Cytokine Profile in Children During the First 3 Months after the Diagnosis of Type 1 Diabetes

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Abstract

Type 1 diabetes is an autoimmune disease with an inflammatory process directed against the β cells in pancreas. This investigation aimed at studying the immune response during the first 3 months after the diagnosis of type 1 diabetes, with focus on the balance of T-helper 1 (Th1)- and Th2-like cytokines, produced spontaneously and in response to relevant autoantigens. Peripheral blood mononuclear cells (PBMCs) were collected from type 1 diabetic children (10–17 years) at 5, 20, 35 and 90 days after diagnosis. Expression of interferon-γ (IFN-γ) and interleukin-4 (IL-4) mRNA were detected by real-time reverse transcriptase polymerase chain reaction and IFN-γ, IL-10 and IL-13 by enzyme-linked immunosorbent assay in cell supernatant after stimulation with a glutamic acid decarboxylase 65 (GAD_{65})-peptide [amino acid (a.a.) 247–279], insulin, the ABBOS-peptide (a.a. 152–169), phytohaemagglutinin and keyhole limpet haemocyanin. Spontaneous and antigen-induced expression and secretion of cytokines were low at the diagnosis of type 1 diabetes. During the first month, after diagnosis, the GAD_{65}-peptide caused an increased ratio of IFN-γ/IL-4 mRNA expression ($P < 0.05$) and increased secretion of IFN-γ ($P = 0.07$). Expression of IFN-γ mRNA did also increase from stimulation with insulin ($P < 0.05$), even though cytokine secretion remained low. Thus, duration after diagnosis as well as metabolic state should be carefully considered both in studies of the pathogenesis of type 1 diabetes and in immune intervention studies at onset.

Introduction

Type 1 diabetes is regarded as an autoimmune disease with an inflammatory process directed against the β cells in pancreas [1, 2]. Destructive insulitis in non-obese diabetic (NOD) mouse and bio-breeding rat has been connected to the expression of proinflammatory cytokines [interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and interferon-α (IFN-α)] and T-helper 1 (Th1) cytokines (IL-2, TNF-β and IFN-γ) which contribute to cell-mediated immunity, e.g. cytotoxic and inflammatory responses mediated by T cells, natural killer (NK) cells and macrophages [3]. On the contrary, the expression of Th2 cytokines (IL-4, IL-5, IL-6, IL-9 and IL-13) connected to antibody production and enhanced eosinophil proliferation, type 3 cytokines (transforming growth factor-β) and anti-inflammatory IL-10 tends to correlate with benign insulitis in these animals [3].

Type 1 diabetes has been shown to be transferable by $CD4^+$ T cells expressing a Th1-like cytokine profile in neonatal NOD mice [4]. On the other hand, treatment with IL-10 [5] and IL-4 [6] or expression of IL-4 in pancreatic β cells of transgenic mice [7] was shown to protect from insulitis and type 1 diabetes in NOD mice. IL-13, produced mainly by activated Th2-like lymphocytes and NK cells [8], shows sequence similarity with IL-4 [9]. The level of IL-13 was significantly decreased in high-risk first-degree relatives of diabetic children in comparison with healthy controls [10], and treatment with IL-13 seemed to prevent the development of insulitis and diabetes in NOD mice [11].

Insulin, probably the most specific autoantigen in the destructive process against β cells preceding the development of type 1 diabetes, is shown to induce cellular responses in both pre-onset [12, 13] and newly diagnosed diabetic patients [13]. Glutamic acid decarboxylase 65 (GAD_{65}) is another autoantigen being associated with the destruction of the β cells and the development of type 1 diabetes [14]. GAD-reactive T cells of a Th1-like phenotype have been obtained after *in vitro* stimulation of peripheral

blood leucocytes from a human leucocyte antigen DR 3/4 heterozygous type 1 diabetic patient [15]. Especially, the part of GAD_{65} [amino acid (a.a.) 247–279] that mimics a peptide of the Coxsackie B virus (a.a. 32–47) has been shown to cause cell proliferation in individuals with an increased risk of developing type 1 diabetes as well as newly diagnosed diabetic patients [16]. We have previously reported that type 1 diabetic children respond to this peptide of GAD_{65} in a Th1-like manner [17].

Thus, evidence that β-cell destruction in type 1 diabetes is of a Th1-like origin is accumulating, whereas it has been suggested that Th2-like cytokine responses may be protective for the disease [18, 19]. The present investigation was performed in order to study cytokine phenotype and kinetics of the immune response during the first 3 months after diagnosis of type 1 diabetes, with focus on the balance of Th1- and Th2-like cytokines, spontaneous and in relation to relevant autoantigens for the disease process of type 1 diabetes.

Materials and methods

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Paque density gradient centrifugation (Pharmacia, Biotech, Sollentuna, Sweden) from sodium-heparinized venous blood samples, obtained from 10 children (five boys and five girls) with diagnosed type 1 diabetes (10–17 years, mean age 14 years). The children constituted the control group in a study of immunological effects of photopheresis treatment (Karlsson Faresjö *et al.*, data to be published). Samples were collected 5, 20, 35 and 90 days after diagnosis. Blood samples taken 5 days after diagnosis were collected at the hospital and the other blood samples were collected when the patients visited the diabetes office, during the morning hours to avoid time of day differences. PBMCs were cryopreserved in liquid nitrogen until use.

Peptides and antigens used for stimulation. The synthetic peptide of human GAD_{65} a.a. 247–279 (NMYAMMIARFKMFPEVKEKGMAALPRLIAFTSE-OH) molecular weight 3823.7 (Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden), the synthetic milk-derived bovine serum albumin peptide ABBOS, a.a. 152–169 (FKADEKKFWGKYLYEIAR) [20] (Department of Medical and Physiological Chemistry) and insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) were used for the stimulation of PBMCs. Phytohaemagglutinin (PHA) (Sigma, Stockholm, Sweden) was used as a positive control, and keyhole limpet haemocyanin (KLH) (Calbiochem, Laboratory Kemi, Stockholm, Sweden) was used as a negative control.

