IFN-γ receptor mRNA, but a 60% reduction in expression of the IL-1 receptor type I (IL-1RI) in INS-1_{res} cells compared with parental INS-1 cells. IL-1β signaling through p38 MAP kinase was found to be normal in INS-1_{res} cells, suggesting that their expression of IL-1RI is sufficient to maintain cytokine action. However, normal IL-1β–mediated translocation of NF-kB and induction of inducible nitric oxide synthase expression and nitric oxide production was severely impaired in the INS-1_{res} cell lines, suggesting a mechanism for the IL-1β resistance. In sum, this study defines a strategy for isolation of cytokine-resistant β-cell lines and provides a new system for studying the mechanisms by which such resistance can be achieved. *Diabetes* 49:562–570, 2000

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Con A, concanavalin A; γ-IFNR, γ-interferon receptor; IFN-γ, γ-interferon; IL-1β, interleukin-1β; IL-1RI, interleukin-1 receptor type I; iNOS, inducible nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; MnSOD, manganese superoxide dismutase; MTT, C,N diphenyl-*N′*-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.

Type 1 diabetes is caused by autoimmune destruction of β-cells within the pancreatic islets of Langerhans. β-Cell destruction is thought to be a T-cell–dependent process. Infiltration of pancreatic islets by mononuclear cells of the immune system, mostly macrophages and T-lymphocytes, precedes β-cell destruction in human subjects with type 1 diabetes and in NOD mice (1). Destruction of β-cells appears to result from direct contact with islet-infiltrating cells and exposure to inflammatory cytokines that they produce (2–5).

Islet transplantation has been considered as an alternative to insulin injection therapy for treatment of type 1 diabetes. Two fundamental obstacles to broad-based application of this approach are *1*) limited tissue supply and *2*) protection of transplanted tissue from immune attack. We and others have been investigating the potential utility of engineered cell lines as surrogates for primary islet cells in treatment of type 1 diabetes (6–8). This has involved development of cell lines capable of glucose-stimulated insulin secretion. In addition, we envision that use of engineered cell lines for diabetes therapy may require their encapsulation in membranes or devices (7). Such devices can provide a cell-exclusive barrier to prevent contact of cellular elements of the immune system and the engrafted tissue. However, it is also expected that by allowing efficient exit of insulin, encapsulation devices will allow entry of small molecular weight mediators of inflammation, such as cytokines and toxic radicals. Thus, our group has also begun to focus on the development of cell lines with resistance to cytokine and oxygen radical-induced damage (9).

Recently, we observed that the rat insulinoma cell line INS-1 is more sensitive to destruction by interleukin-1β (IL-1β) than a second insulinoma line, RIN 1046-38, and its derivatives (9). We also found that RIN cells had higher

endogenous levels of manganese superoxide dismutase (MnSOD) than INS-1 cells. Because it had been suggested that MnSOD might be involved in protection against cytokine-induced damage (10), we investigated the effect of stable overexpression of the enzyme in INS-1 cells. We found that MnSOD-overexpressing cells were completely resistant to IL-1β–induced cytotoxicity, and that this protection seemed to be occurring via a blockade in IL-1β activation of inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production (9). However, INS-1 cells are also susceptible to damage by γ-interferon (IFN-γ), and MnSOD overexpression did not provide protection against this cytokine. Further, MnSOD overexpression provided only partial protection against media from activated peripheral blood mononuclear cells (PBMC), which presumably secrete a combination of IFN-γ, IL-1β, and other cytotoxic factors (9).

The goal of the present study was to test a novel strategy for the development of insulinoma cell lines that are resistant to the toxic effects of both IL-1β and IFN-γ, as well as media from activated PBMC. This involved culturing of INS-1 cells in increasing concentrations of IL-1β or IFN-γ for ~8 weeks and collection of surviving cells, ending at concentrations of 10 ng/ml and 100 U/ml, respectively. These studies define a potential path for the development of cytokine-resistant cell lines that may be useful for cell-based therapy of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Cells and reagents. The rat insulinoma cell line INS-1 (11) was cultured in RPMI 1640 medium containing 10% fetal calf serum, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l Na-pyruvate, 50 μmol/l β-mercaptoethanol, 100 U/ml of penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO) at 37°C and 5% CO_2. Recombinant rat IL-1β was obtained from Endogen (Cambridge, MA). Recombinant rat IFN-γ was obtained from Gibco BRL (Gaithersburg, MD). N^G-monomethyl-L-arginine (L-NMMA) (Sigma) was prepared as a 100 mmol/l solution in phosphate-buffered saline (PBS) and diluted to a final concentration of 1 mmol/l in culture medium.

Selection of resistant cell line. The selection process was initiated by incubation of INS-1 cells in 30-mm culture dishes containing culture medium supplemented with 0.5 ng/ml of rat IL-1β and 5 U/ml of rat IFN-γ. The culture medium was changed every 3 days. Surviving cells were trypsinized, harvested, pooled, and recultured at 0.5 ng/ml IL-1β and 5 U/ml rat IFN-γ. After the cells started to grow, the concentration of cytokines was increased to 1 ng/ml of rat IL-1β and 10 U/ml of rat IFN-γ. The process was repeated at increasing concentration of cytokines (2.5, 5, and 10 ng/ml of rat IL-1β and 25, 50, and 100 U/ml of rat IFN-γ) until the concentration of rat IL-1β and rat IFN-γ reached 10 ng/ml and 100 U/ml, respectively. Cells that were recovered after the entire selection protocol were termed INS-1_{res}. To assess the stability of the resistant phenotype, clonal lines were derived by stable transfection of parental INS-1 cells or INS-1_{res} cells with a plasmid containing the cDNA encoding human proinsulin, as described (12).

MTT viability assay. The C,N diphenyl-*N′*-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay has been described previously (9,13,14). Briefly, cells were trypsinized, counted, and inoculated at 80,000 cells/well in flat-bottom 96-well tissue culture plates (Corning Glass Works, Corning, NY). After 24 h in RPMI culture medium lacking cytokines, replacement medium containing various concentrations of cytokines was added as specified in the figure legends. After 48 h, the test media were discarded and replaced by 115 μl/well of medium with 75 μg/ml of MTT for 1.5 h at 37°C and 5% CO_2. The resulting formazan crystals were solubilized in 115 μl 0.04N HCl in isopropanol. The optical density of the solubilized formazan was read at 575 and 650 nm with a SpectraMax 340 (Molecular Devices, Sunnyvale, CA) plate reader. The reduction in optical density caused by cytokine treatment was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

Preparation of activated supernatants from rat PBMC. Heparinized blood was collected from Wistar rats (Charles River Breeding Laboratories, Wilmington, MA), and rat PBMC were isolated over Histopaque 1077 (Sigma) as described (9). Then, 2×10^6 PBMC/ml were activated by incubation in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml streptomycin for 3 days in the presence of 10 μg/ml lipopolysaccharide (LPS) (*E. coli* 0127:B8; Sigma), 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma), or 10 μg/ml of concanavalin A (Con A) (Sigma). Conditioned media derived from these incubations were added to INS-1 cells for cytotoxicity measurements as described (9).

