Effects of interleukin-12 *in vitro* on pancreatic islets isolated from normal rodents and from non-obese diabetic mice

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Abstract

Administration of the T-helper 1 (Th1) cell promoting cytokine interleukin-12 (IL-12) accelerates the development of autoimmune diabetes in non-obese diabetic (NOD) mice. In this study we examined the effects of IL-12 on isolated islets from NMRI (Naval Medical Research Institute-established) mice, Sprague-Dawley (S-D) rats and NOD mice. NMRI and S-D islets were cultured in medium RPMI 1640 + 10% fetal calf serum and exposed for 48 h to recombinant mouse IL-12 (0, 0.1, 1 and 10 ng/ml). Islet glucose metabolism, as measured by glucose oxidation rate, was suppressed by about 25% in NMRI islets exposed to 10 ng/ml IL-12. In rat islets 0.1 ng/ml IL-12 induced a 20% decrease in glucose oxidation rate. Islets cultured with 10 ng/ml IL-12 showed a decrease in medium insulin accumulation both in mouse and rat. Glucose-stimulated insulin release was lowered in rat islets exposed to 10 ng/ml IL-12, but not affected in NMRI islets. In NMRI islets IL-12 did not influence nitric oxide production as measured by nitrite formation. In rat islets IL-12 induced a decrease in nitrite formation compared with control islets. Islets were isolated from female NOD mice (age 5, 12, 20 and 26 weeks) and examined either immediately or cultured for 7 days with

10 ng/ml IL-12 alone or in combination with 4 ng/ml of the T-cell stimulating cytokine interleukin-2 (IL-2). In the age groups >5 weeks of age the glucose-stimulated insulin release was lower in freshly isolated compared with cultured control islets. IL-2+IL-12 addition induced a small decrease in glucose-stimulated insulin release in islets from 12-week-old animals. With increasing age the DNA content in freshly isolated islets increased due to immune cell infiltration. The DNA content in cultured islets was decreased by 40–60% compared with freshly isolated islets in the age groups over 5 weeks. Islet insulin content was similar in both freshly isolated and cultured islets. None of the cytokines, either alone or in combination, affected islet DNA or insulin content. We conclude that IL-12 has minor suppressive effects *in vitro* on normal rodent islets. It is likely that the reported accelerated diabetes development of IL-12 administration to NOD mice *in vivo* is not mediated by a direct toxic effect to the islets. The suppressed insulin release in NOD mouse islets treated with IL-2+IL-12 suggests, however, that the accelerating effect might partly be attributed to stimulation of immune cells present in the insulinitic lesion.

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Introduction

The disease process leading to human insulin-dependent diabetes mellitus (IDDM) is believed to be of autoimmune origin (Gepts 1965, Eisenbarth 1986), with immune cells infiltrating the islets of Langerhans, i.e. insulinitis, leading to massive β -cell destruction. In animal models of IDDM similar findings have been observed (Nakhooa *et al.* 1978, Makino *et al.* 1980). Results from studies in the non-obese diabetic (NOD) mice have suggested that the autoimmune response is mediated by the T-helper 1 (Th1) subset of T-cells (Rabinovitch 1994). Interleukin-12 (IL-12) is a cytokine playing a central role in promoting a Th1 immune response (Trinchieri 1995). It can thus be envisaged that IL-12 has a key role in launching a Th1 mediated β -cell suppressive immune response in IDDM

(Rabinovitch 1994). In high risk IDDM first degree relatives, IL-12 production *in vitro* was increased, supporting the hypothesis that IL-12 is involved in the pathogenesis of IDDM (Szelachowska *et al.* 1997). In other experiments it has been shown that daily injection of this cytokine caused an increase and acceleration of diabetes development in NOD mice (Trembleau *et al.* 1995). On the contrary, weekly injections of IL-12 prevented diabetes in NOD mice (O'Hara *et al.* 1996). There is also an association between IL-12 gene expression and disease development both in cyclophosphamide induced diabetes in NOD mice (Rothe *et al.* 1996) and during the natural disease process in this strain of mice (Rabinovitch *et al.* 1996) and when administered *in vivo*, the IL-12 antagonist IL-12(p40)₂ prevented cyclophosphamide induced diabetes in NOD mice (Trembleau *et al.* 1996, Rothe *et al.* 1997).

