

# Double-Stranded RNA Cooperates with Interferon- $\gamma$ and IL-1 $\beta$ to Induce Both Chemokine Expression and Nuclear Factor- $\kappa$ B-Dependent Apoptosis in Pancreatic $\beta$ -Cells: Potential Mechanisms for Viral-Induced Insulinitis and $\beta$ -Cell Death in Type 1 Diabetes Mellitus

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Viral infections may trigger the autoimmune assault leading to type 1 diabetes mellitus. Double-stranded RNA (dsRNA) is produced by many viruses during their replicative cycle. The dsRNA, tested as synthetic poly(IC) (PIC), in synergism with the proinflammatory cytokines interferon- $\gamma$  (IFN- $\gamma$ ) and/or IL-1 $\beta$ , results in nitric oxide production, Fas expression,  $\beta$ -cell dysfunction, and death. Activation of the transcription nuclear factor- $\kappa$ B (NF- $\kappa$ B) is required for PIC-induced inducible nitric oxide synthase expression in  $\beta$ -cells, and we hypothesized that this transcription factor may also participate in PIC-induced Fas expression and  $\beta$ -cell apoptosis. This hypothesis, and the possibility that PIC induces expression of additional chemokines and cytokines (previously reported as NF- $\kappa$ B dependent) in pancreatic  $\beta$ -cells, was investigated in the present study. We observed that the PIC-responsive region in the Fas promoter is located between nucleotides -223 and -54. Site-directed mutations at the NF- $\kappa$ B and CCAAT/enhancer binding protein-binding sites prevented PIC-induced Fas promoter activity. Increased Fas promoter ac-

tivity was paralleled by enhanced susceptibility of PIC + cytokine-treated  $\beta$ -cells to apoptosis induced by Fas ligand.  $\beta$ -Cell infection with the NF- $\kappa$ B inhibitor AdI $\kappa$ B<sup>(SA)</sup><sup>2</sup> prevented both necrosis and apoptosis induced by PIC + IL-1 $\beta$  or PIC + IFN- $\gamma$ . Messenger RNAs for several chemokines and one cytokine were induced by PIC, alone or in combination with IFN- $\gamma$ , in pancreatic  $\beta$ -cells. These included IP-10, interferon- $\gamma$ -inducible protein-10, IL-15, macrophage chemoattractant protein-1, fractalkine, and macrophage inflammatory protein-3 $\alpha$ . There was not, however, induction of IL-1 $\beta$  expression. We propose that dsRNA, generated during a viral infection, may contribute for  $\beta$ -cell demise by both inducing expression of chemokines and IL-15, putative contributors for the build-up of insulinitis, and by synergizing with locally produced cytokines to induce  $\beta$ -cell apoptosis. Activation of the transcription factor NF- $\kappa$ B plays a central role in at least part of the deleterious effects of dsRNA in pancreatic  $\beta$ -cells. (*Endocrinology* 143: 1225–1234, 2002)

**I**N ADDITION TO genetic predisposition, environmental factors and viral infections probably play a key role in the pathogenesis of autoimmune diseases such as type I diabetes mellitus (T1DM) (1–3). At least six human and nine animal viruses have been suggested as triggering agents for clinical or experimental T1DM (4). Human pancreatic  $\beta$ -cells can be infected *in vitro* by different enteroviruses, leading to persistent infection, functional impairment, and in some cases cell death (5). Enterovirus antibody levels are increased in children with newly diagnosed T1DM (6), and recent findings indicate that children developing persistent  $\beta$ -cell autoimmunity have higher enterovirus antibody levels than children who remain islet autoantibody negative (7).

During the process of viral replication, double-stranded RNA (dsRNA) is often generated in the cytosol of virus-infected cells, and its presence functions as a cellular alerting signal for the presence of viruses (8). dsRNA, tested in the form of poly(IC) (PIC) triggers diabetes in diabetes-resistant biobreeding rats and accelerates  $\beta$ -cell destruction in diabetes-prone biobreeding rats (9, 10). Under *in vitro* conditions, PIC inhibits glucose-stimulated insulin biosynthesis in mouse islets (11). Exposure of rat pancreatic islets or autofluorescence-activated cell sorting (FACS)-purified  $\beta$ -cells to PIC, in combination with the proinflammatory cytokines interferon- $\gamma$  (IFN- $\gamma$ ) or IL-1 $\beta$ , results in nitric oxide (NO) production,  $\beta$ -cell dysfunction, and death (12, 13). These cytokines are probably released by infiltrating mononuclear cells in the vicinity of  $\beta$ -cells during insulinitis, and their signal transduction at the  $\beta$ -cell level may cross-talk with the signal transduction induced by viral products (12–14).  $\beta$ -Cell death seems to occur by both NO-dependent (in the case of PIC + IL-1 $\beta$ ) and NO-independent (in the case of PIC + IFN- $\gamma$ ) mechanisms (13). PIC, together with cytokines, leads to the expression of genes that may participate in the induction of  $\beta$ -cell death, such as inducible NO synthase (iNOS) and Fas (13, 14). Note that PIC alone induces neither

Abbreviations: AdI $\kappa$ B<sup>(SA)</sup><sup>2</sup>, Mutated nondegradable I $\kappa$ B $\alpha$ ; C/EBP, CCAAT/enhancer-binding protein; dsRNA, double-stranded RNA; FACS, fluorescence activated cell sorting; FasL, Fas ligand; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO 342, Hoechst 33342; IFN, interferon; iNOS, inducible nitric oxide synthase; IP-10, interferon- $\gamma$ -inducible protein-10; IRAP, IL-1 receptor antagonist protein; MCP-1, macrophage chemoattractant protein-1; MIP-3 $\alpha$ , macrophage inflammatory protein 3 $\alpha$ ; NF, nuclear factor; NO, nitric oxide; OD, optical density; PI, propidium iodide; PIC, poly(IC), polyinosinic-polycytidylic acid; rhs, recombinant human soluble; T1DM, type 1 diabetes mellitus; WT, wild-type.

$\beta$ -cell death nor NO formation by  $\beta$ -cells. Activation of the transcription nuclear factor- $\kappa$ B (NF- $\kappa$ B) is required for PIC + IFN- $\gamma$ -induced iNOS expression in  $\beta$ -cells (13, 15), but it remains unclear whether this transcription factor is also required for PIC-induced Fas expression.

Once immune tolerance is broken and the  $\beta$ -cells are recognized as targets by the immune system, the pancreatic islets are progressively invaded by mononuclear cells. Mononuclear cell migration is regulated by chemokines, a large family of small cytokines with four conserved cysteines linked by disulfide bonds (16). The chemokines macrophage chemoattractant protein-1 (MCP-1), regulated upon activation normal T cell expressed and secreted, IFN- $\gamma$ -inducible protein (IP-10), and macrophage inflammatory protein (MIP)-1 $\beta$  are highly expressed in islets obtained from diabetes-prone nonobese diabetic mice at the early stages of insulinitis and probably contribute to mononuclear cell homing and adhesion (17–19). It is noteworthy that the  $\beta$ -cells themselves, on exposure to IL-1 $\beta$  and/or IFN- $\gamma$ , express several chemokines and cytokines, including IP-10, MCP-1, fractalkine, MIP-3 $\alpha$ , and IL-15 (19–21). Expression of these chemokines is regulated to a large extent via NF- $\kappa$ B activation (22, 23).

