Interferon-γ Increases the Sensitivity of Islets of Langerhans for Inducible Nitric-oxide Synthase Expression Induced by Interleukin 1∗

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The purpose of this study was to evaluate the effects of interferon-γ (IFN-γ) alone and in combination with interleukin 1β (IL-1β) on inducible nitric-oxide synthase (iNOS) mRNA and protein expression, nitrite production, and insulin secretion by islets of Langerhans. Treatment of rat islets with IL-1β results in a concentration-dependent increase in the production of nitrite that is maximal at 5 units/ml. Individually, 0.1 unit/ml IL-1β or 150 units/ml rat IFN-γ do not stimulate iNOS expression or nitrite production by rat islets; however, in combination, these cytokines induce the expression of iNOS and the production of nitrite to levels similar in magnitude to the individual effects of 5 units/ml IL-1β. The islet β-cell, selectively destroyed during insulin-dependent diabetes mellitus, appears to be one islet cellular source of iNOS as 150 units/ml rat IFN-γ and 0.1 unit/ml IL-1β induced similar effects in primary β-cells purified by fluorescence-activated cell sorting and in the rat insulinoma cell line, RINm5F. iNOS expression and nitrite production by rat islets in response to 150 units/ml rat IFN-γ and 0.1 unit/ml IL-1β are correlated with an inhibition of insulin secretion and islet degeneration that are prevented by the iNOS inhibitor aminoguanidine. The mechanism by which IFN-γ increases the sensitivity of β-cells for IL-1-induced iNOS expression appears to be associated with an increase in the stability of iNOS mRNA. Last, cellular damage during physical dispersion of islets results in the release of sufficient amounts of IL-1β to induce iNOS expression and nitrite production in the presence of exogenously added rat IFN-γ. The cellular source of IL-1β under these conditions is believed to be resident islet macrophages as depletion of macrophages prior to dispersion prevents IFN-γ-induced iNOS expression and nitrite formation by dispersed islet cells. These studies show that the T-lymphocyte cytokine, IFN-γ, increases the sensitivity of rat islets to the effects of IL-1β on iNOS expression and nitrite production by 10-fold, in part, through the stabilization of iNOS mRNA. Our studies also support an effector role for IFN-γ, in concert with resident islet macrophage release of IL-1β, in mediating β-cell destruction during the development of autoimmune diabetes.

Insulin-dependent diabetes mellitus is an autoimmune disease characterized by the selective destruction of insulin-secreting β-cells found in islets of Langerhans. Many lines of evidence support a role for the involvement of cytokines as effector molecules that participate in the development of diabetes. Mandrup-Poulsen et al. (1) first showed that treatment of isolated rat islets with conditioned media derived from activated mononuclear cells results in a potent inhibition of insulin secretion followed by islet destruction. The active component of this conditioned media was determined to be the cytokine IL-1β (2). IL-1-induced inhibition of insulin secretion is both time- and concentration-dependent and requires mRNA transcription and new protein synthesis (3). Recently, IL-1-induced inhibition of insulin secretion has been attributed to the expression of iNOS and increased production of nitric oxide by β-cells (4, 5).

Southern et al. (6) first demonstrated that treatment of rat islets with IL-1β and tumor necrosis factor results in an inhibition of insulin secretion that is attenuated by the nitric-oxide synthase inhibitor nitro-l-arginine methyl ester. We and others (7–9) have shown that IL-1β-induced inhibition of insulin secretion and IL-1β-induced nitrite production by rat islets are completely prevented by Nω-nitro-l-arginine (NMMA) and aminoguanidine (AG). The expression of iNOS by rat islets has been demonstrated at the level of mRNA and protein (10–12). Immunohistochemical colocalization of iNOS and insulin demonstrates that IL-1β selectively induces the expression of iNOS by β-cells (12). The inhibitory and destructive effects of IL-1β on islet function and viability are mediated, in part, by the ability of nitric oxide to target and inhibit the enzymatic activity of mitochondrial enzymes, including aconitase and the electron transport chain complexes I and II (7, 13, 14). Treatment of rat islets for 18 h with IL-1β results in an 80% inhibition of aconitase activity that is prevented by NMMA (7, 13). IL-1β has also been shown to reduce islet cellular levels of ATP and to inhibit glucose oxidation in a nitric oxide-dependent manner (15).