In vitro stimulation of PBMCs. Frozen mononuclear cells were thawed, directly from −196 °C to +37 °C, in water bath during addition of RPMI 1640 supplemented with 10% fetal calf serum. One million PBMCs (viability approximately 95% for each population) were diluted in 500 µl of AIM V research grade (Gibco, Täby, Sweden) supplemented with 2 mM L-glutamine, 50 µg/l of streptomycin sulfate, 10 µg/l of gentamicin sulfate and 2×10^{-5} M 2-mercaptoethanol (Sigma). One million PBMCs were stimulated with GAD_{65} (a.a. 247–279), the ABBOS-peptide, PHA or KLH, all at a concentration of 5 µg/ml [21], or with insulin at a concentration of 50 µg/ml [22], for 48 h at 37 °C, in 5% CO_2 for further analysis of the expression of cytokine-specific mRNA.

One million PBMCs were as well stimulated with GAD_{65} (a.a. 247–279) and the ABBOS-peptide at a concentration of 50 µg/ml [23], with insulin at a concentration of 500 µg/ml or with PHA and KLH at a concentration of 5 µg/ml, for 96 h at 37 °C, in 5% CO_2, for further analysis of cytokines in the cell-culture supernatants. PBMCs, incubated without antigen but otherwise under the same conditions, were used for the determination of spontaneous expression of mRNA and secretion of cytokines. In samples with limited number of cells, the order of priority for stimulation with antigens was GAD_{65} (a.a. 247–279), the ABBOS-peptide, insulin, PHA and finally KLH.

RNA isolation and cDNA synthesis. Total RNA was isolated from PBMCs with RNeasy 96 (Quiagen, KEBO, Spånga, Sweden), as recommended by the supplier. Total RNA was quantified by optical density at 260 nm. Using equal amounts of total RNA (7 ng/µl) from PBMCs, stimulated under various conditions, mRNA was marked complementary with random hexamers and complementary DNA (cDNA) was synthesized from the mRNA by TaqMan Reverse Transcription with MultiScribe Reverse Transcriptase (Perkin Elmer, Stockholm, Sweden), according to the manufacturer's description. The final cDNA product was stored at −20 °C for subsequent cDNA amplification by PCR.

Real-time PCR. PCR mixture (final volume 25 µl) contained TaqMan cytokine gene expression reagents (Perkin Elmer) with cytokine-specific target primers and probe (IFN-γ; 4308250S or IL-4; 4308243S, FAM dye layer) and endogenous reference primers and probe (rRNA, VIC dye layer) in MicroAmp Optical 96-well reaction plate with optical caps. The reaction mixture was amplified with ABI Prism 7700 Sequence Detector (Perkin Elmer) for 40 cycles with an annealing temperature of 60 °C for IL-4 and IFN-γ. The FAM dye layer yields the results for quantification of the cytokine target mRNA, while the VIC dye layer yields the results for quantification of the 18S ribosomal RNA endogenous control. The passive reference contained the dye ROX in order to normalize for non-PCR-related fluctuations in fluorescence signal. In all experiments, controls without template as well as control RNA (Perkin Elmer) were included.

Calculation of relative quantification values. The relative quantification values for the cytokine gene expression assays were calculated from the accurate C_T, according to the manufacturer's description (protocol P/N 4304671, Perkin Elmer). The accurate C_T represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected, obtained from both dye layers in the assay. C_T value for rRNA (VIC) was substracted from the specific cytokine C_T value (FAM for IFN-γ and IL-4) to calculate $\Delta - C_T$ for the calibrator and samples in each cytokine gene expression assay. $\Delta - C_T$ values for duplicate wells of the calibrator sample for each cytokine were averaged. The average delta (calibrator) value was subtracted from the $\Delta - C_T$ values of samples to calculate their $\Delta\Delta - C_T$ (sample) values. The $\Delta\Delta - C_T$ value for duplicate wells of each cytokine sample was averaged. This operation normalizes the number of target mRNA molecules to the number of rRNA molecules. Relative quantification values were obtained after calculating the two-averaged $\Delta\Delta - C_T$.

Enzyme-linked immunosorbent assay. IFN-γ level, in supernatants from PBMCs stimulated in vitro, was measured by enzyme-linked immunosorbent assay (ELISA) [24] in microtitre wells (Costar 3690, Life Technologies, Stockholm, Sweden) coated with 2 μg/ml of monoclonal mouse anti-human antibody (clone 2571811, R&D Systems, Abingdon, UK) in coating buffer (CLB, Amsterdam, The Netherlands) overnight at room temperature (RT). Wells were blocked with 2% milk in phosphate-buffered saline (PBS) for 1 h at RT. Cell-culture supernatants and standard (recombinant human IFN-γ, R&D) were applied in duplicates and incubated for 1 h at RT. Bound IFN-γ was detected by 0.20 μg/ml of goat anti-human biotinylated polyclonal antibody (R&D) diluted in high-performance ELISA-dilution buffer (CLB) for 1 h at RT and furthermore incubated for 30 min at RT with horseradish peroxidase conjugated to poly-streptavidin (0.1 μM, CLB). A coloured product was formed after incubation with 3,3′,5,5′-tetramethylbenzidine liquid substrate (Sigma-Aldrich, Stockholm, Sweden) for 30 min in the dark and thereafter with 1.8 M H_2SO_4 to stop the reaction. The sensitivity for IFN-γ was 12 pg/ml, according to the manufacturer.

IL-10 and IL-13 levels were measured by commercially available ELISA (CLB Pelikine Compact™, Amsterdam, The Netherlands). Microtitre wells (Costar 3690) were coated with a monoclonal anti-human antibody in coating buffer overnight at RT, whereas non-bound material was removed by washing. Wells were blocked with 5% milk in PBS for 1 h at RT. Cell-culture supernatants and standards (recombinant human IL-10/IL-13) were applied in duplicates and incubated for 1 h at RT. Cytokine was detected by anti-human biotinylated polyclonal antibody diluted in high-performance ELISA-dilution buffer for 1 h at RT and furthermore incubated for 30 min at RT with horseradish peroxidase conjugated to poly-streptavidin. The sensitivity was 2.35 pg/ml for IL-10 and 1.0 pg/ml for IL-13, according to the manufacturer. Antigen-induced cytokine response was presented as pg/ml after subtraction of spontaneously secreted cytokine from PBMCs incubated without antigen, but otherwise cultured under the same conditions.