Besides leading to chemokine formation, the cytokines IL-1 $\beta$  and IFN- $\gamma$  directly induce  $\beta$ -cell dysfunction and death, mostly by apoptosis (14, 24, 25). Cytokine-induced  $\beta$ -cell apoptosis is probably secondary to changes in the expression of a large number of gene modules, including a group of genes regulated by NF- $\kappa$ B (14, 21, 23). Indeed, inhibition of cytokine-induced NF- $\kappa$ B activation by either overexpression of an I $\kappa$ B mutant form (I $\kappa$ B<sup>(SA)2</sup>), or by stable expression of a dominant negative inhibitor of NF- $\kappa$ B, prevents cytokine-induced cell death in, respectively, human islet cells (26), purified rat  $\beta$ -cells (27), and insulin-producing MIN6 cells (28). As discussed above, IL-1 $\beta$  and IFN- $\gamma$  also synergize with dsRNA to induce  $\beta$ -cell death. The mechanism(s) behind these synergistic effects may include both NO generation and dsRNA-dependent protein kinase activation (12, 13, 29), but the role for NF- $\kappa$ B in this process remains unclear.

Against the background outlined above, we presently evaluated whether NF- $\kappa$ B is required for PIC + cytokine-induced Fas expression and  $\beta$ -cell death and whether PIC induces expression of different chemokines and cytokines on pancreatic  $\beta$ -cells. The results obtained suggest that dsRNA, generated during a viral infection, may contribute for  $\beta$ -cell demise by both inducing expression of chemokines, which can contribute to the build-up of insulinitis, and synergizing with locally produced cytokines to induce NF- $\kappa$ B-dependent  $\beta$ -cell apoptosis.

## Materials and Methods

### Culture of primary $\beta$ -cells and insulin-producing cell lines

Pancreatic islets were isolated from male Wistar rats by collagenase digestion and then dissociated into single cells in a calcium-free medium containing trypsin and DNase. Single  $\beta$ -cells were subsequently purified by FACS by using cellular light scatter and flavin adenine dinucleotide-autofluorescence as discriminating parameters. These preparations contain about 90–95% viable  $\beta$ -cells (30). The purified  $\beta$ -cells were cultured in Ham's F-10 medium supplemented with 10 mM glucose, 50  $\mu$ M

3-isobutyl-1-methylxanthine, 1% BSA (Roche Molecular Biochemicals, Mannheim, Germany), 0.1 mg/ml streptomycin (Continental Pharma, Puteaux, Belgium), 0.075 mg/ml penicillin (Laboratoires Diamant, Brussels, Belgium), and 2 mM L-glutamine (Invitrogen, Paisley, UK) (31). For viability experiments (see below), FACS-purified single  $\beta$ -cells ( $3 \times 10^3$  cells per well) were cultured for 6–9 d in Falcon 96-well microtiter plates (Becton Dickinson and Co., Franklin Lakes, NJ) containing 200  $\mu$ l medium. Culture medium was changed every 3 d and fresh cytokines added. For mRNA determination the single  $\beta$ -cells were reaggregated for 3 h in a rotatory shaking incubator (32), cultured for 14–16 h in suspension (culture conditions as described above), and exposed for 6 h to different combinations of cytokines and/or PIC.

Mouse MIN-6 and rat INS-1 cells and RINm5F were kindly provided by Dr. H. Ishihara (University of Tokyo, Tokyo, Japan), Prof. C. B. Wollheim (Center Medical Universitaire, Geneva, Switzerland), and Prof. S. Sandler (Uppsala University, Uppsala, Sweden), respectively. These cells were cultured as previously described (33–35).

### PIC, cytokine, and Fas ligand (FasL) treatment

The effects of cytokines and/or PIC were examined after 6 h and 4 and 6 d of culture in the presence of recombinant murine IFN- $\gamma$  (1000 U/ml, 10 U/ng, Invitrogen), recombinant human IL-1 $\beta$  (50 U/ml, 38 U/ng, a kind gift of Dr. C. W. Reynolds, National Cancer Institute, Bethesda, MD), and synthetic PIC (100  $\mu$ g/ml; Sigma, St. Louis, MO). The concentrations of cytokines were selected based on our previous studies with rodent pancreatic islets and  $\beta$ -cells (13, 20, 36–38), and the concentration of PIC was selected based on data from the literature (12, 13, 39) and our own dose-response studies (data not shown). Culture media were collected after 72 h for nitrite determination (nitrite is a stable product of NO oxidation), which was performed spectrophotometrically at 546 nm wavelength after colored reaction with the Griess reagent (40). Nitrite was not determined at subsequent time points because of the decreased viability of  $\beta$ -cells exposed to PIC and cytokines for 6 or more d (13).

In some experiments the  $\beta$ -cells were pretreated for 1 h or 30 min with, respectively, an IL-1 receptor antagonist protein (IRAP; R & D Systems, Abingdon, UK) or caspase inhibitors before exposure to IL-1 $\beta$  + IFN- $\gamma$  or IFN- $\gamma$  + PIC (same concentrations as above). In the experiments with IRAP (1.25  $\mu$ g/ml), culture medium was changed after 3 d and fresh IRAP and cytokines was added for an additional 3 d (total of 6 d exposure). The concentration of IRAP selected (1,000-fold higher than the concentration of IL-1 $\beta$ ) has been previously shown to protect whole islets against IL-1 $\beta$ -induced dysfunction (41). In the experiments with caspase inhibitors,  $\beta$ -cells were pretreated for 30 min with either caspase 1 inhibitor (Z-YVAD-FMK, 100  $\mu$ mol/liter; Enzyme Systems Products, Livemore, CA), caspase 3 inhibitor (Z-DEVD-FMK, 25  $\mu$ mol/liter; Enzyme Systems Products) or caspase 8 inhibitor (Z-IETD-FMK, 10  $\mu$ mol/liter; Calbiochem, Leuven, Belgium) and then exposed to IL-1 $\beta$  + IFN- $\gamma$  or IFN- $\gamma$  + PIC (same concentrations as above) for 4 d. After 2 d, culture medium was changed and fresh caspase inhibitors and cytokines were added. The concentrations selected for the caspase blockers have been previously shown to block caspase activity in different cell types, including  $\beta$ -cells (12, 42, 43).

For the experiments with recombinant FasL,  $\beta$ -cells were pretreated for 6 h with IL-1 $\beta$ , IL-1 $\beta$  + IFN- $\gamma$ , PIC, IL-1 $\beta$  + PIC, or IFN- $\gamma$  + PIC (same concentration as above) and then exposed to recombinant human soluble (rhs)FasL (100  $\mu$ g/ml; Alexis, L aufelfingen, Switzerland) and enhancer (1 mg/ml; Alexis) for 18 h. The concentrations selected for rhs-FasL and enhancer have been previously shown to induce apoptosis in rat cells (44).