Autoimmune diabetes is associated with a local inflammatory reaction (insulitis) in and around pancreatic islets. In an activated state, T-lymphocytes and macrophages, primary cellular components of islet insulitis, release high levels of IL-1 and IFN-γ, respectively. Although the effects of IL-1 on islet function have been examined in detail, few studies have investigated the effects of IFN-γ on β-cell function and viability. Many lines of evidence support a role for IFN-γ in the devel-

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The abbreviations used are: IL-1, interleukin 1β; IFN-γ, interferon-γ; NMMA, Nω-nitro-l-arginine; AG, aminoguanidine; iNOS, inducible nitric-oxide synthase; IRAP, interleukin 1 receptor antagonist protein; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline.

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opment of autoimmune diabetes. This evidence includes 1) IFN-γ mRNA expression in islets correlates with the development of insulitis and diabetes in the nonobese diabetic mouse (16); 2) transgenic mice expressing IFN-γ under control of the insulin promoter develop insulitis and diabetes (17); and 3) monoclonal antiserum specific for IFN-γ attenuates the development of diabetes in the nonobese diabetic mouse (18, 19). In this study we have examined the effects of IFN-γ alone and in combination with IL-1β on iNOS expression, nitrite formation, and islet function and viability. Alone, IFN-γ does not modulate islet function or viability; however, IFN-γ increases the sensitivity of rat islets to IL-1β by stabilizing IL-1-induced iNOS mRNA expression resulting in the increased production of nitric oxide. The increased sensitivity of rat islets for IL-1 results in the inhibition of β-cell function and islet destruction at concentrations of IL-1β that alone have no effect on islet viability or function.

EXPERIMENTAL PROCEDURES

Materials and Animals—RINm5F cells were obtained from Washington University Tissue Culture Support Center (St. Louis, MO). RPMI Medium 1640 containing 1× glutamine, CMRL-1066, or RPMI Medium 1640 containing 2 mM glutamine, CMRL-1066, was obtained from BioWhittaker (Walkersville, MD), GIBCO BRL (Gaithersburg, MD), or Life Technologies, Inc. (Gaithersburg, MD). Fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin were gifts from Life Technologies, Inc. Aminoguanidine hemisulfate (AG) and collagenase type XI were from Sigma. [3H]-32P[dCTP and enhanced chemiluminescence reagents were purchased from Amerham Corp. Human recombinant IL-1β was from Cistron Biotechnology (Pine Brook, NJ). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rabbit antiserum specific for the C-terminal 27 amino acids of mouse macrophage iNOS was a gift from Dr. Thomas Misko (G. D. Searle, St. Louis, MO). iNOS and cyclophilin cDNAs were gifts from Dr. Charles Rodi (Monsanto Corporate Research, St. Louis, MO) and Dr. Steve Carroll (Department of Pathology, Washington University, St. Louis, MO), respectively. Mouse recombinant interleukin 1 receptor antagonist protein (IRAP) was a gift from Dr. Charles Hall (Cytogen, Kalamazoo, MI). All other reagents were from commercially available sources.

Islet Isolation and Culture—Islets were isolated from male Sprague Dawley rats by collagenase digestion as described previously (20). Following isolation, islets were cultured overnight in complete CMRL-1066 (CMRL-1066 containing 2 mM l-glutamine, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) under an atmosphere of 95% air and 5% CO2 at 37 °C. Islets were isolated by centrifugation (6,000 g, 30 s) and then resuspended to a final concentration of 150–400 islets/ml of complete CMRL-1066 in 96-well microtiter plates with the indicated concentrations of IL-1β and IFN-γ for 24 h at 37 °C. Islets were then incubated at 37 °C for 30 min, the incubation buffer was removed, and glucose-stimulated insulin secretion was initiated by the addition of 200 μl of KRB containing either 3 or 20 mM d-glucose. Islets were then incubated at 37 °C for 30 min, the incubation buffer was removed, and insulin content was determined by radioimmunossay (23).