Measurement of nitrite formation. Nitrite formation was measured as an indication of NO production from cells treated with medium containing cytokines or with conditioned media from activated PBMC as described previously (9).

Insulin secretion experiments. Insulin secretion from INS-1 or INS-1_{res} cells and clones derived from these lines was measured in static incubation assays conducted as described previously (15).

Immunoblot analysis. For analysis of iNOS expression, cells were seeded in six-well plates and treated with complete medium with or without IL-1β (10ng/ml) + IFN-γ (100 U/ml) for 48 h. For analysis of p38 MAP kinase expression and activation, cells in six-well plates were washed once with PBS, cultured in serum-free medium overnight, and then treated with serum-free medium or serum-free medium containing IL-1β or IL-1β + IFN-γ for 20 min. After treatment, cells were 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 A, 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 for 20 min. Supernatant fractions were collected and protein concentrations were determined. Samples were suspended in 2 × (for iNOS assays) or 5 × (for p38 MAP kinase) sample buffer (final concentration in both cases, 60 mmol/l Tris, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 60 mmol/l β-mercaptoethanol, and 100 mmol/l dithiothreitol, pH 6.8), boiled for 5 min, and electrophoresed with 10% precast Tris-glycine gels (Bio-Rad Laboratories, Hercules, CA). Protein was transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked with 4% (for iNOS) or 5% (for p38) dry milk in Tris-buffered saline with Tween (10 mmol/l Tris, 150 mmol/l NaCl, and 0.1% Tween 20, pH 8.0). Blots were incubated with anti-iNOS (Transduction Laboratories, Lexington, KY), anti–p38 MAP kinase (total protein) (New England BioLab, Beverly, MA), or anti–phosphorylated p38 MAP kinase (New England BioLab) antibodies, all at 1:1,000 dilution, according to the manufactures' protocols. The protein bands were visualized with horseradish peroxidase–conjugated goat anti–rabbit IgG (Amersham Life Science, Arlington Heights, IL) and enhanced chemiluminescence (Amersham Life Science). Immunoblots were scanned with a UMAX UC840 scanner (Cupertino, CA).

RNA isolation and analysis. Total RNA was isolated from cells by TRIzol (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. We resolved 10 μg of total RNA on a 1.5% formaldehyde/agarose gel, and 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, Woodbridge, NJ). The radiolabeled probes were prepared from the cDNAs encoding the interleukin-1 receptor type I (IL-1RI) or the γ-IFN receptor (γ-IFNR) (kindly provided by Dr. Anice Thigpen, BetaGene, Dallas, TX). *Xba*I fragments of the cDNAs encoding IL-1RI or γ-IFNR were isolated by gel purification using the DNA-purification kit (Qiagen, Valencia, CA), and radiolabeled with ^{32}P-labeled dCTP by random priming with the redi-prime labeling kit (Amersham Life Science). After hybridization and washing, nylon membranes were exposed to films to create autoradiographs. Signals were quantified by Phosphoimager (Molecular Dynamics, Sunnyvale, CA).

NF-kB mobility shift assay. Nuclear extracts of cells were prepared by procedures adapted from those of Dignam et al. (16) and total protein in the extracts was measured by the method of Bradford (17), using a kit from BioRad. Binding reactions (20 μl total) were performed by incubating 4 μg of protein extract with 0.03 pmol (50,000 cpm) of a ^{32}P-labeled double-stranded oligonucleotide containing the NF-kB 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 bovine serum albumin, and 100 μg/ml poly (dI-dC) as nonspecific competitor DNA. Incubations were performed 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, and 2 mmol/l EDTA), dried, and exposed to Kodak film (Eastman-Kodak, Rochester, NY).

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

RESULTS

Selection of an INS-1 cell population resistant to cytokine-mediated cytotoxicity. Our group and others have shown that the inflammatory cytokines IL-1β and IFN-γ induce cytotoxicity in insulinoma cells (2–5,9,10). In the current study, we tested a selection strategy for obtaining INS-1

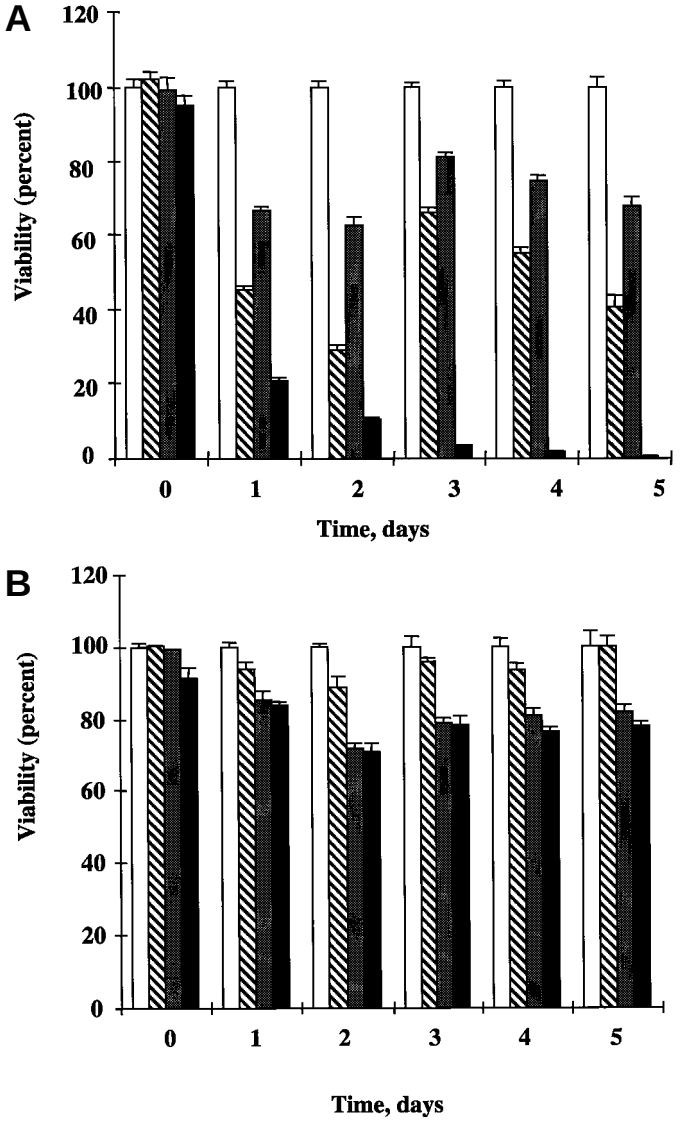

FIG. 1. Viability of parental and selected INS-1 cells after cytokine treatment. INS-1 cells were grown in normal tissue culture medium (*A*) or were selected in increasing concentrations of IL-1β ± IFN-γ (*B*) for a period of 8 weeks. After this procedure, cells were seeded in 96-well dishes and grown overnight in normal medium in the absence of cytokines. They were then cultured for 0–5 days in normal tissue culture medium containing no added cytokines (control), 10 ng/ml of rat IL-1β, 100 U/ml of rat IFN-γ, or both cytokines (as indicated in the figure). After this incubation, cells were subjected to the MTT viability assay, as described in RESEARCH DESIGN ANDMETHODS. Data represent the means ± SE for three independent experiments, each performed in triplicate. □, Medium control; ▨, IL-1β; ▩, IFN-γ; ■, IL-1β + IFN-γ.