### Messenger RNA isolation and RT-PCR

Poly(A)<sup>+</sup> RNA was isolated from  $\beta$ -cells ( $5 \times 10^4$  cells) using oligo(deoxythymidine)25-coated polystyrene Dynabeads (Dynal, Oslo, Norway). The reverse transcription reaction and the subsequent PCR were performed as previously described (38, 45). PCR specificity and efficiency was enhanced by using hot-start PCR with 12-min predenaturation at 95 C and then 32 cycles with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and IP-10, 31 cycles with IL-15, 33 cycles with MCP-1, 40 cycles with fractalkine, and 36 cycles with MIP-3 $\alpha$ , at 94 C for 45 sec, 58 C for 45 sec, and 72 C for 80 sec. The number of cycles was

selected to allow linear amplification of the cDNA under study. The primer sequences used for amplification of rat cDNAs for GAPDH, MCP-1 (20), IP-10, IL-15, fractalkine, and MIP-3 $\alpha$  (21) were as described in the indicated references. The primers for IL-1 $\beta$  determination were as described by Heitmeier *et al.* (46). The ethidium bromide-stained agarose gels were photographed under UV-transillumination using a Digital Science DC120 camera (Kodak, Rochester, NY). The PCR band intensities on the image were quantified by Biomax 1D Image analysis software (Kodak) and expressed in pixel intensities (ODs). The target cDNAs present in each sample were corrected for the respective GAPDH values and normalized using the maximum signal in each amplification as 10 (21). Expression of the housekeeping gene GAPDH is not affected by exposure to cytokines in both whole islets and FACS-purified  $\beta$ -cells (Refs. 13 and 47 and present data).

### Cell transfection and luciferase assay

Studies on Fas promoter activity in single  $\beta$ -cells ( $4 \times 10^4$  cells per condition) were performed by transient transfection with the plasmid pFas-811luc containing the nucleotides  $-811$  to  $+123$  of the rat Fas promoter (wild-type, WT), pFas-223luc, pFas-54luc, and mutants from pFas-811luc in which either the NF- $\kappa$ B binding site or the CCAAT/enhancer-binding protein (C/EBP)-binding site between nucleotides  $-223$  and  $-54$  were inactivated (48).  $\beta$ -Cells were transfected with lipofectamine (Invitrogen, Gaithersburg, MD), and after 4 h they were exposed for 16 h to IL-1 $\beta$  or PIC. Luciferase activities were assayed with the dual-luciferase reporter assay (Promega Corp., Madison, WI) in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The values of the test plasmid were normalized for the luciferase activity value of the cotransfected control plasmid, pRL-CMV (49, 50). The different conditions tested did not affect pRL-CMV activity, which was, respectively, for control and IL-1 $\beta$ - and PIC-treated  $\beta$ -cells (means  $\pm$  SEM of six experiments)  $960 \pm 184$ ,  $1236 \pm 135$ , and  $1063 \pm 330$ .

### Transfection with recombinant adenoviruses

After overnight incubation,  $\beta$ -cells were infected with control virus AdLuc, encoding the luciferase gene (27), or with AdI $\kappa$ B<sup>(SA)2</sup>, expressing a NF- $\kappa$ B-superrepressor (see below). The recombinant replicative-deficient adenovirus containing a mutated nondegradable I $\kappa$ B $\alpha$  (AdI $\kappa$ B<sup>(SA)2</sup>) was prepared as previously described (51), and it was a kind gift from Dr. C. Jobin, Division of Digestive Diseases and Nutrition, University of North Carolina (Chapel Hill, NC).  $\beta$ -Cells were infected at a multiplicity of infection 7.5 for 2 h at 37 C. We have previously shown that AdI $\kappa$ B<sup>(SA)2</sup>, at this multiplicity of infection, prevents cytokine-induced NF- $\kappa$ B activation, iNOS, and Fas mRNA expression and  $\beta$ -cell death without affecting  $\beta$ -cell insulin content or glucose-induced insulin release (27).

### Assessment of $\beta$ -cell viability

The percentage of viable, apoptotic, and necrotic  $\beta$ -cells was determined after 6 d of exposure to cytokines and/or PIC, the time required to detect significant increases in cell death in FACS-purified  $\beta$ -cells (13, 38, 52). For this purpose,  $\beta$ -cells were incubated for 15 min with propidium iodide (PI, 10  $\mu$ g/ml) and Hoechst (HO) 342 (20  $\mu$ g/ml) (53). Under the present experimental conditions (culture in a serum-free medium), cells remain attached to the culture dish and the nuclear remains from cells undergoing apoptosis or necrosis are preserved. This fluorescence assay for single  $\beta$ -cells is quantitative and has been validated by systematic comparisons with electron microscopy observations (36, 53). The method has been successfully used to evaluate apoptosis/necrosis in rat (13, 53), mouse (38, 45), and human (52)  $\beta$ -cells. In each experimental condition, a minimum of 500 cells was counted by two observers, one of them unaware of the sample identity. The necrosis and apoptosis indexes were calculated as [(% necrotic or apoptotic cells in experimental condition  $-$  % necrotic or apoptotic cells in control) / (100  $-$  % dead cells in control)]  $\times$  100 (54). The mean value for, respectively, necrosis and apoptosis in control single  $\beta$ -cells (not exposed to cytokines) after 6 d in culture was  $25\% \pm 1\%$  and  $14\% \pm 2\%$  ( $n = 4$ ).

### Statistical analysis

The results are presented as means  $\pm$  SEM. Statistical differences between the groups were determined by paired *t* test or, when indicated, by ANOVA followed by multiple paired or unpaired *t* tests with the Bonferroni correction.

## Results

### NF- $\kappa$ B and C/EBP binding sites are required for PIC-induced Fas promoter activity

We have previously shown that PIC induces Fas mRNA expression in pancreatic  $\beta$ -cells (13). To delineate the PIC-responsive regions in the Fas promoter, transient transfections were performed with the plasmid pFas-811luc containing the nucleotides  $-811$  to  $+123$  of the rat Fas promoter (WT), pFas-223luc, pFas-54luc (Fig. 1), and mutants from pFas-811luc in which either the NF- $\kappa$ B binding site or the C/EBP-binding site between nucleotides  $-223$  and  $-54$  were inactivated (Fig. 2). The values for relative luciferase activity ratios were (means  $\pm$  SEM of three experiments): WT not exposed to cytokines,  $0.001 \pm 0.001$ ; WT exposed to PIC,  $0.007 \pm 0.001$ ; and WT exposed to IL-1 $\beta$ ,  $0.012 \pm 0.001$ . Thus, PIC induced a nearly 7-fold increase in Fas promoter activity, similar to the induction observed in WT constructs exposed to IL-1 $\beta$  (used as positive control; 48). Both pFas-811luc and pFas-223luc were induced by PIC or IL-1 $\beta$ , but pFas-54luc did not respond to these stimuli (Fig. 1). This suggests that the PIC-responsive region in the Fas promoter is located between nucleotides  $-223$  and  $-54$ . In a subsequent series of experiments, we evaluated whether binding sites for NF- $\kappa$ B and C/EBP, located in this region, were required for PIC-induced Fas expression. These two sites have been previously shown to mediate IL-1 $\beta$ -induced Fas expression in  $\beta$ -cells (48), a finding confirmed in the present series of experiments (Fig. 2). Moreover, site mutations at the NF- $\kappa$ B and