Islet Viability—Islets (25/500 μl of complete CMRL-1066) were cultured for 96 h in 24-well microtiter plates with the indicated concentrations of IL-1β, IFN-γ, and aminoguanidine (AG). Islet degeneration was determined in a double-blind manner by phase-contrast microscopic analysis. Islet degeneration is characterized by the loss of islet integrity, disintegration, and partial dispersal of islets as described previously (14, 24, 25).

Western Blot Analysis—RINm5F cells (400,000/400 μl of complete CMRL-1066) were cultured in 24-well microtiter plates with the indicated concentrations of IL-1β and IFN-γ for 24 h at 37 °C, washed 3 times with 0.1× phosphate-buffered saline (PBS), pH 7.4, followed by the addition of 25 μl of sodium dodecyl sulfate (SDS) sample mix (0.25% Triton-HCl, 20% β-mercaptoethanol, and 4% SDS). The lysed cells were then transferred to 1.5-ml microcentrifuge tubes, and the individual wells of the microtiter plate were rinsed with 15 μl of distilled H2O which was then added to the corresponding lysed samples. The samples were boiled for 4 min followed by the addition of 4 μl of loading dye (0.05% bromphenol blue in 80% glycerol). Rat islets (150/400 μl of complete CMRL-1066) were cultured for 40 h with the indicated concentrations of IL-1β and IFN-γ at 37 °C under an atmosphere of 95% air and 5% CO2. The islets were isolated by centrifugation (6,000 g, 3 min) and washed 3 times with 0.1× PBS. Islets were lysed by the addition of 25 μl of SDS sample mix and 15 μl of distilled H2O, boiled for 4 min, followed by the addition of 4 μl loading dye. Proteins were separated by SDS-gel electrophoresis using standard conditions (26) and transferred to polyvinylidene fluoride membranes (Pharmacia Biotech Inc.) under semi-dry transfer conditions. Blots were blocked overnight in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% nonfat dry milk. Blots were washed one time with TBST and then incubated for 1.5 h at room temperature with rabbit anti-iNOS (1:2000 dilution) in TBST containing 1% nonfat dry milk. Following incubations with the primary antiserum, blots were washed 4 times with TBST and then incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody at a dilution of 1:7000. The blots were washed 3 times in TBST and once in 0.1× PBS at room temperature. Detection of rat iNOS was by enhanced chemiluminescence according to manufacturer’s specifications (Amersham Corp.).

Northern Blot Analysis—RINm5F cells (10 × 106 cells/3 ml of complete CMRL-1066) were cultured for 6 and 12 h at 37 °C with the indicated concentrations of IL-1β and IFN-γ. For the mRNA stability experiments, RINm5F cells (10 × 106 cells/3 ml of complete CMRL-1066) were cultured for 6 h in the presence of the indicated concentrations of IL-1 and IFN-γ. Actinomycin D (1 μM) was then added, and the cells were cultured for an additional 6 h. After culture, the cells were washed 3 times with 0.1× PBS, pH 7.4, and total RNA was isolated using the RNAsesy kit (Qiagen, Inc., Chatsworth, CA). Total cellular RNA (10–20 μg) was denatured and fractionated by gel electrophoresis using a 1.0% agarose gel containing 2.2 mM formaldehyde. RNA was transferred by capillary action in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) to Duralon UV nylon membranes (Stratagene, La Jolla, CA), and the membranes were hybridized overnight with 32P-labeled probe specific for iNOS or cyclophilin (27). The cDNA probe was radiolabeled with [3H]-32P[dCTP by random priming using the Prime-a-Gene nick translation system from Promega (Madison, WI). iNOS cDNA probe corresponds to bases 509–1415 of the rat iNOS coding region. 28 S RNA band or cyclophilin was used as an internal control for RNA loading. Hybridization and autoradiography were performed as described previously (28).