C-peptide. C-peptide was determined by a radioimmunoassay technique based upon the original assay developed by Heding [25]. The detection limit for the assay was 0.03 nmol/l, and the reference value among fasting healthy children and adolescents was 0.18–0.63 nmol/l.

Islet cell antibodies. Islet cell antibodies (ICA) was detected with immunofluorescence on human pancreas sections according to Bottazzo et al. [26]. A monospecific anti-immunoglobulin G (IgG) conjugated with fluorescein isothiocyanate was used for detection. Our method was standardized according to International Juvenile Diabetes Foundation (IJDF) standards for ICA determination. Our laboratory has participated in international ICA workshop with a specificity as well as sensitivity of 100%.

GAD autoantibodies. GAD autoantibodies (GADA) were determined according to Grubin et al. [27] and Petersen et al. [28] using recombinant human ^{35}S-GAD_{65} as tracer according to Falorni et al. [29]. Serum was immunoprecipitated with human ^{35}S-GAD_{65} in duplicates. Antibody-bound ^{35}S-GAD_{65} was separated from unbound antibodies with Protein-A-Sepharose using Millipore Multiscreen Assay System. The antibody-bound ^{35}S-GAD_{65} was counted by a β-counter. Included in each assay were one strongly positive control (IJDF standard) and three negative samples (sera from three healthy controls). Out laboratory participated in the second GADA proficiency test 1996 and reached sensitivity and specificity of 100%.

Insulin antibodies. Insulin antibodies (IA) was detected with a radioligand competition assay according to Palmer et al. [30]. Insulin in the sample was removed by acidification and charcoal extraction. Serum was incubated in duplicates with bovine ^{125}I-insulin and ^{125}I-insulin with an excess of unlabelled insulin. After incubation, free and antibody-bound ^{125}I-insulin was separated with precipitation with polyethylene glycol. Included in each assay were a positive control (pool of sera from three IA-positive patients) and a negative control (with undetectable IA). Results were calculated as the difference between incubation with ^{125}I-insulin and incubation with ^{125}I-insulin and excess of unlabelled antigen. Cut-off was set at mean ± 2SD for healthy controls as 99 nU/ml.

Statistics. As the expression of mRNA for IFN-γ and IL-4 as well as secretion of the measured cytokines was not normally distributed (even after logarithmic transformation), two time-points were compared by Mann–Whitney U-test, while three or more time-points of duration were compared with Kruskal–Wallis test for unpaired observations. Spearman's rank correlation was used when comparing two variables non-parametrically, whereas comparison between paired groups was analysed with Wilcoxon signed

rank test. A probability level of <0.05 was considered to be statistically significant, whereas P-value ≤ 0.1 because of the small material was regarded as a tendency. Calculations were performed with a statistical package STATVIEW 5.0.1 for Macintosh (Abacus Concepts Inc, Berkeley, CA, USA).

Ethics. The study was approved by the Research Ethics Committee of the Faculty of Health Sciences at Linköping University, and all parents or responsible guardians have given their informed consent on behalf of the children.

Results

Spontaneous cytokine secretion

A number of immunological parameters changed during the first 3 months after the diagnosis of type 1 diabetes, as summarized in Table 1. Secretion of IFN-γ tended to increase from the time of diagnosis until 3 weeks duration ($P = 0.07$), but thereafter tended to decrease until 3 months duration ($P = 0.07$) (Fig. 1). IL-13 followed a similar pattern of secretion but without statistically significant changes (Fig. 1). Spontaneous secretion of IL-10 increased from 1 to 3 months duration ($P < 0.05$) (Fig. 1; Table 1).

Expression and secretion of cytokines after *in vitro* stimulation

Stimulation of PBMCs from diabetic children with a peptide of GAD_{65} (a.a. 247–279) that mimics a peptide of Coxsackie B virus protein 2C revealed several changes of the immune response during the course. The GAD_{65}-peptide induced secretion of IFN-γ that tended to increase during the first month after diagnosis ($P = 0.07$) (Fig. 2B). Expression of IFN-γ mRNA, detected by real-time RT-PCR, did as well tend to increase from day 20 to day 35 ($P = 0.06$) (Fig. 2A). On the contrary, expression of IL-4 mRNA significantly decreased during the first 3 months after diagnosis ($P < 0.05$) (Fig. 2A; Table 1). Thus, the ratio of IFN-γ/IL-4 mRNA increased ($P < 0.05$) (Fig. 3A; Table 1) indicating a Th1-deviated cytokine profile in response to the GAD_{65}-peptide. Secretion of IL-10 after GAD_{65}-stimulation tended to decrease from diagnosis to 3 months ($P = 0.06$) (Fig. 2B), which further support an increase in the GAD-induced inflammatory profile. Initially, IL-13 decreased from stimulation with the GAD_{65}-peptide ($P < 0.05$), but thereafter increased with duration of disease ($P = 0.01$) (Fig. 2B; Table 1).

Insulin is probably the most specific autoantigen in the destructive process against β cells preceding the development of type 1 diabetes. Insulin-induced expression of IFN-γ mRNA increased ($P < 0.05$) (Fig. 2C; Table 1) as well as the ratio of IFN-γ/IL-4 mRNA expression ($P = 0.07$) (Fig. 3B) from diagnosis to 3 months duration, in favour of a shift to Th1. Incubation with insulin resulted in inhibition of the cytokine secretion at the time of diagnosis. No major changes were observed after that, although a tendency to increase of IFN-γ ($P = 0.1$) (Fig. 2D) and IL-13 ($P = 0.07$) (Fig. 2D) was noted, whereas secretion of IL-10 tended to decrease with duration of disease ($P = 0.09$) (Fig. 2D). Thus, although not fully consistent, a Th1-like biased immune response after *in vitro* stimulation with insulin was observed with the duration of disease.

