Cytokine regulation of glutamate decarboxylase biosynthesis in isolated rat islets of Langerhans

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Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease in which cytokines are thought to play an important role in β -cell destruction and immune regulation. A major target of β -cell autoimmunity in IDDM is the enzyme glutamate decarboxylase (GAD). We hypothesized that cytokines in the insulinitis lesion modulate the synthesis of GAD. This may, in turn, modify the rate of β -cell destruction. Accordingly we cultured rat islets in the presence and absence of cytokines, and measured synthesis of both isoforms of GAD, GAD65 and GAD67, by [³⁵S]methionine incorporation and immunoprecipitation with a rabbit antiserum that recognizes both GAD65 and GAD67. Incubation of islets with interleukin (IL)-1 β (1 ng/ml, 24 h), tumour necrosis factor α (TNF- α ; 200 units/ml, 24 h) or interferon γ (IFN- γ ; 500 units/ml, 72 h) significantly decreased the synthesis of both GAD65 and GAD67, but reduced neither total protein synthesis nor insulin accumulation in the medium or content. Incubation of islets for 24 h in IFN- α (1000 units/ml),

TNF- β (50 ng/ml), IL 2 (1000 units/ml), IL-4 (100 ng/ml), IL-6 (10 ng/ml), IL-10 (20 ng/ml), IL-12 (10 ng/ml) or transforming growth factor β 2 (TGF- β 2; 5 ng/ml) did not significantly alter GAD65 or GAD67 synthesis. Inhibition of GAD65 and GAD67 protein synthesis by IL-1 β , TNF- α or IFN- γ was reversed by co-incubation with the nitric oxide synthase inhibitor, N^G-monomethyl arginine (NMMA). Expression of both GAD65 and GAD67 mRNA, measured by RNase protection assay, was also decreased by IL-1 β and completely restored to baseline levels by NMMA. Thus the synthesis of both isoforms of islet GAD is selectively decreased in the presence of IL-1 β , TNF- α or IFN- γ by a NO-mediated mechanism, probably at the level of cytokine gene transcription. As GAD autoimmunity has been previously shown to have a pathogenic role in an animal model of IDDM, its inhibition by cytokines might limit the immune response, thereby regulating the rate of β -cell destruction in IDDM.

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is the result of selective autoimmune destruction of insulin-secreting pancreatic islet β cells, a process in which cytokines are believed to play a pivotal role. A molecular target of β -cell autoimmunity in IDDM is the enzyme glutamate decarboxylase (GAD) [1]. GAD is expressed at highest levels in the central nervous system, where it catalyses the conversion of glutamate to the inhibitory neurotransmitter γ -aminobutyric acid (GABA), the main inhibitory neurotransmitter. High levels of GAD expression are also found in the pancreatic islets (reviewed in [2]), where the function of GABA remains uncertain [3]. However, the presence of GABA_A receptors on α (glucagon-containing) and δ (somatostatin-containing) cells of the islets suggests a role for GABA in paracrine signalling [4]. GAD exists as two isoforms, of molecular masses 65 kDa (GAD65) and 67 kDa (GAD67) [5]. GAD65 predominates in human islets [6], and is a target of autoantibodies in human IDDM [7]. In the rat both GAD isoforms are found, and in the mouse GAD67 predominates [2]. Antibodies to GAD have also been demonstrated in two animal models of autoimmune diabetes, the diabetes-prone BioBreeding (BB) rat [8], and the non-obese diabetic (NOD) mouse [9]. In the NOD mouse, intravenous [10] or intrathymic [11] injection of GAD65

results in decreased T-cell proliferative responses to GAD and protection from IDDM, indicating that GAD autoimmunity is important in the pathogenesis of IDDM in this model.