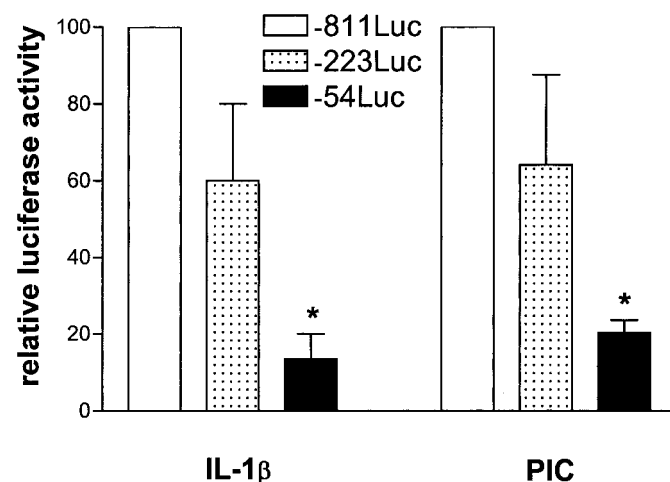


FIG. 1. Fas promoter activity in  $\beta$ -cells after a 16-h exposure to IL-1 $\beta$  (50 U/ml) or PIC (100  $\mu$ g/ml). Studies on Fas promoter activity were performed by transient transfection with the plasmid pFas-811luc, containing the nucleotides  $-811$  to  $+123$  of the rat Fas promoter (WT); pFas-223luc; or pFas-54luc. Data are expressed as percentage of the relative luciferase activity observed in cells transfected with the WT construct and exposed to IL-1 $\beta$  or PIC (considered as 100% in each individual experiment; absolute values provided in *Results*). Results are means  $\pm$  SEM of three experiments. \*,  $P < 0.05$  vs. WT exposed to similar treatment; paired *t* test.

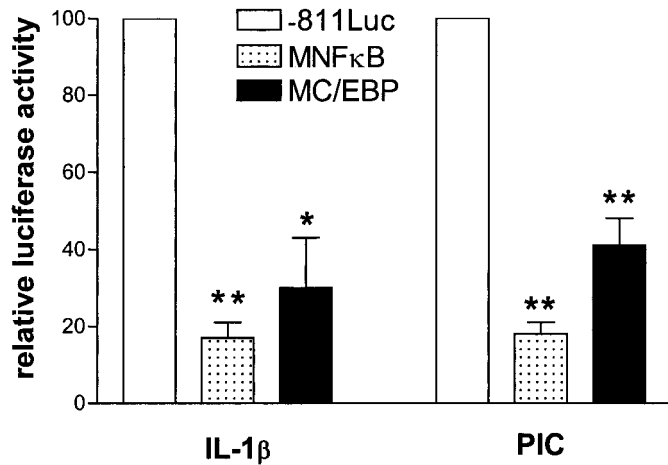


FIG. 2. Effect of site-directed mutations on Fas promoter activity following IL-1 $\beta$  (50 U/ml) or PIC (100  $\mu$ g/ml) treatment of  $\beta$ -cells. Cells were transfected with the plasmid pFas-811Luc (WT) construct described in *Materials and Methods* or with the same construct mutated either at the NF- $\kappa$ B-binding site (MNF- $\kappa$ B) or the C/EBP-binding site (MC/EBP). Data are expressed as percentage of the relative luciferase activity observed in cells transfected with the WT construct and exposed to IL-1 $\beta$  or PIC (considered as 100% in each individual experiment; absolute values provided in *Results*). Results are means  $\pm$  SEM of three experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. WT exposed to similar treatment; paired  $t$  test.

C/EBP binding sites also prevented PIC-induced Fas expression (Fig. 2).

#### Exposure of $\beta$ -cells to PIC plus cytokines renders them susceptible to FasL-induced apoptosis

To evaluate whether PIC and/or cytokine-induced Fas promoter activity (present data) and mRNA expression (13) is paralleled by enhanced sensitivity to FasL-induced apoptosis,  $\beta$ -cells were pretreated for 6 h with IL-1 $\beta$ , IL-1 $\beta$  plus IFN- $\gamma$ , PIC, IL-1 $\beta$  plus PIC, or IFN- $\gamma$  plus PIC and then exposed to rFasL together with an enhancer. Exposure of  $\beta$ -cells to PIC and/or cytokines for 18 h in the absence of FasL plus enhancer or to FasL, enhancer, or FasL plus enhancer in the absence of cytokines did not induce  $\beta$ -cell death (data not shown). On the other hand,  $\beta$ -cells pretreated with either PIC and/or cytokines and then exposed to FasL + enhancer showed a significant increase in the percentage of apoptotic cells (Fig. 3). This indicates that PIC and/or cytokines induce functional Fas expression and lead to  $\beta$ -cell apoptosis in the presence of functional FasL.

#### Effect of I $\kappa$ B<sup>(SA)2</sup> expression on $\beta$ -cell viability

The findings that NF- $\kappa$ B is required for both PIC-induced Fas (present data) and iNOS (13) and is required for IL-1 $\beta$  + IFN- $\gamma$ -induced  $\beta$ -cell death (27) raised the possibility that this transcription factor is also involved in PIC + IFN- $\gamma$ -induced  $\beta$ -cell death. To investigate the role of NF- $\kappa$ B in PIC and cytokine-induced  $\beta$ -cell death, FACS-purified  $\beta$ -cells were infected with recombinant adenovirus expressing a nondegradable mutant form of I $\kappa$ B (AdI $\kappa$ B<sup>(SA)2</sup>) or a control, recombinant adenovirus AdLuc encoding the luciferase gene (27, 51).

Exposure of  $\beta$ -cells to PIC + IL-1 $\beta$ , PIC + IFN- $\gamma$ , or IL-1 $\beta$

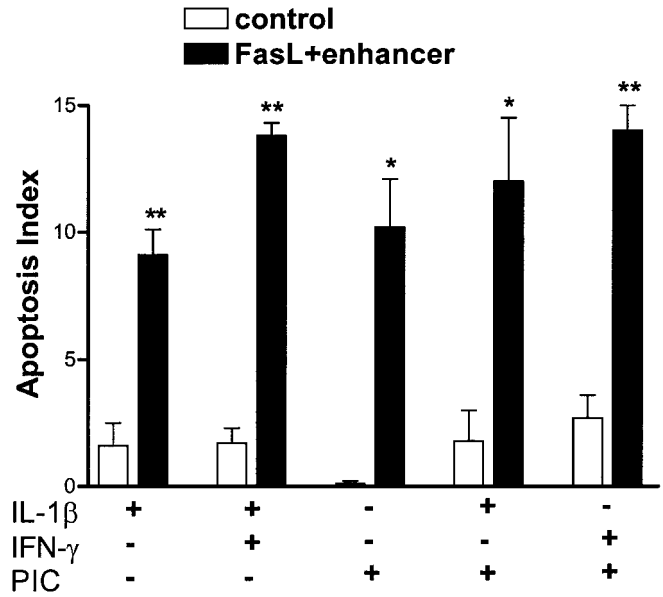


FIG. 3. FasL-induced apoptosis in  $\beta$ -cells pretreated with PIC or cytokines.  $\beta$ -cells were pretreated for 6 h with IL-1 $\beta$ , IL-1 $\beta$  + IFN- $\gamma$ , PIC, IL-1 $\beta$  + PIC, or IFN- $\gamma$  + PIC and then exposed to rFasL and enhancer for 18 h. Apoptosis indexes were calculated as described in *Materials and Methods*. Cell viability was determined with the DNA dyes HO 342 and PI. Results are means  $\pm$  SEM of four to six experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. respective controls (*i.e.* cells exposed to PIC and/or cytokines without FasL and enhancer); paired  $t$  test.