There has been a vivid discussion concerning the role of environmental factors as possible triggers of autoimmune type 1 diabetes. The most likely environmental triggers of type 1 diabetes are viral infections and dietary proteins. Among dietary proteins, mainly cow's milk proteins have been implicated as possible triggers of the autoimmune response resulting in type 1 diabetes. However, the ABBOS-peptide from bovine serum albumin (BSA) elicited only modest cytokine responses. Secretion of IFN-γ by the ABBOS-peptide tended to increase during the first month ($P = 0.08$) (Fig. 2F) as well as the induced expression of IFN-γ mRNA which increased with time of duration ($P < 0.05$) (Fig. 2E; Table 1). Expression of IL-4 mRNA tended to decrease when stimulated *in vitro* with ABBOS ($P = 0.07$) (Fig. 2E), and the ratio of IFN-γ/IL-4 mRNA tended to increase until 1 month duration ($P = 0.06$) (Fig. 3C). Secretion of IL-10 was significantly reduced from diagnosis until 3 months ($P < 0.05$) (Fig. 2F; Table 1), whereas IL-13 increased from 1–3 months after *in vitro* stimulation with ABBOS ($P < 0.05$) (Fig. 2F; Table 1). Thus, the milk-derived peptide from BSA induced a modest Th1- and Th2-like profile that fluctuated with the duration of disease.

The mitogen PHA always induced prominent mRNA expression and cytokine secretion. The expression of IL-4 mRNA decreased ($P = 0.01$) (Fig. 2G; Table 1), whereas IFN-γ mRNA expression ($P < 0.05$) (Fig. 2G; Table 1) as well as the ratio of IFN-γ/IL-4 mRNA ($P < 0.05$) (Fig. 3D; Table 1) increased during the first month after diagnosis. This is in line with the increased Th1-biased cytokine pattern observed for the diabetes-associated antigens during the course of disease. Furthermore, exposure to PHA also caused increased secretion of IFN-γ (Fig. 2H) which tended to be correlated with the expression of IFN-γ mRNA at 1 month duration ($r = 0.6$, $P = 0.08$). In contrast, KLH from mollusc used as a negative control did not cause any pronounced immune response, in PBMCs from type 1 diabetic children (data not shown). Furthermore, control RNA was amplified to the same extent in all reactions performed by real-time RT-PCR.

C-peptide

Endogenous insulin secretion measured as connection-peptide increased significantly 3 weeks after diagnosis to 3 months duration ($P < 0.05$) (Fig. 4A; Table 2). This is probably due to a better metabolic control usually

Table 1 Cytokine profile in children during the first 3 months after the diagnosis of type 1 diabetes*

Cytokine	Spontaneous/*in vitro* stimulated	Expression and secretion of cytokines				Changes in cytokine expression and secretion
		Day 5	Day 20	Day 35	Day 90	
IFN-γ	Spontaneous	19 (0–998)	117 (0–482)	52 (0–251)	16 (0–115)	D5–20 (+), $P=0.07$; D20–90 (–), $P=0.07$
IL-10	Spontaneous	29 (0–311)	89 (0–736)	58 (0–360)	134 (0–573)	**D35–90 (+), $P<0.05$**
IL-13	Spontaneous	13 (0–156)	115 (8.5–317)	85 (4.9–207)	19 (0–278)	Not significant
IFN-γ	GAD_{65} (a.a.247–279)	0 (−295–178)	14 (−71–128)	48 (0–284)	25 (−3.2–1030)	D5–35 (+), $P=0.07$
IFN-γ mRNA	GAD_{65} (a.a.247–279)	1.1 (0.4–3.4)	0.8 (0.5–2.2)	1.4 (0.5–2.6)	1.6 (0.2–4.1)	D20–35 (+), $P=0.06$
IL-4 mRNA	GAD_{65} (a.a.247–279)	1.8 (0.8–22)	1.1 (0.3–2.4)	1.2 (0.5–2.0)	0.9 (0.3–1.8)	**D5–90 (–), $P<0.05$**
IFN-γ/IL-4 mRNA	GAD_{65} (a.a.247–279)	0.9 (0–4.2)	0.9 (0.2–2.9)	1.1 (0.6–2.8)	1.0 (0.2–8.8)	**D20–35 (+), $P<0.05$**
IL-10	GAD_{65} (a.a.247–279)	0.4 (−72–24)	−0.7 (−62–12)	1.9 (−11–145)	−8.0 (−95–6.2)	D5–90 (–), $P=0.06$
IL-13	GAD_{65} (a.a.247–279)	4.3 (−82–119)	−24 (−122–2.2)	39 (−16–130)	30 (−4.8–126)	**D5-20(–) $P<0.05$ D20–90(+) $P=0.01$**
IFN-γ	Insulin	−26 (−50–0)	−66 (−162–0)	−52 (−234–0)	0 (−51–0)	D20–90 (+), $P=0.1$
IFN-γ mRNA	Insulin	0.8 (0.5–1.4)	0.7 (0.2–3.5)	0.9 (0.4–2.5)	1.4 (0.4–4.8)	**D5–90 (+), $P<0.05$**
IL-4 mRNA	Insulin	1.0 (0.3–6.8)	0.7 (0.3–7.8)	0.7 (0.5–0.9)	0.9 (0.4–2.9)	Not significant
IFN-γ/IL-4 mRNA	Insulin	0.8 (0.2–2.4)	1.0 (0.1–4.6)	1.4 (0.7–3.0)	1.0 (0.4–8.2)	D5–90 (+), $P=0.07$
IL-10	Insulin	−7.8 (−154–0)	−50 (−471–0)	−9.2 (−212–82)	−45 (−415–0)	D5–90 (–), $P=0.09$
IL-13	Insulin	−26 (−108–0)	−100 (−205–−8.5)	−85 (−207–−4.9)	−18 (−56–0)	D35–90 (+) $P=0.07$
IFN-γ	ABBOS (a.a. 152–169)	−12 (−26–0)	−26 (−61–106)	23 (−50–51)	0 (−30–1263)	D5–35 (+) $P=0.08$
IFN-γ mRNA	ABBOS (a.a. 152–169)	1.0 (0.5–2.4)	0.7 (0.2–1.6)	0.9 (0.4–1.4)	1.4 (0.2–5.0)	**D20–90 (+), $P<0.05$**
IL-4 mRNA	ABBOS (a.a. 152–169)	1.9 (0.7–5.3)	1.2 (0.4–2.1)	0.8 (0.2–2.3)	0.9 (0.3–3.5)	D5–35 (–), $P=0.07$
IFN-γ/IL-4 mRNA	ABBOS (a.a. 152–169)	0.7 (0.1–1.5)	0.8 (0.1–1.9)	1.0 (0.4–1.8)	1.2 (0.1–17.1)	D5–35 (+), $P=0.06$
IL-10	ABBOS (a.a. 152–169)	0 (−23–154)	−9.4 (−30–3.9)	1.5 (−24–183)	−14 (−134–2.6)	**D5–90 (–) $P<0.05$**
IL-13	ABBOS (a.a. 152–169)	−3.5 (−24–9.9)	−12 (−46–23)	−18 (−43–4.3)	13 (−35–81)	**D35–90 (+), $P<0.05$**
IFN-γ	PHA	5283 (2494–12225)	4093 (2831–6339)	7410 (2932–52999)	23606 (3087–43990)	Not significant
IFN-γ mRNA	PHA	26 (5.7–379)	17 (2.8–46)	30 (1.2–261)	35 (3.0–993)	**D20–35 (+), $P<0.05$**
IL-4 mRNA	PHA	81 (3.2–503)	28 (2.6–176)	37 (3.0–326)	14 (3.4–149)	**D5–35 (–), $P=0.01$**
IFN-γ/IL-4 mRNA	PHA	0.5 (0–9.2)	0.4 (0.1–2.2)	1.3 (0.4–2.4)	1.5 (0.2–13)	**D5–35 (+), $P<0.05$**
IL-10	PHA	1395 (492–4081)	1971 (691–4040)	2177 (539–3898)	2009 (19–4177)	Not significant
IL-13	PHA	1685 (1579–3531)	1407 (869–1792)	1836 (841–2441)	1120 (692–1869)	Not significant