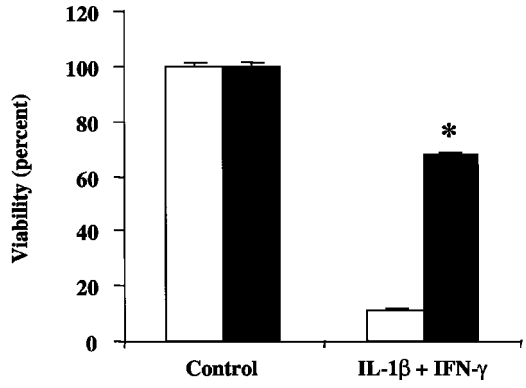

FIG. 2. Parental INS-1 cells do not recover viability upon removal of cytokines. Parental INS-1 cells (□) or INS-1_{res} cells (■) were grown in the absence of cytokines for 96 h (control) or in the presence of 10 ng/ml of rat IL-1β ± 100 U/ml of rat IFN-γ for 48 h, followed by growth in normal medium lacking cytokines for 48 h. Cells were then subjected to the MTT viability assay, as described in RESEARCH DESIGN AND METHODS. Note that parental INS-1 cells did not recover their viability in response to removal of cytokines. Data represent the means ± SE for three independent experiments, each performed in triplicate. *INS-1_{res} cells were more viable than parental INS-1 cells, with $P \leq 0.001$.

cells that are resistant to both of these cytokines. To this end, INS-1 cells were cultured in medium with increasing concentrations of IL-1β + IFN-γ over an 8-week period, beginning at 0.5 ng/ml + 5 IU/ml, and ending at 10 ng/ml + 100 IU/ml, respectively. Cells carried through this entire procedure grew well at the highest cytokine concentrations and were designated INS-1_{res}.

The degree of cytokine resistance achieved by this selection strategy was investigated using an MTT cell viability assay. Parental INS-1 and INS-1_{res} cells were treated with IL-1β (10 ng/ml), IFN-γ (100 U/ml), or IL-1β (10 ng/ml) + IFN-γ (100 IU/ml) after an overnight culture period in the absence of cytokines. In parental INS-1 cells, 1 day of treatment with IL-1β or IFN-γ alone reduced viability to 40 ± 3 and 68 ± 3%, respectively, relative to untreated cells, with similar viabilities at longer time periods of treatment with these cytokines (Fig. 1*A*). The combination of IL-1β (10 ng/ml) + IFN-γ (100 U/ml) was much more potent, causing a sharp drop to 21 ± 0.4% viability after 1 day of exposure, and then a continued decline to near-complete cell destruction (0.3 ± 0.03% viability) after 5 days. In sharp contrast, cell viabilities of INS-1_{res} cells were maintained at 89 ± 1.3, 72 ± 1.1, and 71 ± 2.6% at 2 days, and at 100 ± 1, 82 ± 1, and 78 ± 1% after 5 days of treatment with IL-1β, IFN-γ, or both cytokines, respectively (Fig. 1*B*). These results confirm that INS-1_{res} cells have gained resistance to cytokine-induced cell damage. In the experiments shown in Fig. 1, optical density (MTT staining) increased in both the parental and INS-1_{res} cells as a function of time when cytokines were absent, with a similar plateau being reached at days 4 to 5 in both instances (data not shown). This indicates that the two populations of cells were growing at similar rates under control conditions. Viability (optical density) in the presence of cytokines on a particular day was normalized to the optical density reading for the control cells incubated without cytokines on that same day. Note that this makes the protective effects of the selection process all the more impressive, since almost all parental INS-1 cells are killed after 5 days of cytokine treatment, when the number of cells is highest, while protection is near-complete at this time point in INS-1_{res} cells.

The MTT assay measures the capacity of mitochondria to transport and metabolize the MTT substrate (13,14). We have confirmed this assay as a measure of cell viability in the following three ways: *1*) In a previous publication from our group (9), we showed that cell viability measured with the MTT assay was well correlated with total cell number and viability assayed with an alternative chemical agent, neutral red. *2*) We have evaluated growth of parental and INS-1_{res} cells exposed to IL-1β ± IFN-γ by cell counting. Parental

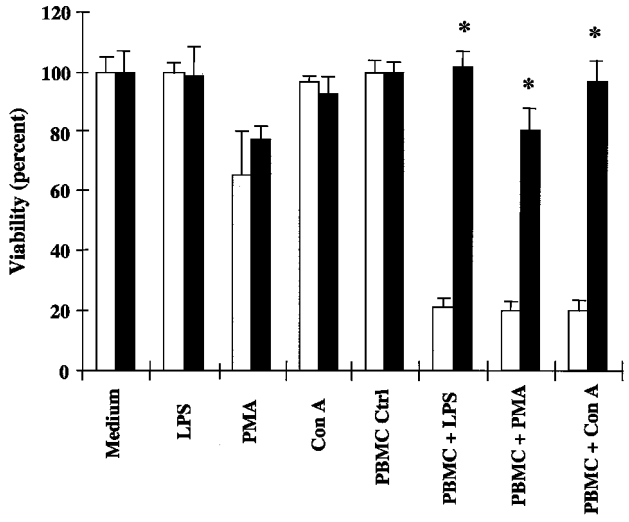

FIG. 3. Viability of parental and selected INS-1 cells after treatment with supernatants from activated PBMC. PBMC were prepared from normal Wistar rats and treated with 10 ng/ml PMA, 10 µg/ml LPS, or 10 µg/ml Con A. The conditioned media from these cells were then collected and added to parental INS-1 cells (□) or INS-1_{res} cells (■) for 48 h. Controls included application of medium alone (medium), medium supplemented with LPS (LPS), medium supplemented with PMA (PMA), medium supplemented with Con A (Con A), or medium from unstimulated rat PBMC (PBMC). The percentage of cells that were viable after these treatments was determined by the MTT assay as described in RESEARCH DESIGN AND METHODS and expressed as a percentage of viable cells after treatment with medium alone. Data represent the means ± SE for three independent experiments, each performed in triplicate. *Conditions for which INS-1_{res} cells are more viable than parental INS-1 cells, with $P < 0.001$.