There are no data regarding the direct effects of IL-12 on islet function. Moreover, the accelerating effects of IL-12 on disease development might be due to either a stimulating effect on cells already present in the insulinitic lesion or a stimulation of the Th1 system in general.

To address these questions we evaluated the effects of IL-12 *in vitro* on isolated islets of Langerhans from normal mice and rats, and we also studied the effects of the cytokine *in vitro* on islets isolated from NOD mice (Strandell *et al.* 1990). In the older age groups the freshly isolated islets of the NOD mice contain a large amount of infiltrating immune cells. In the experiments with NOD mouse islets we also studied a combination of IL-2 and IL-12, since experiments *in vitro* have shown that IL-12 can act in synergy with IL-2 in stimulating proliferation of natural killer cells and lymphokine activated killer cells (DeBlaker *et al.* 1995) as well as T-cells (Armant *et al.* 1996). Proliferation of islet-associated leukocytes, has been reported to occur when islets with insulinitis are cultured together with IL-2 (Nagata *et al.* 1989) so it is possible that these two cytokines are required to maintain an immune response directed against the islet β -cells *in vivo*.

Materials and Methods

Animals, islet isolation and tissue culture

Islets from male Sprague-Dawley (S-D) rats, bred in a local colony (Biomedical Centre, Uppsala, Sweden), and male NMRI (Naval Medical Research Institute-established) mice (BK Universal, Sollentuna, Sweden) were isolated by a collagenase digestion procedure (Sandler *et al.* 1987). Groups of 150–200 islets were kept free-floating in culture medium RPMI 1640 (Sigma Chemicals, St Louis, MO, USA) supplemented with 10% (vol/vol) fetal calf serum (Sigma), benzylpenicillin (100 U/ml) and streptomycin (0.1 mg/ml). The islets were cultured at 37 °C in a gas phase of humidified air + 5% CO₂ for 7 days before use. The culture medium was changed every second day. Islets in groups of 50 were subsequently transferred to new non-attachment culture dishes containing 2.25 ml RPMI 1640 + 0.25 ml fetal calf serum and exposed for 48 h to recombinant murine IL-12 (R&D Systems, Abingdon, Oxon, UK; 0, 0.1, 0.5, 1, 10 ng/ml). After this period islets were used for functional studies, as described below.

In the other experiments islets from female NOD mice were used. The NOD mice were obtained from a local colony established at the Biomedical Centre, Uppsala University in 1988. Originally, three breeding pairs of inbred NOD mice were obtained from the Clea Company (Aobadi, Japan). The colony was re-derived by caesarean delivery in 1992, due to a decreasing diabetes incidence and infection with mouse hepatitis virus. The mice have subsequently been maintained barrier-bred under pathogen-free conditions and the cumulative diabetes

incidence is 65% in the females and <5% in the males at 30 weeks of age. The pancreatic islets from non-diabetic NOD mice aged 5, 12, 20 or 26 weeks were either studied immediately after isolation or cultured for 7 days in the presence of IL-12 (10 ng/ml) and/or recombinant murine IL-2 (R&D Systems; 4 ng/ml). Islets from diabetic mice were not used because of the difficulties obtaining a sufficient number of islets from these animals. Culture medium was changed on days 2, 4 and 6. Control islets were cultured with medium alone without cytokine addition. The number of islets obtained from NOD mice aged 26 weeks were too few to allow culture with cytokines, and therefore only islets cultured under control conditions were included from this age group.