In the NOD mouse, the earliest stage of insulinitis is characterized by infiltration by macrophages, followed by T-cells [12]. Cytokines and other inflammatory mediators released by these cells are probably important both in the regulation of the immune response and in destruction of β cells (reviewed in [13]). Pancreatic histopathology in the human and the NOD mouse has the appearance of cell-mediated immunity, and the cytokines expressed by infiltrating cells in the insulinitis lesion include interleukin 1 β (IL-1 β), interferon γ (IFN- γ), tumour necrosis factor α (TNF- α), IL-4, IL-6 and TNF- β [14]. Additionally IL-2, IL-10 and IL-12 mRNA have been detected in NOD mouse pancreata [15]. There are few data about GAD expression in the insulinitis lesion and its regulation by cytokines. In one study, IL-1 β down-regulated GAD expression in isolated rat islets [16], but the effect of other cytokines was not examined. The free radical nitric oxide (NO) has cytotoxic effects and is released by a variety of cell types, including pancreatic β cells, in response to cytokines. In the β cell, NO is produced in response to cytokines such as IL-1 β and TNF- α [17], and is considered to be an important mediator of functional inhibition and cytotoxicity [13].

We propose that expression of GAD in islets is altered by

Abbreviations used: FBS, fetal bovine serum; GABA, γ -aminobutyric acid; GAD, glutamate decarboxylase; IDDM, insulin-dependent diabetes mellitus; IFN, interferon; IL, interleukin; NMMA, N^G-monomethylarginine; NOD, non-obese diabetic; TGF, transforming growth factor; TNF, tumour necrosis factor.

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cytokines released from inflammatory cells in the insulinitis lesion. As GAD has a demonstrated pathogenic role in IDDM, its modulation by cytokines might thereby regulate the rate of β -cell destruction. In this context we have examined the effect of cytokines and NO on rat islet GAD synthesis. Rat islets were chosen because they express both GAD65 and GAD67 proteins, which are readily detected by immunoprecipitation.

METHODS

Antibodies

Antiserum 1608 was raised in rabbits against a C-terminal fragment of human islet GAD65 encompassing residues 229–585, which is 75% identical with the C-terminus of GAD67. The fragment was expressed as a hexa-histidine fusion protein in *Escherichia coli*, and purified with Ni^{2+} -chelating affinity chromatography. Two isoform-specific rabbit anti-(GAD peptide) antisera were also raised. Antiserum 1872 was against the unique C-terminal GAD65 peptide VLEDNEERMSRLSK (residues 515–528) and antiserum 1881 was against the unique C-terminal GAD67 peptide GVPDSPERRWKLHR (residues 523–536); peptides were synthesized by Chiron Mimotopes (Victoria, Australia). The hybridoma producing the GAD65 monoclonal antibody GAD6 was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, U.S.A.) and GAD67-specific rabbit polyclonal antiserum K2 was from Chemicon International Inc. (Temecula, CA, U.S.A.). Monoclonal anti-rat MHC Class I (RT1.A) antibody CL007A was a gift from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada).

Cytokines

Murine IFN- γ was purchased from Genentech Inc. (San Francisco, CA, U.S.A.); murine IL-1 β and IL-6 were purchased from PeproTech (Rock Hill, NJ, U.S.A.); recombinant human IFN- α /D was purchased from Hoffman-La Roche (Nutley, NJ, U.S.A.); and murine IL-2, IL-4, IL-10 and IL-12, and human TNF- β and transforming growth factor β 2 (TGF- β 2) were purchased from Genzyme (Cambridge, MA, U.S.A.).

Islet isolation

Experimental procedures were approved by the local Animal Ethics Committee. Islets were isolated by the method of Lake et al. [18]. Briefly, 10–12-week-old male Wistar rats were killed by CO_2 inhalation and islets isolated by injection of intraductal 0.7–1.5 mg/ml collagenase (Collagenase P; Boehringer Mannheim GmbH, Mannheim, Germany), digestion for 15 min at 37 °C and purification on a discontinuous BSA (Advanced Protein Products, Brockmoor, West Midlands, U.K.) density gradient, with relative densities of 1.088, 1.069, 1.056 and 1.000. Islets were collected at the 1.069–1.056 interface, washed three times in RPMI and cultured overnight in RPMI supplemented with 2% (v/v) fetal bovine serum (FBS; CSL Ltd., Victoria, Australia).