INS-1 cells do not grow in the presence of the combined cytokines, whereas INS-1_{res} cells grow rapidly (data not shown). *3*) The question raised by the growth curves is whether the cytokines are acting by inhibiting cell growth, as opposed to actual cell killing. To address this, we have treated INS-1 and INS-1_{res} cells with cytokines for 48 h and then removed cytokines for an additional 48 h. As shown in Fig. 2, parental INS-1 cells do not recover their viability when cytokines are removed. These findings confirm that our selection strategy is a method for protecting cells against cytokine-mediated cytotoxicity.

INS-1_{res} cells are resistant to the cytotoxic effects of conditioned medium from activated PBMC. We next determined whether the resistance of INS-1_{res} cells to cytokines can be extended to killing mediated by a complex mixture of cytokines and other toxins. Conditioned media were collected from rat PBMC stimulated by LPS, PMA, or Con A for 72 h, and applied to parental INS-1 or INS-1_{res} cells for 48 h. As shown in Fig. 3, cell viability was improved from 21 ± 3, 20 ± 2, and 20 ± 2% in INS-1 cells to 102 ± 4, 81 ± 7, and 97 ± 7% in INS-1_{res} cells after incubation in media from LPS-, PMA-, or ConA-treated PBMC, respectively. Thus, INS-1_{res} cells gain almost complete resistance to the effects of conditioned medium from activated rat PBMC.

Relative stability of the resistant phenotype in INS-1_{res} cells. The stability of the resistant phenotype in INS-1_{res} cells was investigated by growth of these cells in the absence of cytokines for 6 weeks. After this period, INS-1_{res} cells still retained 113 ± 3, 73 ± 3, and 63 ± 2% viability upon 48 h of exposure to IL-1β, IFN-γ, or IL-1β + IFN-γ, respectively. Similar studies were performed with conditioned media from rat PBMC, revealing viabilities of 103 ± 3, 77 ± 5, or 99 ± 5% for INS-1_{res} cells treated with media prepared from LPS-, PMA-, or Con A-treated PBMC, respectively. These results indicate that resistance to IL-1β–induced killing is a stable feature of INS-1_{res} cells, whereas resistance to cytotoxicity induced by IFN-γ requires continued presence of the cytokine in the tissue culture medium.

iNOS expression and NO production are blocked in cytokine-treated INS-1_{res} cells. IL-1β is known to stimulate NO production in islet β-cells via induction of iNOS, and NO production has been implicated in IL-1β–mediated β-cell destruction (2–5,9,10). We therefore investigated whether this pathway was altered in INS-1_{res} cells. In experiments with parental INS-1 cells, large increases in nitrite production were observed in response to 48 h of culture in IL-1β or IL-1β + IFN-γ, whereas IFN-γ alone had no effect. In contrast, none of these cytokines stimulated NO production in INS-1_{res} cells (Fig. 4*A*). Similarly, NO production was potently stimulated by the addition of conditioned medium from LPS-, PMA-, or Con A-treated PBMC to parental INS-1 cells, but was completely blocked in similarly treated INS-1_{res} cells (Fig. 4*B*). In keeping with these findings, IL-1β + IFN-γ caused a large increase in immunodetectable iNOS in parental INS-1 cells, although this effect was completely blocked in INS-1_{res} cells maintained in

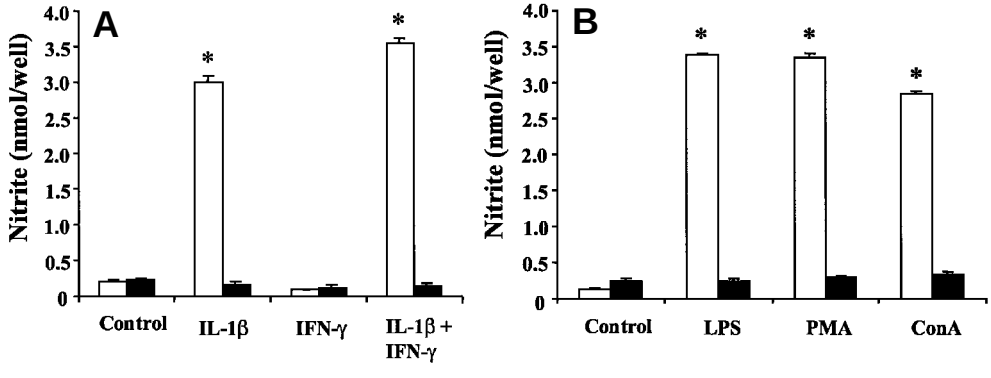

FIG. 4. Nitrite production is blocked in INS-1_{res} cells during stimulation by cytokines or PBMC supernatants. Parental INS-1 cells (□) or INS-1_{res} cells (■) were treated with cytokines (*A*) or media from activated rat PBMC (*B*), as described in the legends of Figs. 1 and 2. After 2 days of treatment with these agents, media were collected and assayed for nitrite accumulation as an index of NO production. Data represent the means ± SE for three independent experiments, each performed in triplicate. *Conditions for which parental INS-1 cells produced more nitrite than INS-1_{res} cells, with $P \leq 0.001$.

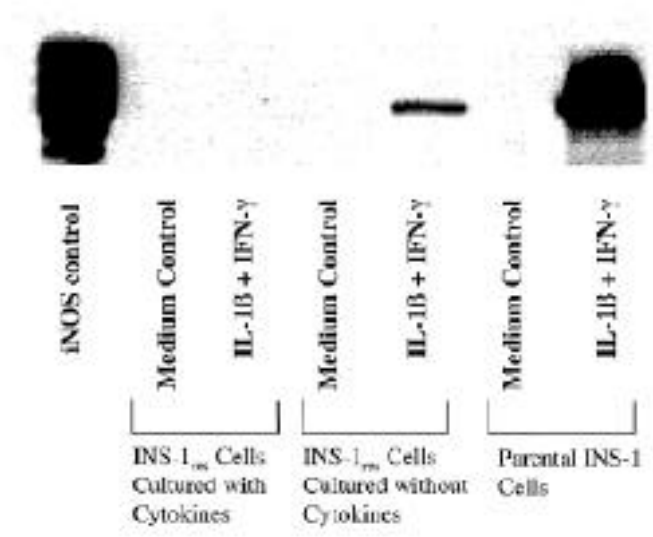

FIG. 5. Cytokine-mediated iNOS expression is impaired in INS-1_{res} cells. Parental INS-1 cells, INS-1_{res} cells grown in tissue culture medium containing IL-1β + IFN-γ, or INS-1_{res} cells grown for 6 weeks in the absence of cytokines were treated for 48 h with 10 ng/ml of rat IL-1β + 100 U/ml of rat IFN-γ. Cell extracts were then subjected to immunoblot analysis, using an antibody recognizing iNOS. Recombinant rat iNOS (iNOS control) was included as a standard. Data are representative of two independent experiments.