+ IFN- $\gamma$  leads to a significant increase in cell death via both necrosis and apoptosis (Fig. 4, A and B). The apoptotic component of cell death was confirmed by additional experiments in which  $\beta$ -cells were exposed to PIC + IFN- $\gamma$  or IL-1 $\beta$  + IFN- $\gamma$  for 4 d in the presence of inhibitors of caspase 1, 3, and 8. Under these conditions, there was a significant decrease in  $\beta$ -cell apoptosis (Fig. 5). Interestingly, when the exposure time to cytokines and caspase inhibitors was prolonged to 9 d, the  $\beta$ -cells that have escaped apoptosis underwent necrosis instead (data not shown).

$\beta$ -Cell infection with AdI $\kappa$ B<sup>(SA)2</sup>, but not AdLuc, prevented both necrosis and apoptosis induced by PIC + IL-1 $\beta$  or PIC + IFN- $\gamma$  (Fig. 4, A and B). Furthermore, and in agreement with our previous observations (27), AdI $\kappa$ B<sup>(SA)2</sup> also prevented IL-1 $\beta$  + IFN- $\gamma$ -induced  $\beta$ -cell death. As a whole, these data suggest that NF- $\kappa$ B activation is required for PIC + IL-1 $\beta$ , PIC + IFN- $\gamma$ , and IL-1 $\beta$  + IFN- $\gamma$ -induced  $\beta$ -cell death.

#### Cytokine- and PIC-induced chemokine and cytokine mRNA expression

Chemokines are another group of NF- $\kappa$ B-dependent genes induced by cytokines in  $\beta$ -cells, with potential relevance for the pathogenesis of T1DM (21, 23). Thus, we next evaluated whether these genes were induced by PIC in pancreatic  $\beta$ -cells. IP-10 mRNA was not detectable in the control condition, but it was induced by PIC, IL-1 $\beta$ , and IFN- $\gamma$ , alone or in combinations (Fig. 6, A and B). Neither IL-1 $\beta$  nor IFN- $\gamma$  alone induced significant IL-15 mRNA expression, but PIC alone induced a clear increase in IL-15 expression (Fig. 6, A and B). This effect of PIC was potentiated by IL-1 $\beta$  and IFN- $\gamma$ . PIC alone failed to induce

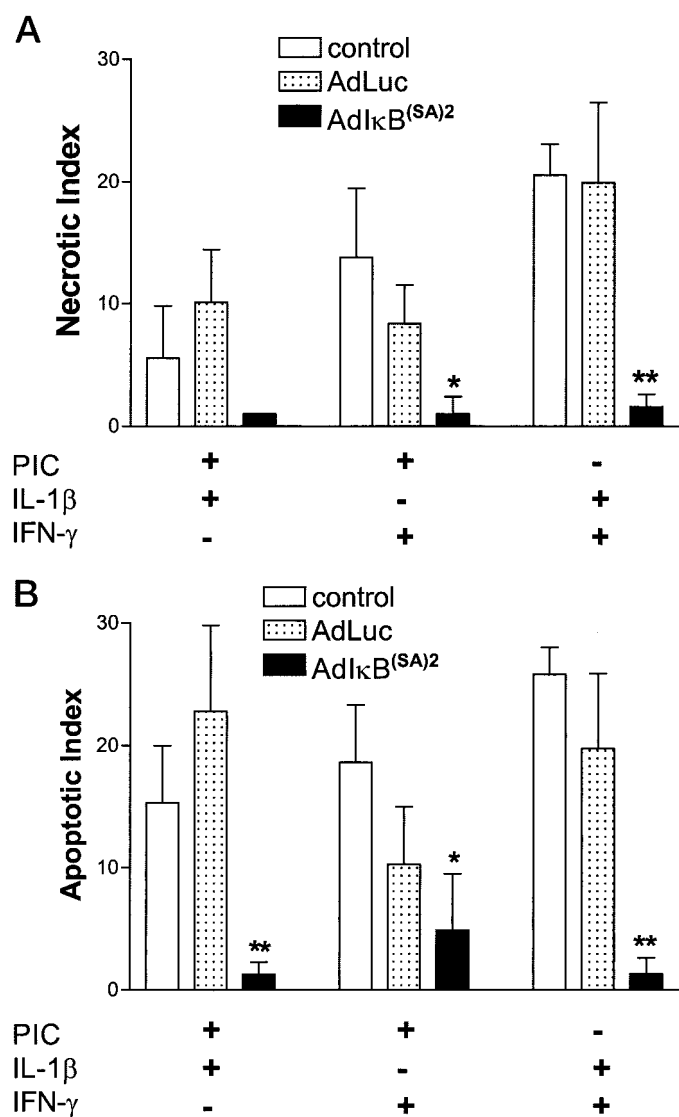


FIG. 4. Effect of NF- $\kappa$ B blocking on cytokine-induced  $\beta$ -cell death.  $\beta$ -Cells were infected with a recombinant adenovirus expressing the nondegradable I $\kappa$ B (AdI $\kappa$ B<sup>(SA)2</sup>) or the control recombinant adenovirus AdLuc. After 2 h of transfection, residual viruses were washed away, and  $\beta$ -cells were cultured for 6 d in the presence of PIC and cytokines. The necrosis (A) and apoptosis (B) indexes were calculated as described in *Materials and Methods*. Cell viability was determined with the DNA dyes HO 342 and PI. Results are means  $\pm$  SEM of four experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. control; ANOVA.

MCP-1 and fractalkine mRNA expression. On the other hand, PIC seemed to increase the expression of both mRNAs when added in combination with IL-1 $\beta$  or IFN- $\gamma$  (Fig. 6, A and B). Similar to IL-1 $\beta$ , but not IFN- $\gamma$ , PIC alone induced MIP-3 $\alpha$  mRNA expression. This effect was not potentiated by the presence of cytokines (Fig. 6, A and B). None of the above-described treatments modified the expression of the housekeeping gene GAPDH (Fig. 6A). The ODs for GAPDH expression were (means  $\pm$  SEM of three experiments): control,  $6.2 \pm 0.2$ ; IL-1 $\beta$ ,  $5.3 \pm 0.7$ ; IFN- $\gamma$ ,  $5.4 \pm 0.9$ ; PIC,  $5.3 \pm 0.6$ ; IL-1 $\beta$  + PIC,  $5.0 \pm 0.6$ ; IFN- $\gamma$  + PIC,  $5.5 \pm 0.3$ ; IL-1 $\beta$  + IFN- $\gamma$ ,  $5.4 \pm 0.5$ .