*mRNA expression and secretion of cytokines [interferon-γ (IFN-γ), interleukin-4 (IL-4), IFN-γ/IL-4 ratio, IL-10 and IL-13] spontaneously and after *in vitro* stimulation with antigens [glutamic acid decarboxylase 65 (GAD_{65}) amino acid (a.a.) 247–279, insulin, the ABBOS-peptide a.a. 152–169 and phytohaemagglutinin (PHA)] are presented as median and range (within brackets). Cytokine expression or secretion is shown at 5, 20, 35 and 90 days (D) after diagnosis of type 1 diabetes. *P*-values represent increased (+) or decreased (–) cytokine response during the first 3 months after the diagnosis of type 1 diabetes (significant changes in bold letters).

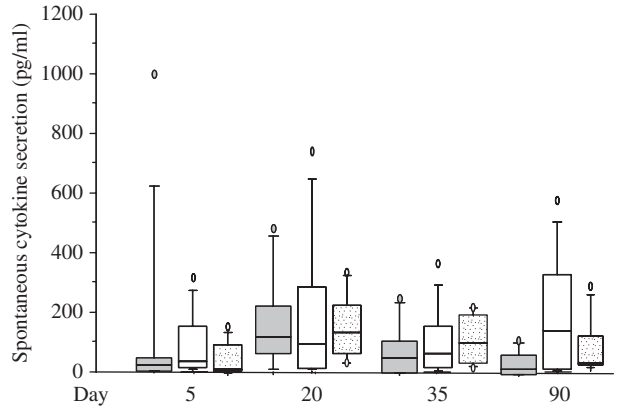

Figure 1 Spontaneous secretion of interferon-γ (grey boxes), interleukin-10 (IL-10) (white boxes) and IL-13 (dotted boxes) detected at 5, 20, 35 and 90 days after diagnosis of type 1 diabetes by enzyme-linked immunosorbent assay (pg/ml) is illustrated by box plots (10th, 25th, 50th, 75th and 90th percentile, and outliers are indicated).

observed during the first months after diagnosis. Secretion of C-peptide tended to be negatively correlated to the expression of IFN-γ mRNA after *in vitro* stimulation with a GAD_{65}-peptide ($r = -0.83$, $P = 0.06$) (Fig. 4B) or with the ABBOS-peptide ($r = -0.83$, $P = 0.06$) 3 months after the diagnosis. On the contrary, secretion of IL-10 from *in vitro* stimulation with insulin tended to be positively correlated to secretion of C-peptide ($r = 0.64$, $P = 0.07$). These findings support a role for a Th1-like profile in the destructive process of the β cells.

Humoral response

Antibodies associated with the progression to type 1 diabetes are directed against autoantigens released from β cells. Insulin antibodies tended to decrease during the first weeks after diagnosis but then increased significantly from 3 weeks after diagnosis ($P < 0.05$), whereas GAD_{65} autoantibodies (GADA) tended to decrease during the first month ($P = 0.08$) (Table 2). There was no correlation observed between the concentration of these antibodies and the expression or secretion of cytokines from *in vitro* stimulation (Fig. 5). Production of islet cell antibodies (ICA) did not change significantly during the first 3 months after diagnosis, (Table 2), but there was a negative correlation to secretion of IL-10 after *in vitro* stimulation with either the ABBOS-peptide ($r = -0.93$, $P < 0.05$) or insulin ($r = -0.90$, $P < 0.05$) at 3 months duration.