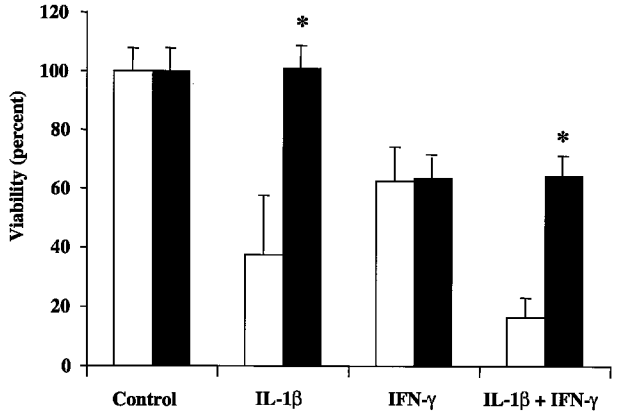

FIG. 6. Effects of individual and combined cytokines on clones derived by stable transfection of parental INS-1 cells or INS-1_{res} cells. Clonal lines derived by transfection as described in the legend of Fig. 6 and in the text were screened for glucose responsiveness. Nine clones derived from INS-1_{res} cells and eight clones from parental INS-1 cells with glucose responses of greater than fivefold were chosen for further study. The glucose-responsive clones derived from parental INS-1 cells (□) or INS-1_{res} cells (■) were grown in the absence of cytokines, and then treated for 48 h in normal medium, or in normal medium containing 10 ng/ml of rat IL-1β, 100 U/ml of rat IFN-γ, or both cytokines. Viability was assessed by the MTT assay, and expressed as percent relative to control cells grown in normal medium. Each clone was assayed in triplicate, and data represent the means ± SE of the eight individual clones derived from parental INS-1 cells and the nine individual clones derived from INS-1_{res}. *Conditions for which clones derived from INS-1_{res} were significantly more viable, with $P < 0.001$.

cytokines, and sharply attenuated in INS-1_{res} cells grown for 6 weeks in the absence of cytokines (Fig. 5).

We do not think that the low level of iNOS expression in INS-1_{res} cells cultured for 6 weeks in the absence of cytokines indicates reversion to an IL-1β–sensitive phenotype, because further culture of these cells in the absence of cytokines for 6 months did not result in restored IL-1β–induced NO production or cytotoxicity (data not shown). These results suggest that INS-1_{res} cells become resistant to the damaging effects of cytokines in part via a block of iNOS expression and NO production. In a previous study, we demonstrated that stable overexpression of MnSOD blocks IL-1β–mediated cytotoxicity and iNOS activation (9), suggesting that upregulation of MnSOD during the selection procedure could have contributed to the block in iNOS. However, immunoblot analysis revealed no differences in MnSOD expression in parental INS-1 and INS-1_{res} cells (data not shown).

Isolation of INS-1_{res} clones by stable transfection with the human insulin gene. We have recently used the technique of stable transfection of the human insulin gene to demonstrate that the INS-1 cells used in our laboratory are comprised of a mixture of glucose-responsive and glucose-unresponsive clones (12). To further investigate the INS-1_{res} cell population, these cells were transfected stably with the human insulin gene, and resultant clones were compared with those isolated after transfection of parental INS-1 cells. There were three reasons for performing this maneuver: *1*) to investigate whether INS-1_{res} cells display the same clonal diversity as the parental population; *2*) to further investigate the stability of the resistance to cytokines in INS-1_{res} cells; and *3*) to increase insulin content in cytokine-resistant cell lines, making these lines potentially more suitable for cell-based insulin replacement in diabetic models.

Transfection of INS-1_{res} or parental INS-1 cells with a plasmid containing the human insulin and neomycin resistance genes resulted in isolation of 51 and 58 individual G418-resistant clones, respectively. Consistent with our previous experiences with parental INS-1 cells (12), a large percentage (~70%) of the INS-1_{res} clones isolated in this manner exhibited poor glucose-stimulated insulin secretion (≤2-fold stimulation at 15 mmol/l glucose compared with 3 mmol/l glucose). A further 15% of the clones showed moderate (two- to threefold) responses to glucose over this concentration range, whereas nine clones exhibited responses of more than fivefold. The average glucose response of the nine strongly responsive clones derived from INS-1_{res} cells was 10.7-fold, similar to the 8.7-fold average response of a comparable group of eight clones derived from parental INS-1 cells.

Stable transfection of INS-1_{res} cells with the human insulin gene and selection of clones was carried out in the absence of cytokines in the growth medium (occupying a period of ~2 months). To investigate whether INS-1_{res} cells retained their resistance to cytokine-mediated killing after this process, the viability of all of the INS-1 and INS-1_{res} clones (58 and 51 clones, respectively) was compared by MTT assay. The average viability of cells treated with the combination of IL-1β and IFN-γ for 48 h was 15 ± 7% for clones derived from parental INS-1 cells, compared with 53 ± 11% for clones derived from INS-1_{res} cells ($P < 0.001$). These results demonstrate that significant cytokine resistance is retained in the INS-1_{res} population even after a round of stable genetic engineering.

INS-1_{res} clones exhibit permanent IL-1β resistance, but reversible IFN-γ resistance. Nevertheless, clones derived from INS-1_{res} cells by stable transfection were on average less resistant to the combination of IL-1β + IFN-γ (53% viability) than the starting INS-1_{res} population (78% viability). To determine whether this is due to loss of resistance to

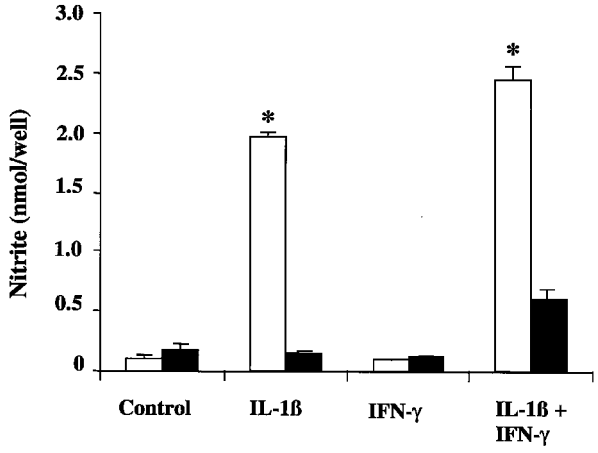

FIG. 7. L-NMMA effects on cytokine-induced nitrite production in a clone derived from parental INS-1 cells. A single clone (834/40) derived by stable transfection of parental INS-1 cells with the human insulin gene was treated for 48 h in normal medium or in normal medium containing 10 ng/ml of rat IL-1β, 100 U/ml of rat IFN-γ, or both cytokines. These incubations were performed in the absence (□) or presence (■) of 1 mmol/l L-NMMA, an inhibitor of iNOS. Data represent the means ± SE for three independent experiments, each performed in triplicate. *Conditions for which parental INS-1 cells produced more nitrite than INS-1_{res} cells, with $P \leq 0.001$.