Islet insulin and DNA content and glucose-stimulated insulin release

After the culture period (S-D and NMRI) or upon medium exchange (NOD), samples were taken from the culture medium for insulin and nitrite determinations (see below). Duplicate (NOD) or triplicate (S-D and NMRI) groups of 10 islets each were transferred to sealed glass vials containing 0.25 ml Krebs–Ringer bicarbonate buffer (Krebs & Henseleit 1932) supplemented with 2 mg/ml BSA (Miles Laboratories, Slough, Bucks, UK) and 10 mM Hepes (Sigma Chemicals), hereafter referred to as KRBH. During the first hour of incubation at 37 °C (O₂:CO₂; 95:5) the KRBH medium contained 1.7 mM glucose. The medium was then gently removed and replaced by 0.25 ml KRBH supplemented with 16.7 mM glucose and the incubation continued for a second hour. The insulin concentration was measured by RIA (Heding 1972). For the RIA, porcine insulin was used for the standard curve, ¹²⁵I-labelled insulin was obtained from EuroDiagnostica (Malmö, Sweden) and the anti bovine insulin antibody was from Biomakor (Rehovot, Israel).

After the incubation, islets were pooled and ultrasonically disrupted in 0.2 ml redistilled water. A 50- μ l fraction of the aqueous homogenate was mixed with 125 μ l acid ethanol (0.18 M HCl in 96% (vol/vol) ethanol) and the insulin was extracted overnight at 4 °C. DNA was measured in another fraction of the water homogenate by means of a fluorophotometrical method (Kissane & Robins 1958, Hinegardner 1971).

Islet glucose oxidation rate

Triplicate groups of 10 islets each were transferred to glass vials (Keen *et al.* 1963) containing 100 μ l KRBH without BSA, but supplemented with D-[U-¹⁴C]glucose (Amersham International, Amersham, Bucks, UK) and non radioactive glucose to a final concentration of 16.7 mM and a specific activity of 0.5 mCi/mM. The islet glucose oxidation rate was subsequently measured as previously described (Andersson & Sandler 1983).

Table 1 Effects of long-term treatment with murine IL-12 on pancreatic islets isolated from Sprague-Dawley (S-D) rats and from NMRI mice on islet DNA, insulin content and medium insulin accumulation. Islets were maintained in medium RPMI 1640 + 10% fetal calf serum for 48 h with IL-12 present at the concentrations indicated. The medium insulin accumulation (ng/48 h × 10 islets) in the control groups were 873 ± 84 (S-D) and 477 ± 78 (NMRI). Values are given as means ± S.E.M. for 17 (rat) or 6 (mouse) experiments

	IL-12 (ng/ml)	DNA content (µg/10 islets)	Insulin content (ng/10 islets)	Insulin/DNA (ng/µg DNA)	Medium insulin accumulation (% of control)
Islets					
S-D	0	0.38 ± 0.02	610 ± 62	1660 ± 190	100 ± 0
S-D	0.1	0.38 ± 0.03	620 ± 65	1820 ± 190	89 ± 6.3
S-D	0.5	0.38 ± 0.03	630 ± 77	1840 ± 270	89 ± 8.1
S-D	1.0	0.40 ± 0.02	620 ± 71	1550 ± 180	93 ± 8.3
S-D	10	0.40 ± 0.02	650 ± 86	1620 ± 170	72 ± 4.1***
NMRI	0	0.24 ± 0.02	350 ± 51	1410 ± 150	100 ± 0
NMRI	1.0	0.29 ± 0.01	350 ± 45	1200 ± 180	88 ± 12
NMRI	10	0.26 ± 0.01	320 ± 53	1260 ± 220	80 ± 7.5*

Symbols (*, ***) denote $P < 0.05$ and $P < 0.001$ respectively, compared with control, using paired Student's *t*-test.

Nitrite determination and medium insulin accumulation

To aliquots of the culture medium (100 µl), 10 µl 0.5% naphthylethylenediamine dihydrochloride together with 5% sulphanilamide (Sigma) and 25% concentrated H_3PO_4 were added (Green *et al.* 1982). The reaction was carried out at 20 °C for 5 min, and the absorbance was measured at 546 nm in a Beckman DU-62 spectrophotometer (Palo Alto, CA, USA) against a standard curve. The detection limit of the assay was 0.25 µM. Medium insulin accumulation was determined by RIA (Heding 1972).

Statistical analysis

A mean was calculated from each duplicate or triplicate group of islets and then considered as one separate observation. Furthermore, every observation represented different islet donors. Values are expressed as means ± S.E.M. and groups of data were compared, using Student's unpaired or paired *t*-test.