Discussion

Type 1 diabetes is an organ-specific chronic autoimmune disease resulting from T-cell-mediated destruction of pancreatic β cells. During the autoimmune process, proteins from β cells presumably become accessible and stimulatory to the immune cells of type 1 diabetic patients. GAD_{65}, located in the islets of Langerhans, is one of these proteins that can stimulate T cells and probably contribute to the pathogenesis [31, 32]. The part of GAD_{65} (a.a. 247–279) that mimics a peptide of the Coxsackie B virus (a.a. 32–47) can cause cell proliferation in individuals with increased risk of developing type 1 diabetes as well as in newly diagnosed diabetic patients [16]. We have previously reported that this GAD_{65}-peptide has the ability to stimulate Th1-like cells in blood to express increased amounts of IFN-γ mRNA in children with recent onset type 1 diabetes [17]. One major finding of the present study is that the GAD_{65}-peptide causes increased mRNA expression and in addition also secretion of cytokines. During the first month after diagnosis, IFN-γ mRNA expression increased, whereas expression of IL-4 mRNA decreased during the same interval, thus showing a Th1-deviated cytokine profile. IFN-γ at the protein level was in line with this, showing a tendency to increased levels during the course. Further support for an increase in the GAD-induced inflammatory profile comes from the observed decrease of the anti-inflammatory IL-10. Regarding IL-10, it should be pointed out that modest responses were observed after *in vitro* stimulation which could be due to that IL-10 is produced mainly by macrophages and therefore not inducible by stimulation with presumed T-cell antigens.

Our finding of lower GAD-induced immune response at the onset of disease compared with subsequent samples is in line with the previous findings of decreased proliferation to peptides of GAD_{65} (a.a. 247–266 and 260–279) at diabetes onset [16, 33, 34]. It was indicated that this was a temporary decline of T-cell proliferation that should not be associated with the β-cell destruction process but with clinical manifestation [34]. This is probably due to the metabolic disturbance at disease onset, and thus after clinical onset then, the metabolic balance is recovered T-cell responses increase. This is in line with our patterns of cytokine secretion.

We have previously observed an inverse relation between the production of autoantibodies against GAD_{65} (GADA) and spontaneous secretion of IFN-γ in high-risk first-degree relatives [22] that may delay the onset of type 1 diabetes [32]. In newly diagnosed diabetic children, we could not observe any correlation between cellular and humoral response to GAD_{65}, although it has been speculated that GAD_{65}-specific autoantibodies can enhance the presentation of an immunodominant T-cell epitope (a.a. 274–286) of GAD_{65} [35].

Specific cellular responses to insulin have in some studies been found in pre-onset [12, 13] and in newly diagnosed diabetic patients [13]. A strong cellular response with increased secretion of IFN-γ to especially the B-chain epitope (a.a. 9–23) of insulin has been demonstrated [36]. In addition, at the onset of type 1 diabetes, Th1-associated