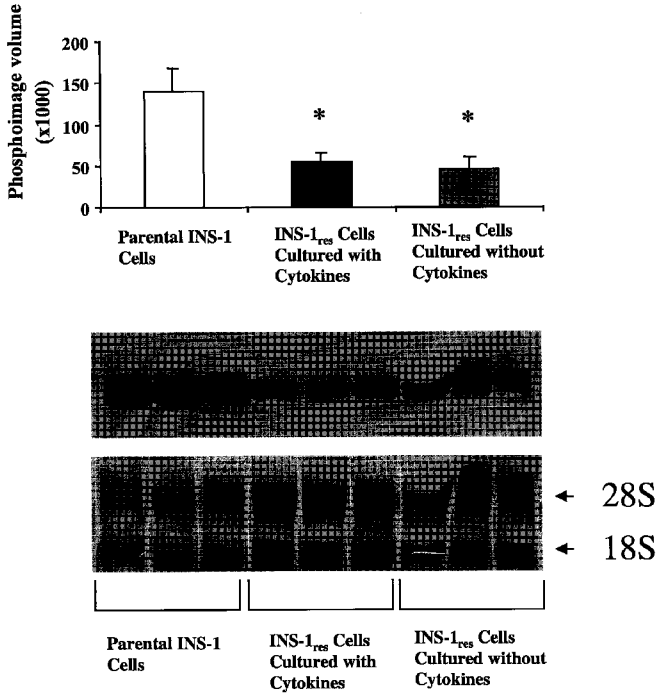

FIG. 8. Levels of IL-1RI mRNA in parental INS-1 cells and INS-1_{res} cells. Parental INS-1 cells, INS-1_{res} cells grown in tissue culture medium containing IL-1β + IFN-γ, or INS-1_{res} cells grown for 4 months in the absence of cytokines were subjected to Northern blot analysis, using the cDNA encoding IL-1RI. The bottom panel is a loading control showing the 28S and 18S ribosomal bands. The middle panel shows the blot hybridized with the IL-1RI probe, and the top panel shows the phosphoimage quantification of the IL-1RI bands. Data in the top panel represent the means ± SE of the representative experiment shown here (total number of experiments for each condition = 3). *INS-1_{res} cells cultured with or without cytokines contain significantly less IL-1RI mRNA, with $P < 0.001$.

one or both cytokines, viability assays were performed on the eight strongly glucose-responsive INS-1 clones and nine glucose-responsive INS-1_{res} clones after treatment with IL-1β, IFN-γ, or the combination of IL-1β + IFN-γ. The nine INS-1_{res} were all 100% resistant to IL-1β–mediated cytotoxicity, but were only 64 ± 6.7 and 64 ± 7.2% viable after treatment with IFN-γ or IFN-γ + IL-1β for 48 h (Fig. 6). Taken together, these data strongly suggest that cytokine-mediated cytotoxicity of the INS-1_{res} clones is attributable to IFN-γ. In contrast to these findings, clones derived from parental INS-1 cells were only 37 ± 20, 63 ± 11, and 16 ± 6.4% viable after treatment with IL-1β, IFN-γ, or IL-1β + IFN-γ, respectively (Fig. 6).

To determine whether resistance to IFN-γ could be reinstated in clones derived from INS-1_{res} cells, all nine lines were recultured in medium containing IL-1β + IFN-γ for 2 weeks. After this period, the transfected INS-1_{res} clones were found to have average viabilities of 95 ± 3.7, 80 ± 4.4, and 81 ± 10% after 48 h of treatment with IL-1β, IFN-γ, or IL-1β + IFN-γ, respectively. This shows that the resistance of INS-1_{res} clones to IFN-γ–mediated damage is reversible and reinducible in response to relatively short periods of culture in the presence and absence of cytokines, whereas resistance to IL-1β is unaffected by these maneuvers.

Selective blockade of IL-1β–mediated cell damage by the iNOS inhibitor L-NMMA. We have previously shown that MnSOD overexpression or application of the iNOS inhibitor L-NMMA is equally effective in preventing IL-1β–mediated killing of INS-1 cells, but these maneuvers fail to inhibit killing by IFN-γ (9). Here we investigated whether the potentiating effect of IFN-γ on IL-1β–mediated cell killing (Fig. 1) could be blocked by the iNOS inhibitor. These studies were carried out on a single clone (834/40) derived by stable transfection of parental INS-1 cells with the human insulin gene. IFN-γ alone did not stimulate NO production from these cells, but IL-1β caused a large increase in NO production that could be completely inhibited by 1 mmol/l L-NMMA (Fig. 7). This effect of the drug corresponded to a near-complete block of IL-1β–mediated cytotoxicity (viability improved from 37 ± 1.2% in the absence of L-NMMA to 86 ± 1.8% in its presence). In contrast, L-NMMA inhibited NO production in response to IL-1β + IFN-γ by 75%, corresponding to an increase in viability from 15 ± 0.9 to 38 ± 2.0%. Thus, the additive cytotoxic effects of IL-1β + IFN-γ can only be partially blocked by 1 mmol/l L-NMMA. This result shows that killing induced by IL-1β is largely via NO production, whereas the potentiating effect of IFN-γ occurs by a distinct mechanism.

Expression of IL-1 type I and IFN-γ receptors in INS-1_{res} cells. To begin to investigate the mechanism of cytokine resistance in INS-1_{res} cells, the levels of mRNA encoding the IL-1RI and the γ-IFNR were compared in parental INS-1 cells, INS-1_{res} cells kept in normal culture medium, and INS-1_{res} cells kept in culture medium containing IL-1β (10 ng/ml) + IFN-γ (100 U/ml). As shown in Fig. 8, INS-1_{res} cells exhibited a 60% decrease in IL-1RI mRNA levels compared with parental INS-1 cells. This reduction was stable after the withdrawal of the cytokines from the culture medium for 4 months. In contrast, levels of γ-IFNR mRNA were found to be unchanged among the three groups of cells (Fig. 9).