Results

Effects of IL-12 on islets isolated from Sprague-Dawley rats and from NMRI mice

Culture of normal rodent islets for 48 h with IL-12 (10 ng/ml) caused a 20–30% reduction in the medium insulin accumulation (Table 1). This effect was not observed at lower concentrations of IL-12. Addition of the cytokine did not affect the islet DNA or insulin content (Table 1). Exposure to IL-12 did not influence the basal insulin secretion at 1.7 mM glucose of the rodent islets (Table 2). The glucose-stimulated insulin secretion at 16.7 mM glucose was decreased from rat islets at

the highest concentration of IL-12, whereas no such significant effect was observed in islets from NMRI mice (Table 2). The highest concentration of IL-12 reduced islet glucose metabolism by about 25%, as measured by the glucose oxidation rate in islets of NMRI mice (Table 2). In rat islets a reduced glucose oxidation rate was observed when 0.1 ng/ml were added, but not with higher concentrations of the cytokine. IL-12 did not affect nitric oxide production from NMRI mouse islets, as measured by nitrite formation (Table 2). However, in rat islets IL-12 decreased nitrite formation as compared with the control group (Table 2).

Effects of IL-12 on islets isolated from NOD mice of different age

From the elderly groups of NOD mice, i.e. at 20 and 26 weeks, the yield of islets after isolation from the pancreas was much reduced, suggesting an ongoing loss of islets *in vivo*. The freshly isolated islets exhibited a progressively increased DNA content with age (Fig. 1). In the islets isolated from NOD mice older than 5 weeks, culture for 7 days decreased the islet DNA content as compared with the value of the corresponding freshly isolated islets (Fig. 1). There were no differences in the islet insulin content, as expressed on a per islet basis, between cultured and freshly isolated islets in any of the age groups (Fig. 2). However, as a consequence of the decreasing islet DNA content in cultured islets, the insulin content per DNA was significantly increased in cultured islets in the age groups above 5 weeks (Fig. 3). The basal insulin release at 1.7 mM glucose was similar in all groups of NOD mouse islets both when examined immediately after isolation and after one week of culture (data not shown). Insulin secretion at 16.7 mM glucose from freshly isolated islets was also similar in the different groups of islets, although

Table 2 Effects of long-term treatment with murine IL-12 on pancreatic islets isolated from Sprague-Dawley (S-D) rats and from NMRI mice on islet insulin release, glucose oxidation rate and medium nitrite accumulation. Islets were maintained in medium RPMI 1640+10% fetal calf serum for 48 h with IL-12 present at the concentrations indicated. Values are given as means ± S.E.M. for 17 (rat) or 8 (mouse) experiments

	IL-12 (ng/ml)	Insulin release (ng/μg DNA × 60 min)		Glucose oxidation rate (pmol/10 islets × 90 min)	Medium nitrite accumulation (pmol/islet × h)
		1.7 mM glucose	16.7 mM glucose		
Islets					
S-D	0	26 ± 5.0	180 ± 23	615 ± 58	0.37 ± 0.08
S-D	0.1	27 ± 5.5	160 ± 19	488 ± 62**	0.20 ± 0.06\$\$
S-D	0.5	27 ± 4.8	200 ± 25	521 ± 78	0.44 ± 0.08
S-D	1.0	24 ± 2.3	160 ± 15	542 ± 43	0.23 ± 0.08\$
S-D	10	24 ± 2.4	130 ± 13\$	558 ± 49	0.03 ± 0.03\$\$\$
NMRI	0	14 ± 1.8	124 ± 11	422 ± 31	0.03 ± 0.02
NMRI	1.0	15 ± 2.0	97 ± 13	338 ± 40	0.0 ± 0.0
NMRI	10	16 ± 0.7	102 ± 12	306 ± 28*	0.0 ± 0.0

Symbols (*,**) denote $P<0.05$ and $P<0.01$ respectively, compared with control, using unpaired Student's *t*-test. Symbols (\$,\$\$, \$\$\$) denote $P<0.05$, $P<0.01$ and $P<0.001$ respectively, compared with control, using paired Student's *t*-test.