Densityometry and Image Analysis—Autoradiograms were scanned
**IFN-γ and IL-1β Stimulate iNOS Expression by β-Cells**

For insulin secretion experiments, rat islets (220 islets/mL complete CMRL-1066) were incubated for 40 h with the indicated concentrations of IL-1β, rat IFN-γ, and AG. Following this incubation period glucose-induced insulin secretion was examined as stated under “Experimental Procedures.” Results are the average ± S.E. of two individual experiments containing three replicates per condition. For islet degeneration experiments, rat islets (25 islets/500 μL complete CMRL-1066) were incubated with the indicated concentrations of IL-1β, rat IFN-γ, and AG for 96 h. Islet degeneration was assessed by phase-contrast microscopy in a double-blind manner. Results are average ± S.E. of four individual experiments. ND, not determined.

### Table I

| Cytokine | AG | Insulin secretion (ng/30 min per 20 islets), Glucose | Percent viable |
|----------|----|----------------------------------------------------|----------------|
| IL-1β    | IFN-γ | 3 min | 20 min | |
| units/mL | µu | | | |
| 0       | 0 | 0.4 ± 0.1 | 11.8 ± 1.3 | 98.4 ± 0.5 |
| 0.1     | 0 | 0.9 ± 0.3 | 13.8 ± 1.2 | 100.0 ± 0.0 |
| 1.0     | 0 | 2.4 ± 0.4 | 7.7 ± 3.2<sup>a</sup> |
| 0.1     | 150 | 1.4 ± 0.5 | 2.2 ± 0.2 | 7.9 ± 4.4<sup>a</sup> |
| 0.1     | 150 | 1.5 ± 0.5 | 8.3 ± 1.6 | 99.0 ± 1.0 |
| 0       | 150 | 0.7 ± 0.2 | 8.4 ± 0.9 | 98.9 ± 0.6 |
| 1.0     | 0 | 1.0 | ND | ND |

*<sup>a</sup>,<sup>b</sup> p < 0.05.

### RESULTS

**Rat IFN-γ Reduces the Concentration of IL-1β Required to Stimulate Rat Islet iNOS Expression by 10-Fold**—To determine if IFN-γ modulates iNOS expression, the effects of rat IFN-γ, alone and in combination with IL-1β, on nitrite production and iNOS expression by isolated rat islets were examined. Incubation of rat islets for 40 h with IL-1β results in a concentration-dependent increase in the production of nitrite (Fig. 1A). IL-1β induces the first detectable increase in nitrite production by rat islets at 0.5 units/mL IL-1β with maximal nitrite production observed at 1 and 5 units/mL IL-1β (data not shown for 1 unit/mL IL-1β). Alone, rat IFN-γ (concentrations from 1.5 to 150 units/mL) does not stimulate nitrite formation by rat islets; however, in the presence of 0.1 unit/mL IL-1β (which alone does not stimulate nitrite formation), rat IFN-γ induces the concentration-dependent production of nitrite by rat islets. The levels of nitrite produced in response to 0.1 unit/mL IL-1β and 150 units/mL rat IFN-γ are similar in magnitude to the levels produced by rat islets treated with 5 units/mL IL-1β. Rat IFN-γ also slightly increases (~8%) the production of nitrite by rat islets stimulated with 5 units/mL IL-1β as compared with 5 units/mL IL-1β alone.

The effects of rat IFN-γ and IL-1β on iNOS expression from the same islets used in Fig. 1A are shown in Fig. 1B. Alone, 0.1 unit/mL IL-1β or 150 units/mL rat IFN-γ do not induce the expression of iNOS by rat islets; however, a combination of 0.1 unit/mL IL-1β and 150 units/mL rat IFN-γ stimulates the expression of iNOS to levels similar in magnitude to the expression of iNOS induced by 5 units/mL IL-1β. Also, the combination of 5 units/mL IL-1β and 150 units/mL rat IFN-γ induce iNOS expression to levels that exceed those induced by the treatment of rat islets with 5 units/mL IL-1β. This effect is consistent with the ability of rat IFN-γ to increase the level of nitrite produced by rat islets in response to maximal concentrations of IL-1β.