Immunoblotting

Islets were lysed in 500 μ l of lysis buffer (10 mM CHAPS, 50 mM Hepes, 150 mM NaCl, 1 mM PMSF, 100 units/ml aprotinin, pH 7.4). Protein concentration was measured by the Bradford method [19] (Bio-Rad, Hercules, CA, U.S.A.), and lysate containing 200 μ g was added to SDS sample buffer and fractionated by SDS/PAGE [10% (w/v) gel] [20]. Proteins were electroblotted on to a nitrocellulose membrane [21], which was then blocked

overnight in 5% (w/v) milk powder in PBS (Blotto). Nitrocellulose strips were incubated in primary antibody, diluted 1:100 in Blotto, for 2 h. In peptide blocking experiments, antibodies were incubated overnight in the presence of 1 mg/ml peptide (final concentration) before use. After four washes in PBS, strips were incubated in anti-rabbit horseradish peroxidase-labelled secondary antibody. Strips were washed five times in PBS, and bands were detected with the enhanced chemiluminescence reagent (Amersham International, Amersham, Bucks., U.K.).

Islet culture, metabolic labelling and detection of GAD and MHC class I

Islets were washed twice in RPMI with 2% (v/v) FBS, and groups of approximately 500 were cultured for 24–72 h in 2 ml of RPMI with 2% FBS in the presence of cytokine. After two washes in methionine-free RPMI with 2% dialysed FBS, islet proteins were biosynthetically labelled by culture for 5 h in 1 ml of methionine-free RPMI with 2% dialysed FBS and 200 mCi of [^{35}S]methionine (Tran ^{35}S -label; ICN, Costa Mesa, CA, U.S.A.), in the presence of the appropriate cytokine. Islets were then washed three times in ice-cold PBS and lysed in 100 μ l of lysis buffer by repeated pipetting followed by leaving for 30 min at 4 °C. Lysates were clarified by centrifugation at 100 000 g for 30 min at 4 °C and then precleared by incubation for 2 h at 4 °C on an orbital plate shaker with 10 μ l (packed bead volume) of Protein A-Sepharose (Pharmacia, Uppsala, Sweden), to which 5 μ l of non-immune rabbit serum had been prebound. Radioactivity in the precleared lysates was measured by β -scintillation counting.

For immunoprecipitations, an equivalent amount of radioactivity in precleared lysate was added to 10 μ l of Protein A-Sepharose, to which 5 μ l of antiserum 1608 or control serum was prebound. After an overnight incubation at 4 °C on the shaker, immunoprecipitates were washed once in high-salt wash buffer (0.5% Triton X-100, 500 mM NaCl, 50 mM NaH_2PO_4 , pH 7.4), twice in PBS plus 0.5% Triton X-100 and once in 0.5% Triton X-100, 4.17 mM Tris, 192 mM glycine, pH 7.4. Immune complexes were eluted from the beads by boiling for 3 min in 20 μ l of double-strength SDS sample buffer and resolved by SDS/PAGE (10% gel). Gels were dried and exposed to a PhosphorImager (Molecular Dynamics) for 3 days. Bands were quantified by densitometry. The region immediately above the GAD67 band, which was free of bands, was used for the background reading, which was subtracted from the specific band density. Results for each band in the cytokine-exposed lane were reported as a percentage of the band intensity in the control culture, to which no cytokine was added. Results are expressed as the means $\pm$ S.E.M. for at least three experiments. In initial dose-response studies, a 2-fold difference in band density was regarded as significant.

In experiments in which MHC class I synthesis was measured, supernatants from immunoprecipitations with antiserum 1608 were incubated overnight at 4 °C with 10 μ l of Protein A-Sepharose to which 2 μ l of anti-(rat MHC class I) antibody CL007A had been prebound. A method identical with that described above was used for elution of immune complexes and detection by SDS/PAGE.

Protein synthesis

Rat islets ($n = 50$) were hand-picked in triplicate into 1 ml of RPMI with 2% FBS containing cytokine. After 3 days of culture

Figure 1 Detection of GAD65 and GAD67 in rat islet lysates

(A, B) Western blot of rat islet lysate, probed with pre-immune sera, and rabbit anti-GAD65 peptide antibody 1872 (A), and anti-GAD67 peptide antibody 1881 (B). Incubations in primary antibody performed in the presence and absence of GAD65 and GAD67 peptides. See text for peptide sequences. (C) Immunoprecipitation of [³⁵S]methionine-labelled rat islet lysate with anti-GAD65/GAD67 antiserum 1608 and with non-immune serum 1867.