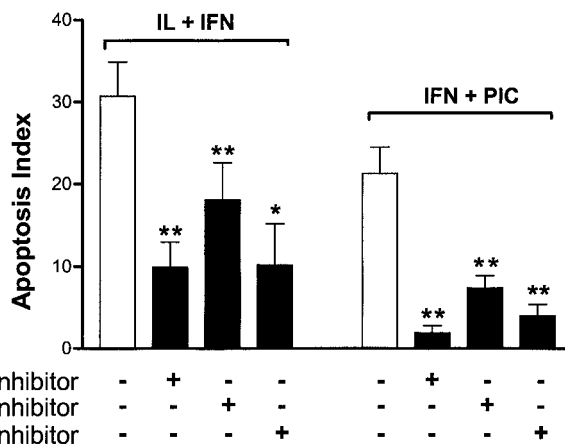


FIG. 5. Effect of caspase inhibitors on PIC and cytokine-induced  $\beta$ -cell apoptosis.  $\beta$ -Cells were pretreated for 30 min with either caspase 1 inhibitor (Z-YVAD-FMK), caspase 3 inhibitor (Z-DEVD-FMK), or caspase 8 inhibitor (Z-IETD-FMK) and then exposed to IL-1 $\beta$  + IFN- $\gamma$  or IFN- $\gamma$  + PIC for 4 d. Apoptosis indexes were calculated as described in *Materials and Methods*. Cell viability was determined with the DNA dyes HO 342 and PI. Results are means  $\pm$  SEM of five experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. respective control (*i.e.* cells exposed to cytokines without caspase inhibitor); ANOVA.

#### Effect of IRAP on PIC-treated $\beta$ -cell NO production and survival

As described above, PIC increases the expression of several chemokines and at least one cytokine (IL-15) in pancreatic  $\beta$ -cells. Recent observations suggested that PIC + IFN- $\gamma$  also induces expression and release of IL-1 $\beta$  by pancreatic  $\beta$ -cells, a phenomenon that could mediate some of the deleterious effects of PIC + IFN- $\gamma$  in  $\beta$ -cells (46). To evaluate this possibility, we initially determined whether PIC + IFN- $\gamma$  induces IL-1 $\beta$  mRNA expression. This was done using primers similar to those described by Heitmeier *et al.* (46). Although we detected a faint IL-1 $\beta$  band in  $\beta$ -cells after 38 cycles of amplification, there was no significant increase in IL-1 $\beta$  mRNA expression following  $\beta$ -cell exposure to IFN- $\gamma$  + PIC, IL-1 $\beta$ , IFN- $\gamma$ , PIC, IL-1 $\beta$  + PIC, or IL-1 $\beta$  + IFN- $\gamma$ , compared with control cells ( $n = 3$ ; data not shown). These data could not, however, exclude that a small fraction of  $\beta$ -cells express IL-1 $\beta$ , mediating some of the biological effects of IFN- $\gamma$  + PIC. To test this possibility, FACS-purified  $\beta$ -cells were incubated for 6 d with PIC and cytokines in the presence or absence of IRAP.  $\beta$ -Cell exposure to IL-1 $\beta$  and IFN- $\gamma$  induced NO production and apoptosis, and this induction was inhibited by IRAP, indicating that the compound was active (Fig. 7, A and B). On the other hand, IRAP prevented neither IFN- $\gamma$  + PIC-induced NO production nor  $\beta$ -cell apoptosis (Fig. 7, A and B). These observations suggest that the PIC effects on  $\beta$ -cells, alone or in combination with IFN- $\gamma$ , are independent of IL-1 $\beta$  production.

#### Effects of PIC on insulin-producing cell lines

The molecular biology experiments described above, and the additional experiments that will be required to clarify PIC signal transduction in  $\beta$ -cells, require large numbers of cells. Because the isolation of primary  $\beta$ -cells is a laborious and time-demanding procedure, we tested whether the insulin-

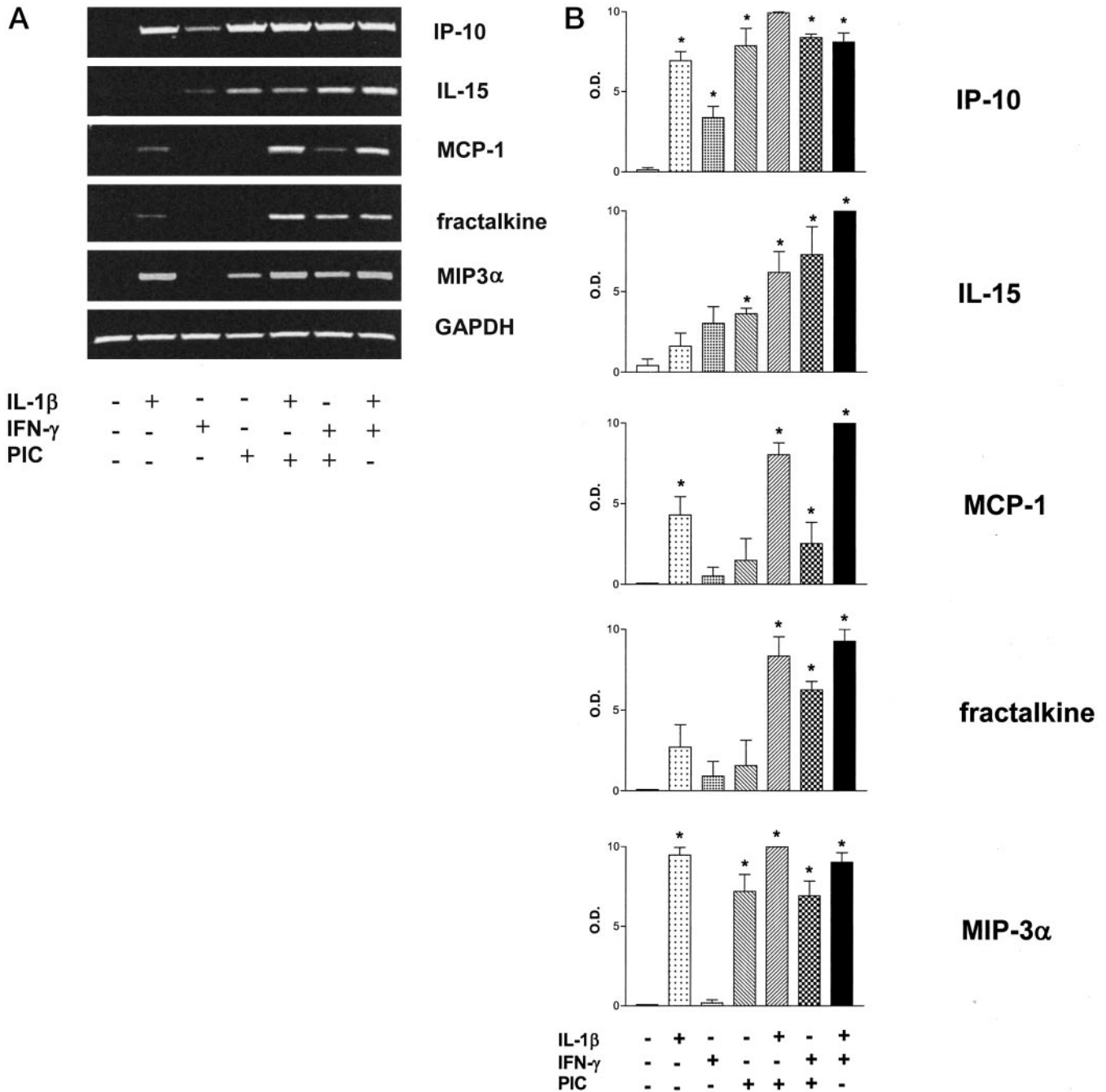


FIG. 6. Chemokine and cytokine expression in PIC-exposed  $\beta$ -cells. A, RT-PCR analysis of IP-10, IL-15, MCP-1, fractalkine, MIP-3 $\alpha$ , and GAPDH mRNA expression by  $\beta$ -cells exposed for 6 h to control condition (no PIC or cytokines added) or IL-1 $\beta$  (50 U/ml), IFN- $\gamma$  (1000 U/ml), or PIC (100  $\mu$ g/ml), alone or in combinations. The cDNA samples were amplified in parallel with GAPDH-specific primers, confirming similar loading in all lanes. The figure is representative of three similar experiments. B, ODs of IP-10, IL-15, MCP-1, fractalkine, and MIP-3 $\alpha$  mRNA expression, corrected for GAPDH and normalized, considering the maximum signal in each amplification as 10. The values are means  $\pm$  SEM of three experiments. \*,  $P < 0.05$  vs. control; paired  $t$  test.