there was a trend for a decline in the eldest age group (Table 3). In the three age groups of islets obtained from NOD mice more than 5 weeks of age, the insulin secretion at 16.7 mM glucose was increased after culture as compared with corresponding freshly isolated islets. Culture of islets from 5- to 20-week-old NOD mice with addition of IL-12 (10 ng/ml) or IL-2 (4 ng/ml) or the two cytokines in combination influenced neither islet DNA nor insulin content in any of the age groups as compared with non-cytokine exposed NOD mouse islets (data not shown). Treatment of islets obtained from 12-week-old animals with IL-12+IL-2 induced a decrease in glucose-stimulated insulin release at 16.7 mM glucose (Table 3), but not in any other age group. Exposure to each cytokine alone did not significantly affect glucose-stimulated insulin

release of the NOD mouse islets (Table 3). Medium nitrite and medium insulin accumulation were similar to control at 2, 4, 6 and 7 days in all the age groups, irrespective of cytokine addition or not (data not shown).

Discussion

The aim of this study was to investigate whether IL-12, a cytokine considered to be crucial for initiating a Th1 cell immune response (Rabinovitch 1994), may directly affect β-cell function. For this purpose we exposed isolated pancreatic islets, from normal rodents and from IDDM-prone NOD mice, to different concentrations of the cytokine *in vitro*.

Figure 1 Islet DNA content in NOD mouse islets from mice at various ages. Solid bars denote freshly isolated islets, hatched bars denote islets cultured for 7 days. Bars are mean ± S.E.M. * and ** denote $P<0.05$ and $P<0.01$ respectively, compared with corresponding freshly isolated islets, using Student's unpaired *t*-test. Figures inside brackets denote number of observations.

Figure 2 Islet insulin content in NOD mouse islets from mice at various ages. Solid bars denote freshly isolated islets, hatched bars denote islets cultured for 7 days. Bars are mean ± S.E.M. Figures inside brackets denote number of observations.

Figure 3 Islet insulin/DNA content in NOD mouse islets from mice at various ages. Solid bars denote freshly isolated islets, hatched bars denote islets cultured for 7 days. Bars are mean $\pm$ S.E.M. * and ** denote $P < 0.05$ and $P < 0.01$ respectively, compared with corresponding freshly isolated islets, using Student's unpaired *t*-test. Figures inside brackets denote number of observations.

We found that IL-12 caused a decreased medium insulin accumulation from the S-D and NMRI islets. This decrease was paralleled by a decrease in the glucose-stimulated insulin release from the rat islets, but not from the NMRI mouse islets. Although a decrease in the glucose oxidation rate was also observed in S-D and NMRI islets when cultured with 0.1 and 10 ng/ml IL-12 respectively, this seemed not to correlate directly to similar changes in glucose-stimulated insulin release, suggesting a dissociation of insulin secretion from glucose metabolism. Thus, we do not think that the decreased insulin release is dependent on an inhibition of glucose stimulation. Previously we have observed that cytokines can induce a dissociation between islet metabolism and insulin release in pancreatic islets. Rat islets exposed to IL-6 showed an increase in glucose oxidation rate and increased ATP hydrolysis but a decrease in insulin secretion (Sandler *et al.*

1990). Moreover, a combination of interferon (IFN)- γ and tumour necrosis factor (TNF)- α led to a reduced medium insulin accumulation, but had no effect on the glucose oxidation rate (Sternesjö *et al.* 1995).

In the islets of S-D rats the decreased glucose-stimulated insulin release was not accompanied by an increase in nitric oxide production as has been seen after exposure to the cytokine IL-1 β (Welsh *et al.* 1991). The present observation is similar to the observation that treatment of islets with IL-4 induces a decrease in insulin release rate, but does not affect nitrite formation (Sandler & Sternesjö 1995). Our current finding is in line with the notion that cytokines can induce inhibitory actions on β -cell preparations, which are not related to nitric oxide (Eizirik *et al.* 1994, Rabinovitch *et al.* 1994, Dunger *et al.* 1996). Interestingly, we found in the present study that IL-12 reduced nitrite accumulation compared with the non-cytokine exposed control group. This could suggest that IL-12 suppresses nitric oxide production either by the constitutive nitric oxide synthase or by cells expressing the inducible form of the enzyme.