they were washed twice with methionine-free RPMI plus 2% dialysed FBS. They were then cultured for 5 h in methionine-free RPMI with 2% dialysed FBS, 50 μ Ci of [³⁵S]methionine and cytokine. After being labelled they were washed three times in ice-cold PBS and lysed by leaving in 100 μ l of 10% (w/v) SDS for 30 min. Particulate matter was removed by centrifuging for 3 min at 11000 *g* and 4 °C. Incorporation of radioactivity into protein was measured as trichloroacetic acid-precipitable radioactivity. Lysate (10 μ l) was pipetted on to a filter paper disc, which was then dried for 10 min. The disc was then soaked in 2 ml of 10% (w/v) trichloroacetic acid for 10 min. The trichloroacetic acid was aspirated and the disc was soaked twice for 10 min in 95% (v/v) ethanol, dried, and radioactivity measured by β -scintillation counting.

Insulin accumulation in the medium, and content

Islets were isolated and precultured in RPMI with 2% FBS for 24 h. Islets ($n = 50$) were hand-picked into triplicate wells of a 24-well plate containing 2 ml of RPMI with 2% FBS and cytokine. Islets were cultured for 24 h in the presence of IL-1 β (1 ng/ml) and TNF- α (200 units/ml), and accumulated insulin was measured in the supernatant by radioimmunoassay (Phadeseph Insulin RIA; Pharmacia). For IFN- γ , in which islets were incubated for a total of 72 h, the medium was changed at 48 h to allow the final 24 h insulin accumulation to be measured. Insulin content was assessed by sonicating islets (10 pulses, Branson Sonifier) in 50 mM K₂HPO₄. Insulin in the sonicates was measured by radioimmunoassay.

GAD65 and GAD67 expression by RNase protection assay

Islets ($n = 3000$) were cultured for 24 h in Petri dishes in 3 ml of RPMI with 2% FBS in the presence of medium alone or IL-1 β and (3) IL-1 β plus *N*^G-monomethylarginine (NMMA). They were then washed twice in ice-cold PBS, pelleted by centrifugation and resuspended in 4 M guanidinium isothiocyanate containing 25 mM sodium acetate and 1 mM EDTA. Total RNA was isolated with a modified guanidinium/CsCl ultracentrifugation method as previously described [22]. Purified ³²P-labelled mouse brain GAD cRNAs were hybridized with total RNAs from rat islets and rat brain (positive control), and tRNA (negative control), at 50 °C for at least 10 h. Hybrids were digested with 2 μ g/ml RNase A (X-A grade; Sigma, St. Louis, MO, U.S.A.) and 0.1 μ g/ml RNase T1 (Sigma) for 30 min at 22 °C. The digestion products were analysed by denaturing urea gel electrophoresis, and the dried gels were imaged on the PhosphorImager. Protected RNA species were identified by comparison with an undigested probe and the signal produced by hybridization with rat brain GAD mRNAs. The integrity of the islet mRNAs was checked with a cRNA for mouse β -actin, which was hybridized and processed with the same procedure.

Statistical analysis

The significance of the ratio of band intensity in cytokine-treated lanes compared with control lanes was determined by Student's *t*-test after logarithmic transformation of data. Differences in protein synthesis and insulin accumulation in the medium and

Figure 2 Effect of cytokines on GAD synthesis in rat islets

Islets were incubated with cytokine, followed by [35 S]methionine labelling. GAD65 and GAD67 were immunoprecipitated with antiserum 1608. Incubation (24 h) of islets in IL-1 β (A) and TNF- α (B); 24, 48 and 72 h incubation of islets with IFN- γ (C). Supernatants from immunoprecipitations in (C) were reprecipitated with MHC class I antibody CL007A (D). Immunoprecipitates were eluted, resolved by SDS/PAGE and bands were detected by autoradiography. Abbreviation: u, units.

content were compared by Student's *t*-test on raw data; $P < 0.05$ was regarded as significant.