producing cell lines INS-1, RINm5F, and MIN6 present a similar response to PIC + cytokines as described above for primary  $\beta$ -cells. Surprisingly, PIC + IFN- $\gamma$  failed to induce iNOS expression and NO production in INS-1 cells (Fig. 8), RINm5F, or MIN6 cells (data not shown), but it induced iNOS mRNA expression (13) and a nearly 10-fold increase in nitrite production by primary  $\beta$ -cells (Fig. 7A). Thus, it seems that clonal insulin-producing cell lines do not respond to

PIC + IFN- $\gamma$ , at least regarding NO production, in a way similar to primary  $\beta$ -cells.

### Discussion

Most cell types recognize the presence of a viral infection by detecting dsRNA molecules synthesized during the replication process of distinct DNA and RNA viruses (8). The

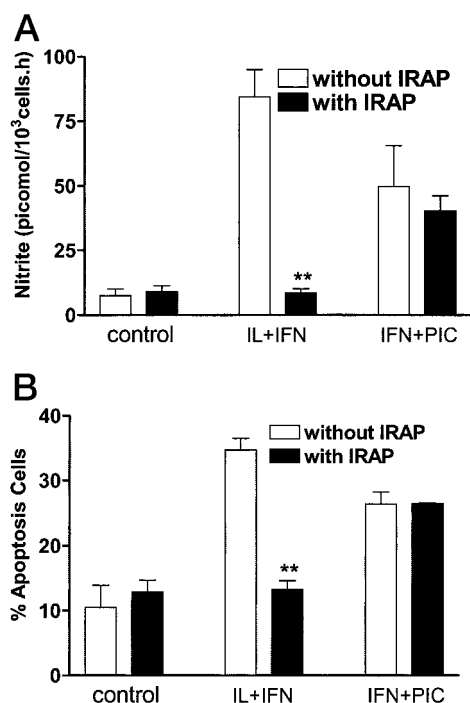


FIG. 7. Effect of IRAP on PIC + IFN- $\gamma$ -induced nitrite production and cell death. A, PIC + IFN- $\gamma$  and IL-1 $\beta$  + IFN- $\gamma$ -induced nitrite production by  $\beta$ -cells cultured for 72 h in the presence or absence of IRAP. B, Prevalence of apoptosis in  $\beta$ -cells exposed for 6 d to control condition or PIC + IFN- $\gamma$  and IL-1 $\beta$  + IFN- $\gamma$  in the presence or absence of IRAP. Cell viability was determined with the DNA dyes HO 342 and PI. Results are means  $\pm$  SEM of three experiments. \*\*,  $P < 0.01$  vs. the same condition without IRAP; paired  $t$  test.

infected cells react by triggering components of the innate immune response, paving the way for the subsequent adaptive immune response (55, 56). The early responses include decreasing the rate of host cell protein synthesis, thus inhibiting viral replication, and in some cases self-elimination of the infected cells via apoptosis. The target cell also produces antiviral (type I) interferons and other cytokines, helping the naive cells to face the viral invasion and, in some cases, to clear it without cell death (55). Most studies on dsRNA effects have been performed in cell lines, often derived from the immune system, and relatively little is known about the responses of primary and non- or poorly dividing cells to dsRNA. It is an intriguing possibility that the same mechanisms that allow some primary cell types to eliminate viruses in a cytokine-dependent, noncytopathic bystander manner may cause the death of other cell types (57). This is of special interest regarding  $\beta$ -cells. Indeed, viruses and their products probably play a role in the pathogenesis of T1DM (4), and locally produced cytokines, such as IL-1 $\beta$  and IFN- $\gamma$ , may act in synergism with dsRNA to induce NO formation and  $\beta$ -cell death in the course of insulinitis (12, 13). In this context, it is noteworthy that PIC does not induce similar effects on diverse clonal insulin-producing cell lines, compared with  $\beta$ -cells (present data). This raises the possibility that the ability of primary  $\beta$ -cells to mount a complex molecular response to viral products is part of the most differentiated functions of  $\beta$ -cells.

PIC induces both iNOS and Fas mRNA expression in

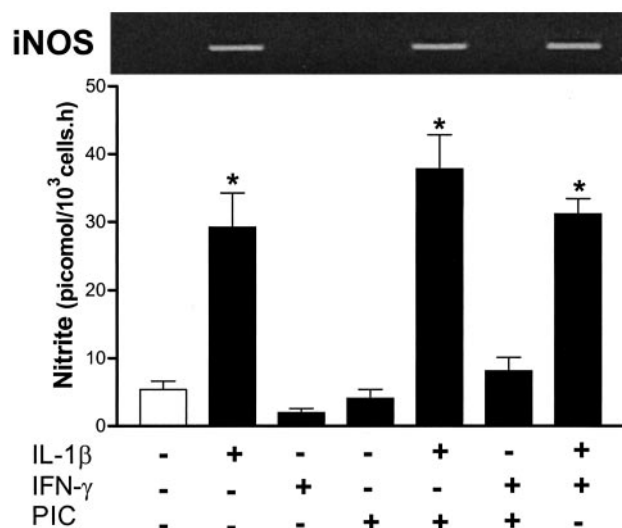
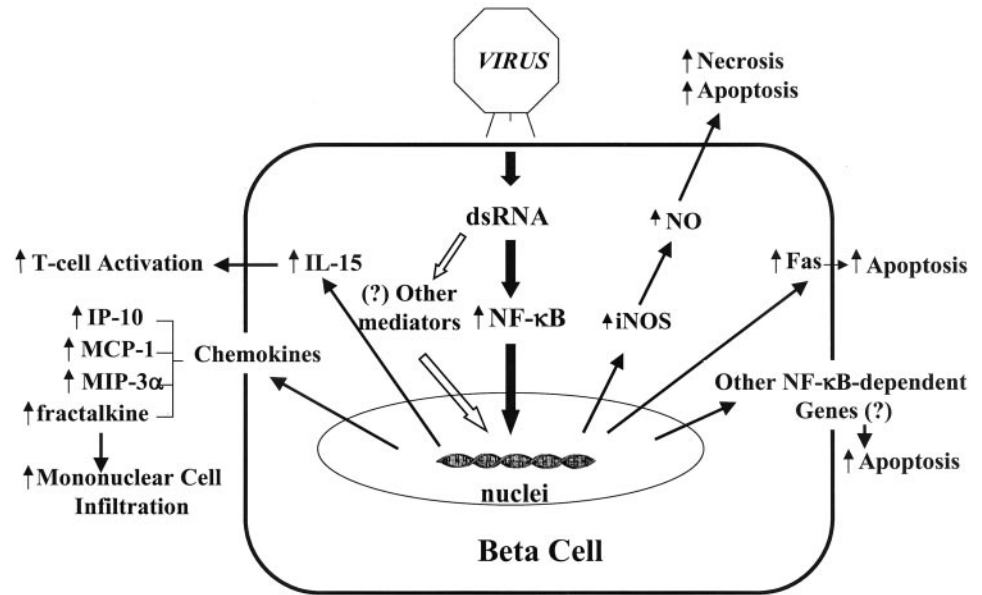


FIG. 8. Effects of PIC and/or cytokines on INS-1 cells. INS-1 cells were cultured for 24 h in the presence or absence of PIC and/or cytokines. The iNOS mRNA expression was determined by RT-PCR (upper part of the figure), but NO production was assessed by medium nitrite accumulation (lower part of the figure). Nitrite results are means  $\pm$  SEM of six experiments, and the PCR shown is representative for two similar experiments. GAPDH mRNA expression was similar in the different experimental groups (not shown). \*,  $P < 0.01$  vs. control (i.e. cells not exposed to PIC or cytokines).