These results indicate that rat IFN-γ, in combination with IL-1β at concentrations that alone do not induce iNOS expression, stimulate the expression of iNOS by rat islets to levels that are similar to the individual effects of maximal concentrations of IL-1β. For convenience, we have defined 0.1 unit/mL IL-1β as submaximal and 1 and 5 units/mL IL-1β as maximal concentrations of IL-1β.

**The Combination of Rat IFN-γ and Submaximal Concentrations of IL-1β Inhibit Glucose-stimulated Insulin Secretion and Induce Islet Degeneration in a Nitric Oxide-dependent Manner**—Our previous studies have shown that nitric oxide mediates the inhibitory effects of IL-1β on glucose-stimulated insulin secretion (8). The effects of rat IFN-γ and IL-1β on insulin secretion by rat islets were examined to determine if nitric oxide production, stimulated by submaximal concentrations of IL-1β in the presence of rat IFN-γ, is associated with an inhibition of insulin secretion. Treatment of rat islets with the maximal concentration of IL-1β results in a complete inhibition of glucose-stimulated insulin secretion (Table I). We have previously shown that IL-1β-induced inhibition of insulin secretion is prevented by the NO inhibitors NNMMA and AG and that NO inhibitors do not modulate glucose-stimulated insulin secretion in the absence of cytokines (8, 9). Incubation of islets for 40 h with a combination of rat IFN-γ and a submaximal...
concentration of IL-1β also results in a complete inhibition of insulin secretion that is prevented by AG (Table I). Individually, submaximal IL-1β or rat IFN-γ do not inhibit glucose-stimulated insulin secretion. The lack of an inhibitory effect of submaximal IL-1β or rat IFN-γ on insulin secretion is consistent with the inability of these cytokines to stimulate nitrite production by rat islets. These findings indicate that treatment of rat islets with a submaximal concentration of IL-1β, in the presence of rat IFN-γ, results in an inhibition of insulin secretion that is mediated by the production of nitric oxide.

The effects of rat IFN-γ, alone and in combination with IL-1β, on islet viability are also shown in Table I. Incubation of islets for 96 h with a maximal concentration of IL-1β results in the complete degeneration of islets. Islet degeneration is characterized by loss of islet integrity and islet dispersion (14, 24, 25). The destructive effects of IL-1β on islet viability are completely prevented by the iNOS inhibitor aminoguanidine (AG), indicating that nitric oxide participates in IL-1β-induced islet degeneration. Alone, rat IFN-γ (150 units/ml) or a submaximal concentration of IL-1β do not induce islet degeneration; however, in combination these cytokines stimulate islet degeneration to levels similar to the individual effects of maximal concentrations of IL-1β alone. The destructive effects of submaximal concentrations of IL-1β, in combination with rat IFN-γ, are completely prevented by AG. These findings indicate that islet degeneration stimulated by rat IFN-γ and submaximal concentrations of IL-1β is mediated by the production of nitric oxide.

**Effects of Rat IFN-γ and IL-1β on iNOS Expression and Nitrite Formation by FACS Purified α- and β-Cells—Islets contain a heterogeneous population of both endocrine and non-endocrine cells, of which the insulin-secreting β-cell is selectively destroyed during the development of autoimmune diabetes. To determine if rat IFN-γ increases the sensitivity of β-cells for IL-1β-induced iNOS expression, we have examined the effects of rat IFN-γ alone, and in combination with IL-1β, on nitrite production and iNOS expression by primary rat α- and β-cells purified by FACS. As shown in Fig. 2, treatment of primary β-cells with submaximal IL-1β in the presence of rat IFN-γ stimulates the production of nitrite to levels similar in magnitude to the effects of maximal concentrations of IL-1β alone. Also, nitrite production by primary β-cells incubated with maximal concentrations of IL-1β, or the combination of maximal IL-1β and rat IFN-γ, are virtually identical.