RESULTS

GAD expression in rat islets

GAD65 and GAD67 were identified in rat islet lysates by immunoblotting (Figures 1A and 1B). A band representing GAD65 was recognized by the GAD65 peptide antiserum 1872, but not by the GAD67 peptide antiserum 1881. Preincubation of antiserum 1872 with the GAD67 peptide had no effect on the lower band, but preincubation with the GAD65 peptide abolished it. Similarly, a higher-molecular-mass band representing GAD67 was recognized by the GAD67 peptide antiserum 1881; this band was blocked by preincubation of antiserum 1881 with the GAD67 but not the GAD65 peptide.

Immunoprecipitation with the rabbit antiserum 1608, raised against the C-terminal fragment of GAD65 similar to GAD67, revealed two bands of similar molecular mass to those detected by immunoblotting with specific antisera, which were not recognized by the non-immune serum 1867 (Figure 1C).

Modulation of GAD protein synthesis by cytokines

In initial experiments designed to determine cytokine dose-response relationships, islets were incubated for 24 h in a range of cytokine concentrations. IL-1 β caused a dose-dependent decrease in both GAD65 and GAD67 synthesis at 0.01, 0.1 and 1 ng/ml. At these doses, GAD67 synthesis was reduced to 60%, 13% and 18% of control levels and GAD65 synthesis to 49%, 8% and 19% respectively (Figure 2A). IL-1 β at 0.001 ng/ml did not inhibit GAD synthesis. TNF- α at 50, 200 and 1000 units/ml caused a dose-dependent decrease of GAD65 synthesis to 51%, 35% and 21% respectively of control levels but had no significant effect on GAD67 in these initial experiments (Figure 2B). IFN- γ at 200 or 500 units/ml over 24 h had no consistent effect on GAD65 or GAD67, but 200 units/ml IFN- γ inhibited GAD

synthesis in a time-dependent manner when islets were incubated for 48 and 72 h (Figure 2C). To demonstrate a biological effect of IFN- γ , supernatants from the anti-GAD immunoprecipitates were reprecipitated with the rat MHC class I-specific monoclonal antibody CL007A (Figure 2D). IFN- γ caused a time-independent increase in MHC class I synthesis to 264%, 364% and 313% of control levels at 24, 48 and 72 h respectively. GAD synthesis was not altered by more than 2-fold compared with the control by IL-2 (50, 500 and 1000 units/ml), IL-4 (0.1, 10 and 100 ng/ml), IL-6 (0.2, 2 and 20 ng/ml), IL-10 (0.2, 2 and 20 ng/ml), TGF- β 2 (0.001, 0.05, 0.5 and 5 ng/ml) or IL-12 (0.1 and 1 ng/ml), but GAD65 and GAD67 synthesis was reduced to 49% and 46% respectively by the highest concentration of IL-12 (10 ng/ml).

To quantify and confirm the effect of these cytokines on GAD synthesis, we performed replicate ($n = 3-6$) experiments with a single dose and duration of incubation. Statistically significant reductions in GAD synthesis were observed when islets were incubated in IL-1 β , TNF- α and IFN- γ . With IL-1 β (1 ng/ml, 24 h), GAD65 and GAD67 band intensity was decreased to $24 \pm 8.7\%$ ($P < 0.01$) and $32 \pm 6.3\%$ ($P < 0.005$) of control respectively, with TNF- α (200 units/ml, 24 h) to $26 \pm 6.4\%$ ($P < 0.05$) and $73 \pm 6.8\%$ ($P < 0.05$) and with IFN- γ (500 units/ml, 72 h) to $56 \pm 7.9\%$ ($P < 0.05$) and $65 \pm 6.4\%$ ($P < 0.05$). GAD65 and GAD67 were not significantly altered by either IFN- α (1000 units/ml), TNF- β (50 ng/ml), IL-2 (1000 units/ml), IL-4 (100 ng/ml), IL-6 (10 ng/ml), IL-10 (20 ng/ml) or TGF- β 2 (5 ng/ml), and the modest effect of IL-12 (10 ng/ml) that we previously observed was not statistically significant.