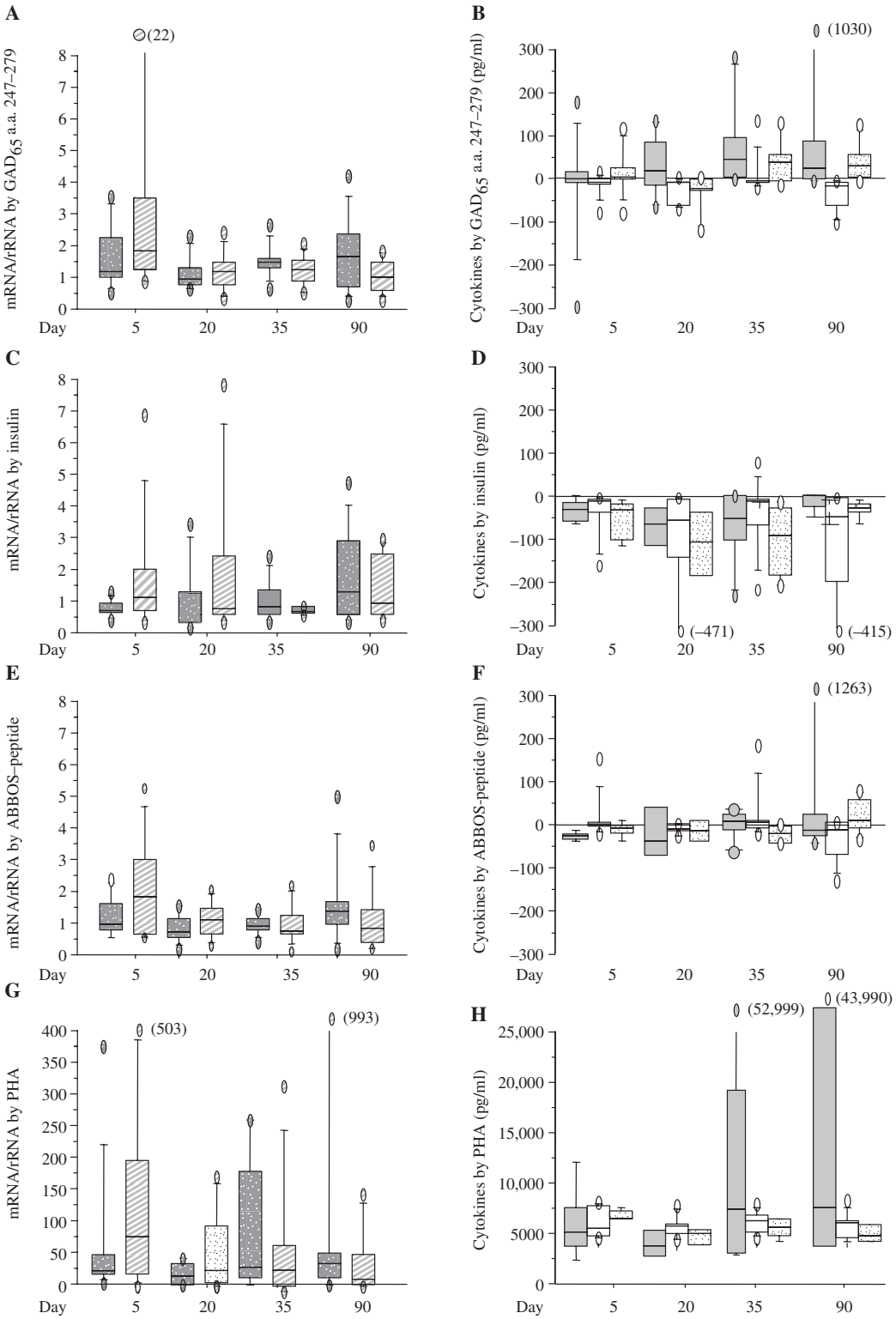

Figure 2 Expression of interferon-γ (IFN-γ) mRNA (dark boxes with white spots) and interleukin-4 (IL-4) mRNA (striped boxes) after *in vitro* stimulation with a peptide of glutamic acid decarboxylase 65 (GAD_{65}) [amino acid (a.a.) 247–279] (A), insulin (C), the ABBOS-peptide (E) and the mitogen phytohaemagglutinin (PHA) (G) detected as relative quantity in comparison with rRNA by real-time reverse transcriptase polymerase chain reaction. Secretion of IFN-γ (grey boxes), IL-10 (white boxes) and IL-13 (white boxes with dark spots) after *in vitro* stimulation with the GAD_{65}-peptide (B), insulin (D), the ABBOS-peptide (F) and PHA (H), after subtraction of spontaneous secreted cytokine are detected at pg/ml by enzyme-linked immunosorbent assay. Expression and secretion of cytokines illustrated by box plots (10th, 25th, 50th, 75th and 90th percentile, and outliers are indicated) was measured 5, 20, 35 and 90 days after the diagnosis of type 1 diabetes.

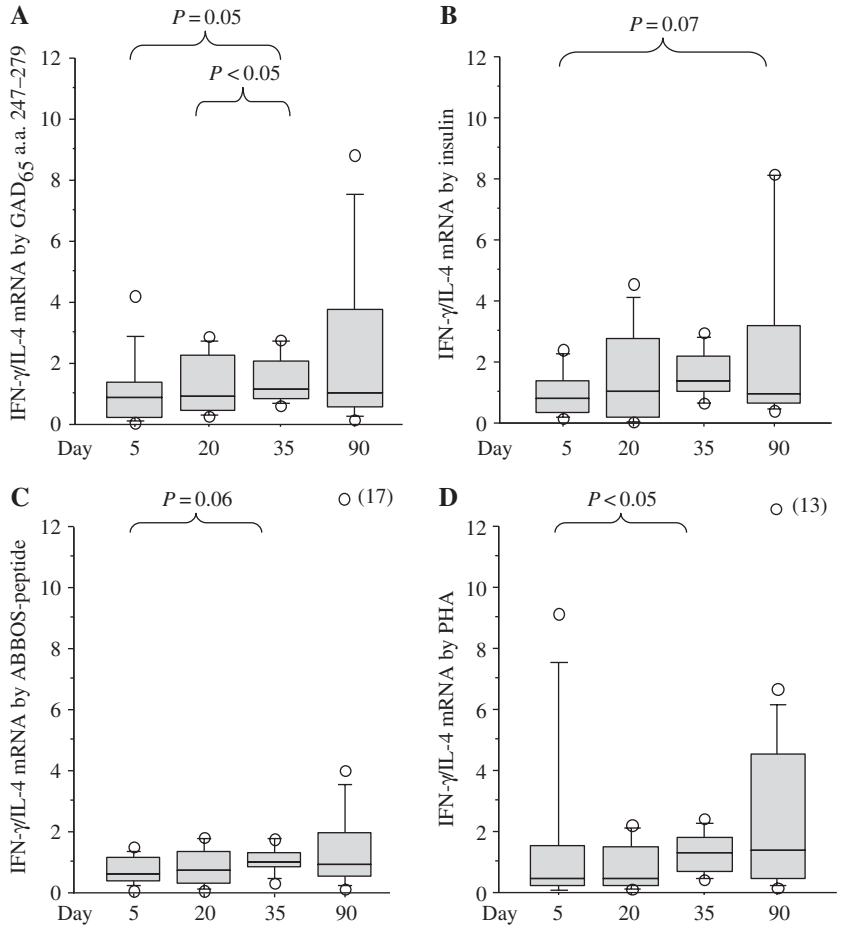

Figure 3 Ratio of interferon-γ (IFN-γ)/interleukin-4 (IL-4) mRNA expression after *in vitro* stimulation with a peptide of glutamic acid decarboxylase 65 (GAD_{65}) [amino acid (a.a.) 247–279] (A), insulin (B), the ABBOS-peptide (C) and phytohaemagglutinin (PHA) (D) from diagnosis until 3 months duration (5, 20, 35 and 90 days) is illustrated by box plots (10th, 25th, 50th, 75th and 90th percentile, and outliers are indicated). Expression of IFN-γ and IL-4 mRNA, from *in vitro* stimulation with antigens after subtraction of spontaneously expressed mRNA is calculated as the relative amount of expressed cytokine in relation to rRNA detected by real-time reverse transcriptase polymerase chain reaction.

IgG class 1 antibodies against insulin have been detected [37]. We found that *in vitro* stimulation with insulin caused increased IFN-γ mRNA expression as well as an increased ratio of IFN-γ/IL-4 mRNA, during the first 3 months after the diagnosis of type 1 diabetes. Contrary, we found low response to insulin *in vitro*, at the level of translation into protein, especially at diagnosis of the disease. Secretion of IFN-γ was initially found to be low but increased with the duration of disease, even though secretion of IFN-γ never reached the same level as expression of IFN-γ mRNA. In fact, several recent studies have failed to detect any T-cell proliferation against insulin or insulin peptides in both diabetic and prediabetic patients [38–40]. We have also previously reported about the phenomena of low IFN-γ response against insulin in both high-risk individuals and newly diagnosed diabetic children, in comparison with other antigens e.g. the GAD_{65}-peptide, as observed by sensitive enzyme-linked immunospot technique [22]. Furthermore, a lack of differences in proliferative response to insulin was noted between controls and new-onset diabetic patients [38]. It has also been reported that neonatal tolerance of rats by murine insulin protects from diabetes without any humoral response and IL-4 secretion observed [41]. This could be due to regulatory T cells that protect from diabetes rather than inducing a non-pathogenic Th2-like response [41].