NF-kB and p38 MAP kinase signal transduction in INS-1_{res} cells. To investigate whether the decline in expression of IL-1RI was linked to reduced signal transduction in

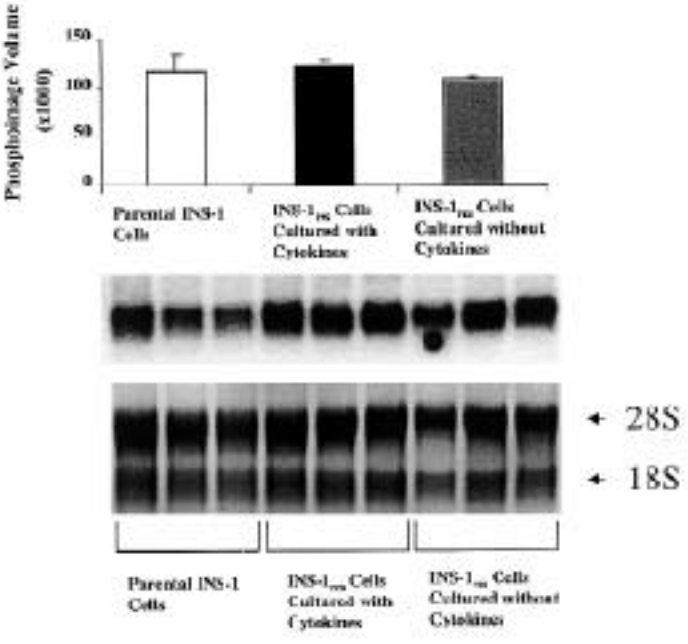

FIG. 9. Levels of γ-IFNR mRNA in parental INS-1 cells and INS-1_{res} cells. Parental INS-1 cells, INS-1_{res} cells grown in tissue culture medium containing IL-1β + IFN-γ, or INS-1_{res} cells grown for 4 months in the absence of cytokines were subjected to Northern blot analysis, using the cDNA encoding the γ-IFNR. The bottom panel is a loading control showing the 28S and 18S ribosomal bands. The middle panel shows the blot hybridized with the γ-IFNR probe, and the top panel shows the phosphoimage quantification of the γ-IFNR receptor bands. Data in the top panel represent the means ± SE of the representative experiment shown (total number of experiments for each condition = 3).

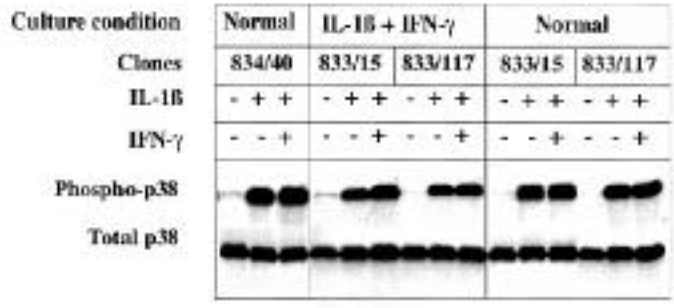

FIG. 10. Normal activation of p38 MAP kinase in clones derived from INS-1_{res} cells. A glucose-responsive clone derived by stable transfection of parental INS-1 cells (834/40) and two glucose-responsive clones derived by stable transfection of INS-1_{res} cells (833/15 and 833/117) were studied. The clones derived by stable transfection of INS-1_{res} cells were grown for 6 months in the absence or presence of 10 ng/ml of rat IL-1β + 100 U/ml of rat IFN-γ. After this growth period, cells were treated in serum-free medium overnight, and then treated with fresh serum-free medium lacking cytokines or containing 10 ng/ml of rat IL-1β or 10 ng/ml IL-1β + 100 U/ml of rat IFN-γ for 20 min and harvested for immunblot analysis with an antibody recognizing total p38 MAP kinase (total p38) or phosphorylated (activated) p38 MAP kinase (phospho-p38). Data are representative of two independent experiments.

INS-1_{res} cells, p38 MAP kinase and NF-kB activation were measured in clonal INS-1 cell lines derived from parental cells (line 834/40) or INS-1_{res} cells (lines 833/117 and 833/15). As shown in Fig. 10, p38 levels were identical in all of the cell lines. In addition, activation of p38 by short-term addition of IL-1β or IL-1β + IFN-γ was completely normal in INS-1_{res}–derived cells, even when such cells were grown in high concentrations of cytokines for 6 months. These results show that the reduced expression of IL-1RI documented in INS-1_{res} cells in Fig. 8 does not impede IL-1β signaling through p38. IL-1β is also known to cause translocation of the transcription factor NF-kB. In contrast to our findings with p38, NF-kB translocation was strongly impaired in clones derived from INS-1_{res} cells relative to the control cells derived from parental INS-1 cells (Fig. 11). The reduction in IL-1β–induced NF-kB activation observed in the resistant lines was stable, i.e., was not affected by the long-term presence or absence of high concentrations of cytokines. Similar results were shown earlier for iNOS induction (Fig. 5). Thus, NF-kB translocation and iNOS expression are well correlated with the degree of sensitivity of the clones to cytokine killing, suggesting that downregulation of this arm of IL-1β signaling is the mechanism by which IL-1β–resistant clones have been selected.

DISCUSSION

In this study, we have described a method for selecting insulinoma cell lines that are resistant to cytokine-mediated cell killing. We show that addition of increasing concentrations of IL-1β and IFN-γ to the tissue culture medium selects for INS-1 cells that are resistant to the cytotoxic effects of these cytokines. The selected cells are also resistant to the toxic effects of medium from activated PBMC. Resistance to IL-1β is a highly stable feature of the selected cells, whereas IFN-γ resistance appears to be transient and reinducible.

The stable resistance to IL-1β appears to be caused by perturbation of a signaling pathway that links the IL-1RI to iNOS, via the transcription factor NF-kB. Thus, long-term culture of INS-1 cells in the presence of high levels of IL-1β + IFN-γ results in a sharp reduction in acute IL-1β–mediated activation of NF-kB and iNOS expression. This inability to normally activate NF-kB and iNOS is stably maintained in INS-1_{res} cells that are cultured in the absence of cytokines for up to 6 months, and such cells also retain complete resistance to IL-1β–induced, but not IFN-γ–induced, cell killing. Similarly, stable transfection of INS-1_{res} cells with the human insulin gene, and isolation and expansion of clonal lines in the absence of cytokines in the tissue culture medium results in isolation of clones that are completely resistant to IL-1β but that have lost much of their resistance to IFN-γ. That the killing mediated by IL-1β is linked to iNOS production is further supported by our studies with the iNOS inhibitor L-NMMA. Killing of unselected INS-1 cells by IL-1β alone is almost completely prevented by L-NMMA treatment, while the drug is only partially effective at reversing the cytotoxic effects of combined addition of IL-1β + IFN-γ and is without effect when IFN-γ alone is added. Thus, long-term culture of INS-1 cells with IL-1β + IFN-γ leads to stable disruption of an as-yet-unidentified component of the signaling pathway that allows IL-1β to activate NF-kB and iNOS expression.

