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Short Communication

Cellular interferon-γ and interleukin-13 immune reactivity in type 1, type 2 and latent autoimmune diabetes: Action LADA 6

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ABSTRACT

Objective: Type 1 diabetes and latent autoimmune diabetes in adults (LADA) are thought to result from immune-mediated β-cell destruction. It remains unclear why LADA is clinically less severe compared to type 1 diabetes. This study aimed to compare the pro-inflammatory (interferon-γ, IFN-γ) and anti-inflammatory (interleukin-13, IL-13) T-cell responses in humans with LADA and type 1 diabetes.
Research design and methods: IFN-γ and IL-13 T-cell responses to a panel of 16 (auto)-antigens were tested using an enzyme linked immune-spot technique and peripheral T-cells from 35 patients with type 1 diabetes, 59 patients with type 2 diabetes, 23 LADA patients, and 42 control subjects.
Results: LADA and type 1 diabetes patients did not display any statistically significant differences in the frequency of IFN-γ or IL-13 responses to auto-antigenic stimuli, positive control or mitogen. Overall very low T cell reactivity to autoantigens was detected in all groups. IL-13 responses but not IFN-γ responses to recall antigen tetanus toxoid were higher in healthy control subjects compared to patients with type 1 or type 2 diabetes or LADA ($P < 0.05$). Diabetes, independent of type, was associated with weaker response to recall antigen tetanus toxoid.
Conclusions: LADA patients are indistinguishable from type 1 diabetes patients for cellular IFN-γ and IL-13 responses upon mitogen and recall antigen stimulation. These results extend previous findings showing that systemic cytokine/chemokine and humoral responses in type 1 diabetes and LADA are similar.

1. Introduction

The term latent autoimmune diabetes of adults (LADA) was introduced in 1995 by P.Z. Zimmet to define a subgroup of adult diabetes patients who were classified clinically as type 2 diabetes subjects but tested positive for GAD auto-antibody [1]. Five years after diagnosis, 80% of LADA patients progress to insulin dependence [2,3]. When LADA patients progress to insulin treatment their phenotype is similar to type 1 diabetes. LADA also resembles type 1 diabetes immunogenetically. LADA patients can share genetic similarities with type 1 diabetes and type 2 diabetes patients, but the predominant genetic association is with type 1 diabetes [4]. On the other hand, LADA patients have increased frequency of HLA-DQB1 and PTPN22 risk genotypes and alleles which distinguish them from patients with type 1 diabetes diagnosed after 35 years of age [5]. Immunologically type 1 diabetes and LADA patients have similar characteristics of auto-antibodies [6–9] and systemic cytokines such as IL-1RA, IL-6, and TNF-α [10].

Only few data are available comparing T-cell reactivity from patients with LADA, type 1 and type 2 diabetes. In a small Chinese study T-cell reactivity to GAD65 was compared between LADA and type 2 diabetes. There was low level reactivity for both IFN-γ and IL-4, and the number of IFN-γ producing T-cells was higher in patients with LADA [11]. A study by Brooks-Worrell et al. reported that unfractionated mononuclear blood cells from LADA and type 1 diabetes patients responded more strongly with proliferation to islet proteins blotted onto nitrocellulose than cells from patients with type 2 diabetes [12]. The quality of the immune response in terms of cytokines produced was not determined.

In the present study we aimed to evaluate autoimmune T cell responses in LADA and type 1 diabetes patients in comparison to patients with type 2 diabetes. We recruited patients from the

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Action LADA trial that have been diagnosed with diabetes within 5 years [13]. In addition we evaluated healthy unrelated control subjects performing enzyme linked immunosorbent-spot assays (ELISPOT).

2. Materials and methods

2.1. Subjects

The study population consisted of 159 German individuals from the Action LADA cohort [10] and included 35 patients with type 1 diabetes, 59 with type 2 diabetes, 23 with LADA, and 42 healthy control subjects. Diabetes patients had been diagnosed with diabetes for a maximum of 5 years. Design and characteristics of the Action LADA study have been described in detail before [10,13]. LADA was defined as diabetes occurring at the age of 30–70, not requiring insulin for the first 6 months after diagnosis and having autoantibodies to GAD65. The study protocol was approved by the Local Ethics Committee in accordance with the declaration of Helsinki. All patients gave written informed consent for the study.

2.2. Enzyme-linked immunosorbent spot assay

ELISPOT was performed as described [14,15] using the U-CyTech Assays (Utrecht, The Netherlands) for IFN-γ and IL-13. Briefly, venous blood was drawn into K^+–EDTA tubes, shipped to the institute and stored over night at RT. Antigens were pipetted into the appropriate wells of 48-well plates. To each antigen-containing well 3.5×106 PBMC's were added and the plate was incubated at 37 °C for 18 h. Next, 0.5 ml of supplemented RPMI 1640 containing 10% human AB serum were added to each well and the plates incubated for additional 22 h.

Following the stimulation the non-adherent PBMC's were collected, washed, suspended in 300 μl medium, and transferred as 100 μl aliquots in triplicate wells into 96-well Nunc Maxisorp plates (Merk, UK). U-CyTech IFN-γ and IL-13 ELISPOT assays were used to determine the number of IFN-γ and IL-13 producing cells. Detected spots were counted using the automated reader system Bioreader 3000LC (BioSys, Germany).