We also investigated islets isolated from NOD mice with a particular interest in islets obtained from elderly mice (20 and 26 weeks), which contain a substantial number of immune cells upon isolation. This was reflected in the present study by an elevated DNA content in the freshly isolated islets. It is possible that addition of IL-12 to such islets might maintain an anti- β -cell immune response *in vitro*. As observed earlier (Strandell *et al.* 1990), we found that after culture the DNA content diminished, probably reflecting depletion of immune cells from the islets and this coincided with a restoration of glucose-stimulated insulin release. In the present study we show that this is also the case for islets from mice as old as 26 weeks of age.

In islets isolated from 12-week-old NOD mice, culture with IL-2 plus IL-12 induced a decrease in glucose-stimulated insulin release as compared with no cytokine addition. One possibility is that this age-dependent effect on insulin release is due to a change in immune cell

Table 3 Insulin release at 16.7 mM glucose of isolated pancreatic islets from NOD mice of different ages. Islets were maintained in medium RPMI 1640+10% fetal calf serum for 48 h with cytokines present as indicated. Islets were incubated for the first hour in 1.7 mM glucose and in 16.7 mM glucose during the second hour. Values are given as means $\pm$ S.E.M. for 3–8 experiments

	IL-12		Insulin release (ng/10 islets $\times$ 60 min)			
	(4 ng/ml)	(10 ng/ml)	5 weeks	12 weeks	20 weeks	26 weeks
Islets						
Fresh	—	—	4.1 $\pm$ 0.9	6.9 $\pm$ 1.6	9.3 $\pm$ 1.6	4.7 $\pm$ 0.6
Cultured	—	—	7.3 $\pm$ 1.2	14.3 $\pm$ 3.6\$	17.0 $\pm$ 3.4*	15.5 $\pm$ 3.3*
Cultured	+	—	9.2 $\pm$ 1.6	15.4 $\pm$ 4.9	14.7 $\pm$ 3.1	ND
Cultured	—	+	8.0 $\pm$ 0.8	16.4 $\pm$ 5.6	16.0 $\pm$ 2.4	ND
Cultured	+	+	8.5 $\pm$ 0.7	10.9 $\pm$ 3.2#	13.0 $\pm$ 2.6	ND

Symbols (*,\$) denote $P < 0.05$ compared with freshly isolated islets, using unpaired or paired Student's *t*-test respectively. Symbol (#) denotes $P < 0.05$ compared with non-cytokine treated cultured islets, using paired Student's *t*-test. ND denotes not determined.

composition in the insulinitis as animals grow older, and that cells responsive to IL-2 plus IL-12 are mainly present at around 12 weeks of age. Interestingly, the age of NOD mice used *in vivo*, when IL-12 was found to accelerate diabetes onset, was between 8 and 14 weeks old (Trembleau *et al.* 1995). It has been reported that mono-nuclear cells isolated from the pancreas of NOD mice proliferate when exposed to IL-2 (Wood *et al.* 1988). In another study in which NOD islets with insulinitis were cultured with IL-2, islet-associated leukocytes proliferated and the islets were destroyed after 3–4 days in culture (Nagata *et al.* 1989). Furthermore, splenic lymphoid cells from BB (BioBreeding) rats cultured with IL-2 have also been reported to have a lytic effect on islets in a ^{51}Cr -release assay (Pukel *et al.* 1987). In these studies 2-mercaptoethanol, a compound enhancing lymphocyte proliferation *in vitro*, was added to the culture medium. We observed no effect on islet function by IL-2 itself, which is in line with previous studies with isolated rat islets (Mandrup-Poulsen *et al.* 1986).

In conclusion, we found that IL-12 has suppressive effects on islet function of normal mice. These effects were minor compared with the previously observed effects of IL-1 β . We therefore believe that the reported accelerating effects of IL-12 on the development of NOD mouse diabetes is not to any great extent dependent on a direct toxicity of IL-12 towards islets. The fact that IL-12+IL-2 had suppressive effects on islets from 12-week-old NOD mice suggests, however, that the effect of IL-12 *in vivo* might partly be attributed to a stimulatory effect on immune cells already present in the insulinitic lesion.

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