The milk-derived BSA-peptide ABBOS induced a modest cytokine secretion. We have previously reported that ABBOS can induce a weak Th1- and Th2-like profile to the same extent in diabetic and healthy children [23]. Furthermore, the mitogen PHA induced expression and secretion for all studied cytokines. This mitogen stimulated the T cells to increased expression and secretion of IFN-γ, decreased IL-4 mRNA expression and a low stable secretion of IL-13 during the first month after the diagnosis of diabetes. This is comparable to the cytokine pattern observed for the studied diabetes-associated antigens, in contrast to the absence of response observed to the control antigen, KLH.

At the onset of disease, the spontaneous secretion of cytokines (IFN-γ, IL-10 and IL-13) was low, which could be a sign of the metabolic disturbance. In subsequent samples, spontaneous cytokine secretion increased with duration of type 1 diabetes.

In summary, we found that cytokine responses, both at the level of transcription as mRNA and after translation into protein, were low recently after the diagnosis of type 1

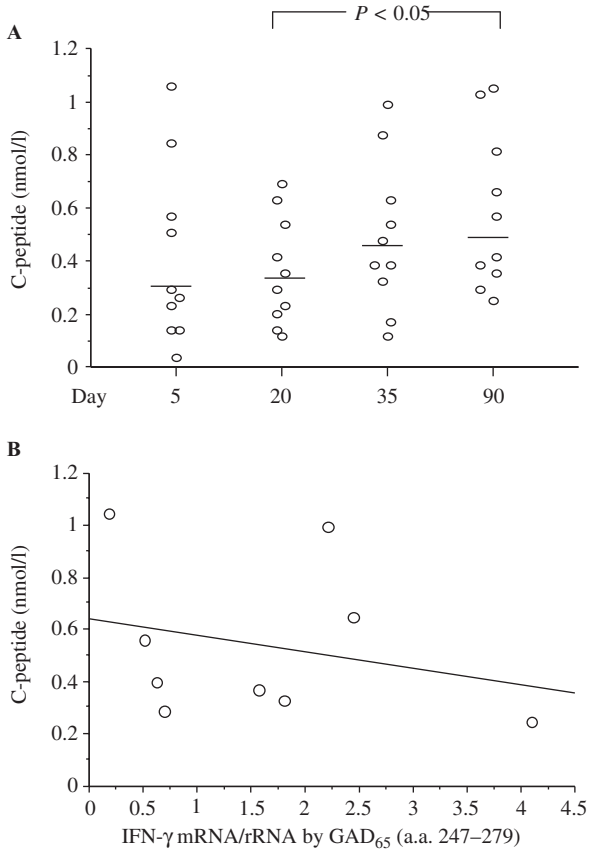

Figure 4 Secretion of C-peptide (nmol/l) increased from 3 weeks (day 20) to 3 months (day 90) after diagnosis of type 1 diabetes ($P < 0.05$) (A). A trend to negative correlation was observed between an increased T-helper 1-like immune response [interferon-γ (IFN-γ) mRNA expression] by the glutamic acid decarboxylase 65 (GAD_{65})-peptide [amino acid (a.a.) 247–279] and endogenous insulin secretion (C-peptide) 3 months after diagnosis of type 1 diabetes ($r = -0.83$, $P = 0.06$) (B).

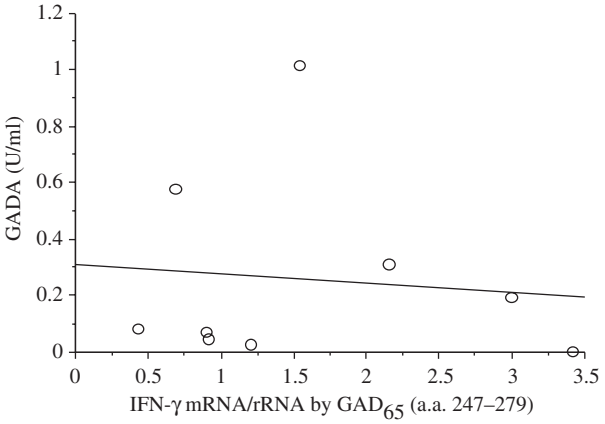

Figure 5 No correlation was observed between cellular response [interferon-γ (IFN-γ) mRNA] to GAD_{65} (a.a. 247–279) and humoral response [glutamic acid decarboxylase autoantibodies (GADA), U/ml] ($r = -0.1$, $P = 0.8$).

diabetes and thereafter increased. The most prominent response was Th1 biased and directed against the putative autoantigen GAD_{65} (a.a. 247–279). We therefore suggest that duration after diagnosis, as well as metabolic state, should be carefully considered both in studies of the pathogenesis of type 1 diabetes and in immune intervention studies at onset.

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Table 2 Antibodies and C-peptide*

Antibodies and C-peptide	Diabetes duration: Day 5	Day 20	Day 35	Day 90	Changes in concentration
C-peptide	**0.30** (0.04–1.05)	**0.33** (0.12–0.69)	**0.44** (0.12–0.99)	**0.48** (0.25–1.05)	**D20–90 (+), $P < 0.05$**
IA	**4.3** (0.8–19.7)	**2.9** (0.2–11.2)	**3.2** (1.7–18.6)	**9.1** (0–41.4)	D5–20 (–), $P = 0.07$; **D20–90 (+), $P < 0.05$**
GADA	**0.09** (0–1.0)	**0.10** (0–1.0)	**0.07** (0–1.0)	**0.13** (0–1.0)	D20–35 (–), $P = 0.08$
ICA	**80** (40–80)	**80** (80–80)	**80** (60–80)	**60** (20–80)	Not significant

*Secretion of C-peptide (nmol/l), antibodies directed against insulin (IA, nU/ml), glutamic acid decarboxylase 65 (GAD_{65}) autoantibodies (GADA, U/ml) and islet cell antibodies (ICA, IJDF) are illustrated as median and range (within brackets). *P*-values represent increased (+) or decreased (–) response at 5, 20, 35 and 90 days (D) after diagnosis of type 1 diabetes (significant changes in bold letters).

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