The effects of rat IFN-γ and IL-1β on iNOS expression correlate with the effects of these cytokines on nitrite production by primary β-cells. As shown in Fig. 2B, submaximal concentrations of IL-1β or rat IFN-γ do not stimulate the expression of iNOS by primary β-cells. However, in combination, these cytokines stimulate the expression of iNOS to levels that are slightly higher than the effects of maximal concentrations of IL-1β on iNOS expression by primary β-cells. Also shown in Fig. 2 are the effects of IL-1β and rat IFN-γ on nitrite formation and iNOS expression by primary α-cells. Individually or in combination, IL-1β and rat IFN-γ do not stimulate the production of nitrite or the expression of iNOS by primary α-cells. These experiments demonstrate that the combination of submaximal concentrations of IL-1β in the presence of rat IFN-γ stimulates the expression of iNOS by primary β-cells, suggesting that the β-cell is one islet cellular source of iNOS under these conditions.

**Time-dependent Effects of Rat IFN-γ and IL-1β on Nitrite Formation and iNOS mRNA Expression and Stability—**We have examined the time-dependent production of nitrite and iNOS mRNA accumulation using RINm5F cells. RINm5F cells represent a homogeneous population of β-cells that respond to IL-1 and IFN-γ in a manner similar to the effects of these cytokines on iNOS expression by intact islets. As shown in Fig. 3A, a maximal concentration of IL-1β stimulates the time-dependent production of nitrite that is first apparent at 6 h, then progresses linearly from 6 to 24 h, with little increase in the level of nitrite from 24 to 48 h. Individually, rat IFN-γ or submaximal concentrations of IL-1β do not stimulate the production of nitrite by RINm5F cells at any time point examined; however, the combination of these cytokines induces the time-dependent production of nitrite by RINm5F cells that is similar to the effects of maximal concentrations of IL-1β alone. Nitrite production induced by the combination of submaximal concentrations of IL-1β in the presence of rat IFN-γ is first detected 6 h after the addition of cytokines (control, 2.9 ± 0.6 pmol/2000 cells versus IL-1β + IFN-γ, 4.3 ± 0.6 pmol/2000 cells) and increases linearly from 6 to 24 h. The rate by which IL-1β alone or the combination of IL-1β and rat IFN-γ stimulate nitrite formation by RINm5F cells was determined by linear regression of nitrite data from 6 to 24-h time points shown in Fig. 3A.

The rate of nitrite formation induced by the combination of submaximal IL-1β and rat IFN-γ is reduced compared with the individual effects of maximal IL-1β (2.5 pmol of nitrite/h versus 3.6 pmol nitrite/h, respectively). Also, the maximal level of nitrite produced in response to submaximal IL-1β and rat IFN-γ is ~20–30% less than that induced by maximal IL-1β alone.

Although nitrite production by RINm5F cells in response to submaximal concentrations of IL-1β in combination with rat IFN-γ is similar to the effects of maximal IL-1β (in terms of the time dependence), the effects of these two conditions on iNOS mRNA accumulation are different. As shown in Fig. 3B, iNOS mRNA accumulation in response to a maximal concentration of

![Fig. 2. Effects of rat IFN-γ and IL-1β on nitrite formation and iNOS expression by primary α- and β-cells purified by FACS.](image-url)
IL-1β is 2-fold higher than the effects of submaximal concentrations of IL-1β and rat IFN-γ following a 6-h incubation. However, following a 12-h incubation, the levels of iNOS mRNA that accumulate in response to submaximal concentrations of IL-1β and rat IFN-γ are nearly identical to the levels observed following a 6-h exposure, whereas maximal IL-1β-induced iNOS mRNA accumulation is reduced to near background levels.