Throughout these experiments a 130 kDa protein was detected when islets were incubated in IL-1 β , TNF- α or IFN- γ (representative experiment shown in Figure 2A). This band did not seem to contain GAD65 or GAD67, as it was not precipitated by the GAD65-specific monoclonal antibody GAD6 or by the GAD67-specific polyclonal antiserum K2. We speculated that this protein may represent an autoantigen that is expressed in the presence of cytokines. Accordingly, 10 sera from islet cell antibody-positive subjects who either subsequently developed IDDM ($n = 6$) or had impaired first-phase insulin release to an

Table 1 Effect of cytokines and combinations of cytokines on total protein synthesis

Islets ($n = 50$) were cultured in cytokine (1 ng/ml IL-1 β , 200 units/ml TNF α , 500 units/ml IFN- γ), alone or in combination, for 24 or 72 h. Trichloroacetic acid-precipitable radioactivity was measured in islet lysates after a 5 h incubation with [35 S]methionine. P related to the difference between incubation in medium alone and with cytokine. Abbreviation: n.s., not significant.

	Culture duration (h)	Radioactivity ($10^{-3} \times$ d.p.m.; mean $\pm$ S.E.M.)	P
Medium alone	24	126.9 $\pm$ 20.7	
IFN- γ	24	88.2 $\pm$ 10.9	n.s.
TNF- α	24	124.8 $\pm$ 12.5	n.s.
IL-1 β	24	89.4 $\pm$ 22.8	n.s.
Medium alone	72	71.2 $\pm$ 5.7	
IFN- γ	72	66.5 $\pm$ 9.8	n.s.
TNF- α	72	57.4 $\pm$ 4.4	n.s.
IL-1 β	72	54.2 $\pm$ 2.1	0.05
IFN- γ + TNF- α	72	15.4 $\pm$ 7.8	< 0.01
IL-1 β + IFN- γ	72	26.1 $\pm$ 3.0	< 0.005
IL-1 β + TNF- α	72	35.3 $\pm$ 3.8	< 0.01

Table 2 Insulin accumulation in the medium, and content

Islets ($n = 50$) were cultured in IL-1 β , TNF- α and IFN- γ for the durations indicated. Accumulation and content of insulin over 24 h were measured by radioimmunoassay.

	Culture duration (h)	Insulin accumulation (m-units/ml)	Insulin content (m-units/ml)
Medium alone	24	1.35.9 $\pm$ 0.11	16.3 $\pm$ 3.38
TNF- α	24	2.18 $\pm$ 0.24*	19.7 $\pm$ 1.78
IL-1 β	24	1.46 $\pm$ 0.13	17.3 $\pm$ 0.96
Medium alone	72	1.80 $\pm$ 0.18	13.6 $\pm$ 2.35
IFN- γ	72	1.55 $\pm$ 0.23	20.9 $\pm$ 3.09

* $P < 0.05$ compared with medium alone.

intravenous glucose tolerance test ($n = 4$) were used in immunoprecipitations of [35 S]methionine-labelled islets. None of these sera immunoprecipitated a 130 kDa band from lysates of islets cultured in 200 units/ml IFN- γ for 24 h, despite a prominent 130 kDa band precipitated from islets with antibody 1608 (results not shown). These results indicate that the 130 kDa protein is not an IDDM-related autoantigen in these preclinical or high-risk subjects.

Effect of cytokines on total protein synthesis

To examine whether inhibition of GAD synthesis by cytokines was due to a reduction in total protein synthesis, we measured total [35 S]methionine incorporation after incubation of islets in cytokines. Total protein synthesis measured in this manner was not significantly affected by incubation of islets in IL-1 β (1 ng/ml, 24 h), IFN- γ (500 units/ml, 72 h) or TNF- α (200 units/ml, 24 h). With prolonged (72 h) incubation at these doses, IFN- γ and TNF- α did not significantly reduce total protein synthesis but IL-1 β caused a modest decrease. However, combinations of these cytokines caused significant decreases in total protein synthesis (Table 1).

Insulin accumulation in the medium, and content

We next measured insulin accumulation in the medium and content by using identical cytokine doses and duration with those that inhibited GAD synthesis. Insulin accumulation was not significantly altered by IL-1 β (1 ng/ml, 24 h) or IFN- γ (500 units/ml, 72 h), but was significantly increased by TNF- α (200 units/ml, 24 h). Insulin content was not altered by any of these cytokines (Table 2).