Interestingly, the selection procedure used in this study results in a stable decrease in expression of the IL-1RI mRNA. Since the receptor mRNA levels remain low even after removal of cytokines from the tissue culture medium, this could represent one explanation for the resistant phenotype. We think that this is unlikely, however, for two reasons: First, expression of IL-1RI is very low in many cell

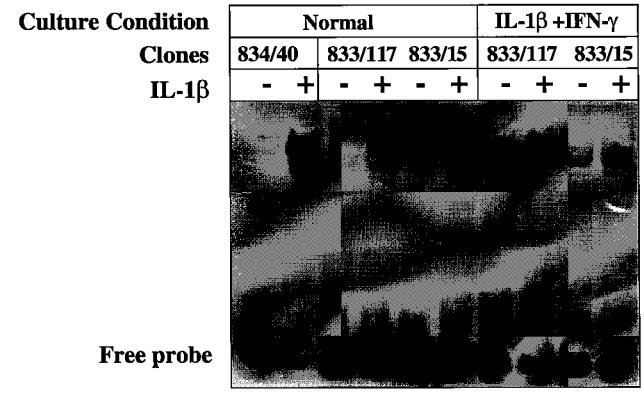

FIG. 11. Activation of NF-kB is impaired in clones derived from INS-1_{res} cells. A glucose-responsive clone derived by stable transfection of parental INS-1 cells (834/40) and two glucose-responsive clones derived by stable transfection of INS-1_{res} cells (833/15 and 833/117) were studied. The clones derived by stable transfection of INS-1_{res} cells were grown for 6 months in the absence or presence of 10 ng/ml of rat IL-1β + 100 U/ml of rat IFN-γ. After this period, cells were treated with 10 ng/ml of rat IL-1β or were left untreated for 1 h. Cells were harvested and nuclei isolated for electrophoretic mobility shift assay analysis with an oligonucleotide probe containing the consensus binding sequence for NF-kB, as described in RESEARCH DESIGN AND METHODS. Data are representative of two independent experiments.

types that are fully responsive to IL-1β, with some cells containing as few as 10 copies of the receptor (18). Despite the fact that IL-1RI mRNA levels are reduced in INS-1_{res} cells compared with the parental INS-1 cells, the receptor still appears to be expressed at substantial levels and is easily detected. Second, signaling through another IL-1RI–mediated pathway is undiminished in INS-1_{res} cells. IL-1β is known to cause activation of a wide variety of protein kinases, including p38 MAP kinase (18,19). We show that INS-1_{res} cells contain normal levels of p38, and that the protein is phosphorylated normally in response to acute administration of IL-1β, regardless of whether the cells were grown in the absence or presence of cytokines in their tissue culture medium. The specific point at which the NF-kB/iNOS signaling pathway has been interrupted in INS-1_{res} cells remains to be defined, but it appears that it will be downstream of events common to the p38 MAP kinase activation pathway. Activation of NF-kB by IL-1β and IL-1RI is complex and involves signaling between a set of proteins that interact with the IL-1RI, including an accessory protein (IL-1AcP), IRAK, and TRAF6, and a complex of proteins involved in NF-kB activation that includes NIK, IKK1, and IKK2 (18,19). Interestingly, in a previous study, we showed that stable overexpression of MnSOD in INS-1 cells also leads to complete blockade of IL-1β–mediated cell killing, with an attendant block of IL-1β–mediated induction of iNOS and NO production (9). However, this effect of MnSOD does not appear to be mediated by inhibition of NF-kB activation (20), suggesting that intervention in IL-1β/iNOS signaling pathway can be achieved at more than one point. The precise molecular mechanisms involved in achieving stable resistance to IL-1β in MnSOD-expressing cells and INS-1_{res} cells are the subject of ongoing investigation.

In contrast to our findings of stable resistance to IL-1β in INS-1_{res} cells and their clonal derivatives, IFN-γ resistance appears to require the continued presence of cytokines in the culture medium. Moreover, once lost, IFN-γ resistance can be regained by simple reculturing of cells in medium containing cytokines. IFN-γ signals through a pathway that is distinct from that used by IL-1β, involving binding to its heterodimeric receptor, association of Janus kinases, and subsequent phosphorylation, dimerization, and translocation of the transcription factor STAT-1 (21,22). The transient nature of IFN-γ resistance cannot be explained by changes in expression of γ-IFNR mRNA, since levels were unchanged in INS-1_{res} cells grown in the presence or absence of cytokines relative to parental INS-1 cells. Thus, our findings suggest that long-term exposure of cells to IL-1β and IFN-γ induces or reduces the expression of a gene or genes that control resistance to the cytoxic effects of IFN-γ and that such effects are reversed by removal of the cytokines from the tissue culture medium. The fact that IFN-γ resistance can be so readily manipulated by the alteration of culture conditions suggests that relevant genes can be identified in the future by subtractive hybridization, differential display cloning, or related strategies. Assuming that a specific gene or genes sufficient for conferring IFN-γ resistance can be identified, such reagents could provide a means for installing permanent resistance to the cytotoxic effects of this cytokine in insulinoma cells.

In autoimmune diabetes, β-cell destruction occurs as a consequence of infiltration of mononuclear cells into pancreatic islets. Each of the inflammatory cytokines tumor necrosis factor-α, IFN-γ, and IL-1β are produced by infiltrating cells and have been implicated as synergistic mediators of β-cell killing (2–5). The studies described here can be viewed as a general strategy for defining mechanisms of resistance to cytokine-induced cytotoxicity. The long-term goal of our research is to treat diabetes by transplantation of engineered cell lines. The three key features of our cell transplant approach will be as follows: *1*) genetically engineered cells that secrete insulin in response to glucose and its potentiators; *2*) a retrievable microporous device that will prevent contact of cellular elements of the immune system (e.g., T-cells) with the engrafted tissue; and *3*) genetic engineering of cell lines such that they are able to withstand attack by smaller elements of the immune system that can pass through the microporous membrane of the device (7). The current study represents a step toward achieving the last of the three goals. Importantly, the selection strategy described in this study does not impair the robust glucose-stimulated insulin secretion found in subcloned cell lines derived from INS-1 cells. Further studies will be required to determine if the stable IL-1β resistance observed in INS-1_{res} cells in vitro is maintained in cells that are transplanted into immunocompetent animals within encapsulation devices, and if so, whether this will result in a measurable increase in the duration of cell survival. Further work will also be required to define the genes involved in the transient IFN-γ resistance, and to develop cells in which the expression of such genes is stably altered to create a permanently resistant line.

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