The persistence of iNOS mRNA accumulation following a 12-h exposure of RINm5F cells with submaximal concentrations of IL-1β in combination with IFN-γ compared with maximal concentrations of IL-1β alone (Fig. 3B) suggests that IFN-γ may stabilize IL-1-induced iNOS mRNA. To examine this question, an analysis of iNOS mRNA stability using the transcriptional inhibitor actinomycin D was performed. In Fig. 3C, RINm5F cells were incubated for 6 h in the presence of IFN-γ and maximal or submaximal concentrations of IL-1β or with maximal concentrations of IL-1β alone. Actinomycin D was then added, and the RINm5F cells were cultured for 6 additional h. As shown in Fig. 3C, ~70% IL-1β-induced iNOS mRNA is degraded in the 6-h incubation following the addition of actinomycin D; however, only ~30% iNOS mRNA is degraded in the presence of maximal concentrations of IL-1 in combination with IFN-γ, and only ~40% iNOS mRNA is degraded in the presence of submaximal concentrations of IL-1 in combination with IFN-γ. These data suggest a role for IFN-γ in the stabilization of IL-1β-induced iNOS mRNA that results in the persistence of iNOS mRNA accumulation after a 12-h exposure to these cytokines (Fig. 3B). Whereas maximal IL-1β-induced iNOS mRNA accumulation is ~2-fold greater than for submaximal IL-1β in the presence of INF-γ, the increase in iNOS mRNA stability afforded by IFN-γ ultimately results in similar levels of iNOS protein expression (data not shown) and nitrite production under both conditions.

Rat IFN-γ-induced iNOS Expression by Islet Cells Requires the Endogenous Release of IL-1—Islets contain resident macrophages that are known to express and release IL-1. We have previously shown that endogenous release of IL-1 within islets results in an inhibition of insulin secretion that is mediated by β-cell expression of iNOS (12). To further examine the extent to which the presence of IFN-γ is able to increase the sensitivity of islets to IL-1, islets were dispersed into individual cells and treated with varying concentrations of IFN-γ or with a maximal concentration of IL-1β alone (Fig. 4). Islet dispersion involves the treatment of intact islets with trypsin, an experimental manipulation that results in the destruction of 2–3% islet cells (based on trypan blue exclusion, data not shown). As shown in Fig. 4A, treatment of dispersed islet cells with rat IFN-γ stimulates nitrite formation and iNOS expression (inset) in a concentration-dependent manner. The interleukin-1 receptor antagonist protein (IRAP), which competes with IL-1 for receptor binding (33), completely prevents IFN-γ-induced nitrite production and iNOS expression by dispersed islet cells. These findings indicate that sufficient levels of IL-1β are released during dispersion to stimulate iNOS expression in the presence of IFN-γ.

The cellular source of the IL-1 released during islet cell dispersion is believed to be the resident islet macrophage. To provide evidence for the intra-islet macrophage as a source of IL-1, the effects of macrophage depletion on IFN-γ-induced iNOS expression and nitrite formation by dispersed islet cells were examined. Macrophage depletion was accomplished by culturing intact islets for 7 days at 24 °C. This culture condition has previously been shown to deplete over 95% of the islet lymphoid population (24). As shown in Fig. 4B, IFN-γ no longer stimulates iNOS expression (inset) or nitrite production by islet
effects of human IFN-γ on nitrite production by human islets. Human islets (200 islets/400 μl of complete CMRL-1066) were cultured in the presence or absence of human recombinant IL-1β and human recombinant IFN-γ for 48 h as indicated. Nitrite levels were determined in culture supernatants as described under "Experimental Procedures." Results are average ± S.E. of four independent experiments from four independent human islet isolations.