2.3. Stimuli

We used 16 different stimuli including mitogens, recall- and auto-antigens, islet hormones, and peptides. Medium alone was used as negative control. PI, a mixture of phorbol–myristate–acetate (PMA) [10 ng/ml] and ionomycin [1 μM] (Sigma, Germany), was used as positive control, while tetanus toxoid (TT) [1.5 Lf/ml] (SVM, The Netherlands) was included as a recall antigen. The other stimuli were human GAD65 [1 μg/ml] (Diamyd, Sweden), proinsulin [10 μg/ml] (Eli Lilly and Company, USA), insulin [10 μg/ml] (Novo-Nordisk, Denmark), ghrelin [5 μg/ml] (American Peptide, USA), hsp60 [0.5 μg/ml] (gift from Peptor, Israel), DiaPep277 [10 μg/ml] (gift from Peptor, Israel); GAD and IA-2 peptides according to Arif et al. [16] – GAD_3.1 (aa 335–352) [5 μg/ml], GAD_4.11 (aa 554–575) [5 μg/ml], GAD_4.7 (aa 270–292) [5 μg/ml], human pIA-2 (p25, p26) [10 μg/ml], pIA-2_R2 (aa 853–872) [5 μg/ml], pIA-2_R3 (aa 752–775) [5 μg/ml], pIA-2_R5 (aa 709–736) [5 μg/ml], and insulin peptide ins B11-23 (aa 11–23) [5 μg/ml] (all peptides were synthesized at the Leiden University Medical Center, The Netherlands).

2.4. Statistics

ELISPOT results are reported as stimulation index (SI), dividing mean spots upon stimulation by mean background [BG] spots as described [16,17]. Descriptive and inferential statistics were applied where appropriate applying SAS Enterprise Guide v4.2 (SAS Institute, USA) and GraphPad Prism v.4 (GraphPad Software, USA). Continuous variables are displayed as medians and interquartile ranges (IQR = Q3 [75%] – Q1 [25%]). To determine differences within all groups we used Kruskal–Wallis test and Mann–Whitney *U* test to compare single groups. For the evaluation of categorical data with two or more classes Fisher's exact test was applied. Results were considered significant when the *P* value was <0.05.

3. Results

The baseline characteristics for sex, age, BMI, diabetes duration, and blood glucose of the groups are presented in Table 1. All groups differed in sex, BMI and blood glucose (for all $P < 0.0001$). In addition, diabetes groups had different diabetes duration ($P < 0.05$).

ELISPOT response to 16 stimuli was evaluated using IFN-γ and IL-13 ELISPOT assays. Immune responses in BG samples were low (median spot numbers [IQR] for IFN-γ = 1 [1.7] and for IL-13 = 0.7 [1.2]), T-lymphocyte responses to mitogen PI were high (Fig. 1A). No differences between the groups in the IFN-γ and IL-13 response to the PI mitogen (Fig. 1A) or background responses were present.

Statistically significant differences between the groups were observed in the anti-inflammatory (IL-13) immune response to the recall-antigen TT (Fig. 1B) and to the peptide IA-2_R3 (Table 2). TT responses were lower in the diabetes groups than in the control group ($P < 0.05$, Kruskal–Wallis test). Type 1, type 2 diabetes and LADA patients had similar responses to TT (Fig. 1B). Upon further analysis applying linear regression we found that IFN-γ and IL-13 responses to tetanus toxoid were not influenced by diabetes duration ($r = -0.00277$; $P = 0.9766$), confirming our previous findings [15]. Furthermore, tetanus response did not significantly relate to age ($r = -0.1207$; $P = 0.1949$) or BMI ($r = -0.0247$; $P = 0.7924$).

For IA-2_R3 the immune response (IL-13) was slightly stronger in the type 2 diabetes group compared to the other groups ($P < 0.05$, Kruskal–Wallis test). Again, T1D and LADA were indistinguishable. For the majority of antigenic stimuli responses were low and mostly did not exceed background responses in line with

Table 1
Baseline characteristics of the study participants.

Characteristic	Control	T2D	T1D	LADA	*P* value
Subjects, *n*	42	59	35	23	
Men, *n* (%)	17 (40.5)	43 (72.9)	23 (65.7)	6 (26.1)	<0.0001
Median age (IQR), years	48.2 (24.6)	49.9 (19.2)	44.3 (13.8)	49.1 (17.2)	n.s.
Median BMI (IQR) (kg/m^2)	23.12 (6.94)	30.3 (7.1)	25.1 (6.3)	26.6 (7.5)	<0.0001
Median glucose (IQR) (mg/dL)	83.0 (14.5)	122.0 (54.0)	126.5 (59.0)	123.0 (52.0)	<0.0001
Median diabetes duration (IQR) months	–	12.5 (34.5)	4.0 (24.0)	27.0 (32.0)	<0.05

Data are presented as number (*n*) and percentage or as medians and interquartile range (IQR).
P-values are corresponding to the comparison of all groups using Kruskal–Wallis test or Fisher's exact test.
T1D – type 1 diabetes; T2D – type 2 diabetes; LADA – latent autoimmune diabetes of adults; n.s. – not significant.