FACS-purified  $\beta$ -cells, and iNOS expression depends on NF- $\kappa$ B activation (13). An increased Fas expression in  $\beta$ -cells, via interaction with FasL expressed in infiltrating mononuclear cells, may contribute to  $\beta$ -cell apoptosis in both experimental models and clinical T1DM (58–61). We presently examined the transcriptional regulation of the Fas gene by experiments in which the rat Fas promoter-luciferase constructs were transfected into purified primary rat  $\beta$ -cells. A PIC-responsive region, located between nucleotides -223 and -54, was identified. Inactivation of two adjacent NF- $\kappa$ B and C/EBP sites in this region prevented PIC-induced Fas promoter activity in  $\beta$ -cells. This indicates that, as previously described for IL-1 $\beta$  (Ref. 48 and confirmed in the present study), both factors are required for induction of Fas expression by PIC in  $\beta$ -cells.

The findings that NF- $\kappa$ B is required for both PIC-induced Fas (present data) and iNOS (13) and is required for IL-1 $\beta$  + IFN- $\gamma$ -induced  $\beta$ -cell death (27), raised the possibility that this transcription factor is also involved in PIC + IFN- $\gamma$ -induced  $\beta$ -cell death. To investigate this issue, FACS-purified  $\beta$ -cells were infected with a AdIKB<sup>(SA)2</sup> (27). AdIKB<sup>(SA)2</sup> prevented  $\beta$ -cell death by both apoptosis and necrosis following prolonged culture in the presence of either PIC + IL-1 $\beta$  or PIC + IFN- $\gamma$ . Induction of  $\beta$ -cell apoptosis by PIC + IFN- $\gamma$  is mostly independent of iNOS expression and NO formation, but iNOS activation is required for PIC + IL-1 $\beta$ -induced  $\beta$ -cell death (13). INOS expression in  $\beta$ -cells depends on NF- $\kappa$ B activation (13, 49), and it is prevented by AdIKB<sup>(SA)2</sup> (27). Thus, it is conceivable that the protective effects of AdIKB<sup>(SA)2</sup> against PIC + IL-1 $\beta$  are, at least in part, owing to inhibition of NO formation. This, however, cannot be the explanation for the protective effects against PIC +

FIG. 9. Proposed model for the role of dsRNA in the early stages of  $\beta$ -cell dysfunction and death in T1DM.



IFN- $\gamma$  because the proapoptotic effect of these agents is not mediated by NO (13).

Chemokines are another group of cytokine-induced and NF- $\kappa$ B-regulated peptides, with a role in both innate and adaptive immune responses to viral infections (16, 62, 63). We presently observed that PIC increases the expression of mRNAs for several chemokines in pancreatic  $\beta$ -cells, including IP-10, fractalkine, and MIP-3 $\alpha$ . PIC alone did not increase MCP-1 expression, but it synergized with IFN- $\gamma$  to increase expression of MCP-1 mRNA. PIC increased IL-15 expression, an effect potentiated by both IL-1 $\beta$  and IFN- $\gamma$ . IP-10 is a CXC chemokine, reported to attract monocytes, T lymphocytes, and NK cells (16). MCP-1 is a CC chemokine, which attracts monocytes and T lymphocytes and may thus contribute to the migration of mononuclear cells to pancreatic islets in early T1DM (19). MIP-3 $\alpha$  attracts dendritic cells and induces adhesion of memory T cells to the adhesion molecule ICAM-1 (64, 65). Fractalkine plays a role in the chemoattraction and adhesion of IL-2-activated NK-cells and CD8<sup>+</sup> T cells (66). IL-15 is a cytokine that enhances the cytolytic function of T and NK cells and induces IFN- $\gamma$  production by NK cells. It is also a potent growth factor of T, B, and NK cells (67). IL-15 and the chemokines described above may play a role in diverse autoimmune diseases (17), including autoimmune diabetes in NOD mice (17–19). The fact that dsRNA triggers the expression of these chemokines in purified  $\beta$ -cells reinforces the hypothesis that chemokines, produced by the target of insulinitis, *i.e.* the  $\beta$ -cells, may contribute to amplify and exacerbate the local inflammatory process (19–21).

It has been recently proposed that PIC + IFN- $\gamma$ -induced pancreatic  $\beta$ -cell damage is mediated via IL-1 $\beta$  production by  $\beta$ -cells (46). We could not reproduce these findings. Thus, there was no significant increase in IL-1 $\beta$  mRNA expression following  $\beta$ -cell exposure to PIC and/or cytokines, alone or in combinations. Moreover, addition of an IRAP protected against IL-1 $\beta$  + IFN- $\gamma$ -mediated  $\beta$ -cell death but not against PIC + IFN- $\gamma$ -mediated  $\beta$ -cell apoptosis (Fig. 7). This, taken

together with the different patterns of mRNA expression induced by PIC and IL-1 $\beta$  (Ref. 13 and present data), suggests that PIC effects on  $\beta$ -cells, alone or in combination with IFN- $\gamma$ , are mostly independent of IL-1 $\beta$  production by the  $\beta$ -cells.

On the basis of the present and previous data (13), we propose a model for the effects of dsRNA in the early stages of insulinitis (Fig. 9). In the course of viral infection, dsRNA is accumulated in the cytoplasm of  $\beta$ -cells, leading to activation of the transcription factor NF- $\kappa$ B. NF- $\kappa$ B translocation to the nuclei leads to iNOS expression and consequent NO production, contributing to both  $\beta$ -cell apoptosis and necrosis. There are probably several additional NF- $\kappa$ B genes involved in  $\beta$ -cell apoptosis (23), but their nature remains to be characterized. Activated NF- $\kappa$ B also stimulates Fas expression, which may sensitize  $\beta$ -cells to apoptosis following interaction with FasL expressed on the surface of activated mononuclear cells. Attraction and activation of these mononuclear cells to the islets may depend on dsRNA-induced local production of chemokines and IL-15. These invading immune cells secrete cytokines such as IL-1 $\beta$ , IFN- $\gamma$ , and TNF- $\alpha$ , causing additional production of chemokines, further increasing insulinitis and the consequent dysfunction and damage of  $\beta$ -cells. In this proposed model, NF- $\kappa$ B activation plays a central role for  $\beta$ -cell demise. It will be of interest to determine whether blocking NF- $\kappa$ B *in vivo* protects  $\beta$ -cells against immune-mediated diabetes triggered in animal models as a consequence of viral infection.

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