Role of NO

We next explored the role of NO as a potential mediator of inhibition of GAD synthesis by IL-1 β , TNF- α or IFN- γ by using the NO synthase inhibitor NMMA. When islets were incubated with IL-1 β (1 ng/ml, 24 h), TNF- α (200 units/ml, 24 h) or IFN- γ (500 units/ml, 72 h), a reduction in GAD65 and GAD67 synthesis was observed. However, co-incubation of islets with these cytokines and NMMA (0.5 mM) consistently restored GAD synthesis to control levels (Figure 3).

GAD65 and GAD67 mRNA expression

To determine whether cytokine-induced down-regulation of GAD and its reversal by NMMA occurs at the level of protein synthesis or the level of mRNA, we measured the expression of GAD65 and GAD67 RNA by RNase protection assay. With IL-1 β as a representative cytokine, islets were cultured for 24 h in medium alone or with cytokine (1 ng/ml), in the absence and presence of NMMA (0.5 mM). When probed with GAD65 cRNA, expression of both GAD65 and GAD67 mRNA was reduced when islets were cultured with IL-1 β , and restored by co-culture with NMMA (Figure 4). Expression of β -actin mRNA was not altered by IL-1 β in the presence or absence of NMMA.

DISCUSSION

Several cytokines have been shown to inhibit islet function *in vitro* and may mediate β -cell destruction in human and animal IDDM. The most potent is the macrophage product IL-1 β [23]. In one study, culture of rat islets in IL-1 β caused a bimodal response, with an initial enhancement followed by a reduction in insulin release and content, that was dependent on dose and ambient glucose concentration [24]. In contrast, TNF- α or IFN- γ did not affect glucose-stimulated insulin release but potentiated the effect of IL-1 β when administered in combination [25]. In addition to causing functional inhibition of islets, cytokines are cytotoxic to β cells *in vitro*. In one study, exposure of rat islet cells to IFN- γ , TNF- α , TNF- β or IL-1 β alone did not cause cytotoxicity except for a small dose-dependent effect of IFN- γ . However, combinations of these cytokines were cytotoxic [26]. In another study [27], prolonged exposure to IL-1 β led to β -cell destruction. Our experiments, in which total islet protein synthesis was measured as [35 S]methionine incorporation, show that a short (24 h) incubation of islets in IL-1 β , TNF- α or IFN- γ , and a prolonged (72 h) incubation in TNF- α or IFN- γ , does not inhibit protein synthesis. However, a prolonged (72 h) incubation in IL-1 β causes a modest decrease in protein synthesis, and combinations of IL-1 β , TNF- α or IFN- γ cause a significant decrease. In contrast, culturing islets in the presence of IL-1 β , TNF- α or IFN- γ under conditions that do not affect total protein synthesis reduces the synthesis of both GAD isoforms, indicating that GAD synthesis is more sensitive to the inhibitory effects of these cytokines than is overall protein synthesis. Biosynthetic labelling and immunoprecipitation, and mRNA

Figure 3 Effect of NMMA on inhibition of GAD synthesis in the presence of 1 ng/ml IL-1 β (A), 200 units/ml TNF- α (B) and 500 units/ml IFN- γ (C)

Islets were cultured for 24 h (A, B) or 72 h (C) in medium alone (left lanes), cytokine (middle lanes) and cytokine with 50 mM NMMA (right lanes). GAD65 and GAD67 were detected by biosynthetic labelling with [35 S]methionine and immunoprecipitation with antiserum 1608. Results are representative of three separate experiments. Abbreviation: U, units.

quantification, were employed as more sensitive indicators of alterations in GAD synthesis in these experiments than measurement of GAD enzymic activity [16] or detection of GAD by immunoblotting. As β -cell destruction in human and rodent IDDM is a gradual process, presumably involving extended exposure to cytokines, it is possible that the degree of inhibition of GAD expression *in vivo* is more pronounced than in these experiments.