**DISCUSSION**

In this report we have examined the effects of IFN-γ, alone and in combination with IL-1, on iNOS expression and nitrite production by both rat and human islets of Langherans. We demonstrate that rat IFN-γ increases the sensitivity of rat islets for IL-1-induced iNOS expression by 10-fold. Alone, concentrations of IL-1β as low as 0.1 units/ml (0.57 pm) or 150 units/ml rat IFN-γ do not induce iNOS expression or nitrite production by rat islets, RINm5F cells, or primary β-cells purified by FACS; however, in combination, these cytokines induce the expression of iNOS and the production of nitrite to similar levels induced by maximal concentrations of IL-1β. Also, IL-1β, alone, or in combination with rat IFN-γ, does not stimulate nitrite formation or iNOS expression by primary α-cells, the other major endocrine cell type found in islets. These results are consistent with previous studies that have identified the β-cell as the islet cellular source of iNOS in response to IL-1β (12, 13) and provide the first direct evidence that IFN-γ modulates the function and viability of primary β-cells by increasing the sensitivity of β-cells for IL-1β-induced
iNOS expression. The combination of IL-1β and rat IFN-γ also results in inhibition of insulin secretion and islet destruction that are prevented by the iNOS inhibitor AG. These findings indicate that nitric oxide participates in the inhibitory and destructive effects of submaximal concentrations of IL-1β plus rat IFN-γ on insulin secretion and islet destruction.

The mechanism by which IFN-γ increases the sensitivity of β-cells for iNOS expression and nitrite production in response to IL-1β appears to be associated with an increase in the stability of iNOS mRNA. Consistent with previous studies (34), maximal concentrations of IL-1β induce an 8-fold increase in the accumulation of iNOS mRNA following a 6-h exposure; however, iNOS mRNA accumulation is reduced to near background levels following a 12-h incubation. In contrast, nearly equivalent levels of iNOS mRNA accumulate in RINm5F cells treated for 6 or 12 h with submaximal concentrations of IL-1β in combination with rat IFN-γ. Stability studies indicate that iNOS mRNA induced by IL-1β and rat IFN-γ is approximately 2-fold more stable than the individual effects of IL-1β. These findings indicate that IFN-γ may induce the expression of factors, and/or activate factors that stabilize iNOS mRNA, thus preventing iNOS mRNA degradation or that IFN-γ may inhibit the activity of factors that are required for down-regulating iNOS mRNA expression, and/or the degradation of iNOS mRNA. These possibilities are currently under investigation.

It is clear from studies with rat islets that IFN-γ reduces the concentration of IL-1β required to stimulate iNOS expression by β-cells; however, it is important to evaluate the effectiveness of human IFN-γ on iNOS expression by human islets treated with maximal and submaximal concentrations of IL-1β to determine if human islets respond in a similar manner. In this study we show that IL-1β, at a concentration as low as 1 unit/ml (5.7 pM), is able to stimulate high levels of nitrite production by human islets in the presence of human IFN-γ (750 units/ml). We also show that in the presence of 75 units/ml human IFN-γ, as little as 10 units/ml IL-1β is required to induce a 2-fold increase in the level of nitrite production. These results indicate that IFN-γ reduces the concentration of IL-1β required to stimulate iNOS expression by human islets in a manner similar to IFN-γ’s effects on rat islets.

We have also evaluated the effects of endogenous IL-1 release on iNOS expression and nitrite production by rat islets. Macrophages are believed to play a primary role in the development of autoimmune diabetes. Islets contain approximately 10–15 resident macrophages. Macrophage depletion, by silica treatment or feeding a diet deficient in essential fatty acids, reduces the concentration of IL-1 required to stimulate iNOS expression and nitrite production by target cells, leading to target cell damage. Under these conditions, macrophage production of nitric oxide may participate in target tissue damage; however, as our studies indicate, cytokine release by activated resident macrophages and cytokine-induced iNOS expression by target cells may be the more important mechanism associated with tissue damage. If our hypothesis is correct, human macrophage production of nitric oxide is not required for target cell damage. This interpretation is consistent with the difficulties in demonstrating human macrophage production of nitric oxide. In conclusion, our studies show that the T-cell cytokine, IFN-γ, directly inhibits the function and viability of islets by reducing the concentration of IL-1 required to stimulate iNOS expression and nitric oxide production by β-cells. These findings support an effector role for IFN-γ, in concert with IL-1, in mediating the initial destruction of β-cells during the development of autoimmune diabetes.

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