Although GAD synthesis was decreased by IL-1 β , TNF- α and IFN- γ , expression of several specific proteins was either increased or unchanged. A 130 kDa protein, precipitated by a polyclonal antibody raised against the C-terminus of GAD65, was markedly induced by all cytokines that decreased GAD synthesis. The identity of this protein is unknown, although the fact that it was not recognized by several anti-GAD antibodies indicates that it is unlikely to contain GAD. Expression of this protein was not altered by the addition of NMMA (results not shown), indicating that it is induced by a factor that is proximal to NO in the IL-1 β signal transduction pathway. We also demonstrated that IFN- γ induces MHC class I, a finding that has been observed in mouse islets [28]. A number of proteins have been shown to be induced by cytokines such as IL-1 β ; these are thought to represent proteins involved in signal transduction, proteins induced by signal transduction, or cellular stress proteins [29]. Despite inhibition of GAD synthesis, there was no corresponding de-

Figure 4 Effect of NO synthase inhibitor (NMMA) and IL-1 β on GAD65 and GAD67 mRNA

RNase protection assay with GAD65 (top), GAD67 (middle) and β actin (lower) cRNA probes.

crease in insulin content or accumulation in the medium under the same conditions that inhibited GAD synthesis.

Cytokine-mediated inhibition of insulin release [17] and β -cell cytotoxicity [30] has been shown to be mediated by the free radical gas NO, which is produced by the oxidation of L-arginine to citrulline catalysed by nitric oxide synthase [31]. The inducible form of nitric oxide synthase, which is involved in cytokine-mediated cytotoxicity, is expressed in pancreatic β cells but not in α cells [32]. NO is also synthesized in macrophages and endothelial cells, from where it may diffuse rapidly to affect β cells. NO is thought to inactivate iron-containing molecules such as aconitase of the citric acid cycle [33]. A number of compounds inhibit NO synthesis, including NMMA, L-N G -nitroarginine and L-N G -nitroarginine methyl ester. Inhibition of NO synthase reverses IL-1 β -induced β -cell toxicity and inhibition of insulin release, suggesting that NO is involved in a single common pathway in cytokine-induced changes in the β cell. Oxygen radicals such as superoxide and the hydroxyl radical may also play a part in cytokine-mediated β -cell dysfunction and destruction. This finding is supported by the observation that exposure of rat islets to combinations of IL-1 β , TNF- α and IFN- γ resulted in increased levels of malondialdehyde, an end product of lipid peroxidation [34]. Furthermore the use of free radical scavengers protected islets from cytokine-induced damage [35]. Our finding that NMMA reverses inhibition of GAD synthesis by IL-1 β , TNF- α and IFN- γ demonstrates that NO plays a key role in cytokine-induced inhibition of GAD synthesis. The RNase protection assay showed that GAD65 and GAD67 mRNA is down-regulated by IL-1 β and is returned to baseline levels by co-culture with NMMA. Thus the NO-mediated inhibition of GAD synthesis by IL-1 β occurs at the level of transcription.

What is the significance of down-regulation of GAD by specific cytokines in β -cell autoimmunity? The demonstrated central role of GAD autoimmunity in the pathogenesis of IDDM indicates that its modulation may influence the rate of β -cell destruction. Some evidence suggests that the level of GAD expression is associated with the rate of β -cell destruction. In a human study, diazoxide, which inhibits insulin secretion and GAD expression in islets, improved endogenous insulin production after diagnosis of IDDM [36]. It has been postulated that down-regulation of the target autoantigen might also be relevant in the protective effect of subcutaneous insulin in preclinical IDDM in the mouse [37] and human [38], where inhibition of β -cell autoantigens might result from β -cell 'rest'. GAD expression is increased in states associated with the development of diabetes in animal models: in one study, infection of mice with Coxsackie virus resulted in increased GAD expression in islets, the appearance of GAD antibodies and the development of diabetes [39]. Interestingly, administration of IL-1 β , TNF- α and IFN- γ to rodent models of IDDM has been shown to reduce insulinitis and diabetes in some studies [40–42]. The mechanism for this paradoxical protection is likely to be complex, depending on the multiple direct and indirect effects of these cytokines. It is possible, however, that cytokine-mediated down-regulation of the target autoantigen is one mechanism by which the immune response is inhibited.

In conclusion, we have demonstrated inhibition of GAD expression at the mRNA and protein level in rat islets by IL-1 β , TNF- α or IFN γ . This effect is mediated by NO, probably at the level of transcription, and may be a response that partly limits β -cell destruction in IDDM.

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