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The cytokine production of peripheral blood mononuclear cells reflects the autoantibody profile of patients suffering from type 1 diabetes

Jana Labikova *, Jana Vcelakova, Tereza Ulmannova, Lenka Petruzelkova, Stanislava Kolouskova, Katerina Stechova

Paediatric Department, University Hospital Motol and 2nd Faculty of Medicine, Charles University in Prague, V Úvalu 84, 150 06 Praha 5, Czech Republic

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ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disorder characterised by the immune-mediated destruction of insulin-producing pancreatic beta cells. The inflammatory process appears to be primarily mediated by pro-inflammatory Th1 lymphocytes, while the role Th17 cells in T1D is currently being investigated. T1D is characterised by the presence of autoantigen-specific autoantibodies.

This study was conducted using patients with confirmed T1D and healthy control subjects. We examined the effect of the patient's autoantibody profile on peripheral blood mononuclear cell (PBMC) cytokine production following stimulation with the major diabetogenic autoantigens GAD65 and IA2. IFN-gamma and IL17 production was detected by ELISPOT and the ratio of basic cellular populations in PBMCs was measured by flow cytometry.

We demonstrated a significant interaction between the patient's autoantibody profile and mode of stimulation. This suggests that autoantigen stimulation has a different effect on different groups of patients depending on their autoantibody profile. An increased production of IL17 was found in patients with high IA2 autoantibodies compared to patients with low levels of autoantibodies and healthy controls regardless of the mode of stimulation. The titre of IA2 autoantibodies positively correlates with the proportion of Tc lymphocytes and negatively correlates with the proportion of Th lymphocytes.

Our results show that a patient's autoantibody profile reflects the type of cellular immune responses. It seems that the high titre of IA2 autoantibodies is related to increased production of IL17 and an increased proportion of Tc lymphocytes. This finding may be useful in designing immunointervention studies to prevent T1D.

1. Introduction

Type 1 diabetes (T1D) is a serious, organ-specific, autoimmune disease that is characterised by an irreversible destruction of pancreatic beta cells by the immune system. This process results in absolute insulin deficiency. Both genetic predisposition and environmental factors influence the development of this disease. Beta-cell destruction is mediated primarily by cellular components of the immune system. Th1 response is considered to be crucial for this process. Autoimmune destruction of beta cells can be identified by the detection autoantibodies in the serum of individuals prior to the onset of clinical symptoms.

After the initiation of autoimmune processes, the pancreas is infiltrated by immune cells, which consist of primarily T and B lymphocytes, macrophages and dendritic cells [1]. When 80–90% of beta cells are destroyed, the remaining cells are not able to sustain the insulin requirement, and the patient begins to experience clinical symptoms of T1D [2]. The destruction of islet cells results in the further release of autoantigens, T cell activation, epitope spreading and amplification of the autoimmune process. Key players in the autoimmune destruction of beta cells are Th1 cells, which are characterised by the production of interferon (IFN)-gamma and IL-2. Conversely, Th2 cells produce IL-4, which is protective [3]. Recently, Th17 cells have been shown to mediate some autoimmune diseases (Rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease) [4]. However, the role of Th17 cells in T1D pathology is still unclear. It seems that Th17 cells are not

* Corresponding author. Tel.: +420 224 432 066/89; fax: +420 224 432 221.
E-mail addresses: jana.labik@seznam.cz (J. Labikova), Jana.Vcelakova@fnmotol.cz (J. Vcelakova), tulma@centrum.cz (T. Ulmannova), lenkapetruzelkova@gmail.com (L. Petruzelkova), stanislava.kolouskova@lfmotol.cuni.cz (S. Kolouskova), Katerinastechova@seznam.cz (K. Stechova).

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necessary for the initiation of T1D, but they may be involved in later stages of the autoimmune process [5]. Major autoantigens identified in human T1D are GAD65 (glutamine acid decarboxylase-65), IA2 (insulinoma antigen-2), insulin and ZnT8 (zinc transporter-8). Both T cell responses as well as specific autoantibodies against these autoantigens can be detected in patients with T1D [6].

There have been many attempts to prevent or at least delay the onset of T1D by various immunointervention approaches. The major disadvantage of nonspecific immune intervention is potentially causing undesirable immune suppression. Thus, it would be ideal to induce a specific tolerance to autoantigens without suppressing the rest of the immune system. Different methods of autoantigen administration (intravenous, intranasal, DNA vaccine) are effective in animal models [7–9]. However, clinical trials have not been very successful yet. Many studies thus far have only used one antigen to induce tolerance. The concept of a single antigen vaccine is to use that antigen to induce a shift from a Th1 response to a Th2 response resulting in bystander suppression of the autoimmune process. The preventive DPT-1 (diabetes prevention trial-1) study did not have an overall positive effect following oral administration of insulin. However, later analysis revealed that positive results were achieved in the subgroup of patients with high titres of anti-insulin autoantibodies [10].

Therefore, we investigated whether the autoantibody profile in T1D patients also reflects the cellular response to T1D-associated autoantigens. This study was conducted on patients with a confirmed T1D diagnosis. The patient's PBMCs were stimulated with three different concentrations of antigens (GAD65, IA2), and their IFN-gamma and IL17 production was detected using an ELISPOT assay. We also measured the ratios of different cell types in unstimulated cells. The proportion of T cells, B cells, NK cells, T regulatory cells (Tregs) and monocytes was determined.

2. Materials and methods

2.1. Study subjects and ethics

Peripheral blood samples were obtained from 40 T1D children and 11 healthy controls. Titres of GAD65 and IA2 autoantibodies and the levels of glycosylated haemoglobin were assessed from the samples. The patients were divided into four groups according to their autoantibody profile (Table 1). The level of autoantibodies was assessed by RIA (RSR RIA GAD Ab kit, Cardiff, UK). The cut-off for a positive result in this assay was 1 U/ml for both IA2 and GAD65 autoantibodies. We considered the titre of autoantibodies to be high when it exceeded 3 U/ml, which would exclude marginally positive results. Characteristics of the groups are summarised in Table 1. The University hospital Motol ethics committee granted ethical approval for this study, and informed consent was obtained.

2.2. Methods

2.2.1. PBMC isolation and stimulation

Approximately 15 ml of peripheral blood was collected into Li-heparin tubes (Vacuette GBO, Austria). PBMCs were isolated from whole blood by Ficoll density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare, Sweden) and were frozen in 10% DMSO (Sigma, St. Louis, MO, USA). Cryopreserved cells were stored in −80 °C and used within 6 months. After thawing, the cells were left overnight in an incubator to eliminate any cells damaged during freezing. Following the overnight rest, the cells were resuspended in fresh RPMI-1640 medium (GIBCO, Life technologies, UK) supplemented with 20% FBS (Sigma), L-glutamine and antibiotics (1 μl/ml penicillin, 1 μg/ml streptomycin, 10 μl/ml 200 mM L-glutamine; all were purchased from Sigma). The concentration of viable cells was assessed by Trypan Blue staining. Subsequently, the cells were stimulated with three different concentrations of synthetic GAD65 (whole protein) and IA2 (peptide a.a. 853–872, SFYLK (Nleu)VQTQETRTLTQFHF, molecular weight 2489) autoantigens (both ProImmune, Oxford, UK). The concentrations used for the stimulation are listed in Table 2. The selection of autoantigens used in this study is based on the Immunology of diabetes society T cell workshops [11,12] as well as on our own experience [13,14]. The whole molecule of GAD65 was successfully used in several studies [13,15] and is also approved for use in antigen-specific clinical trials [16]. The immunogenicity of IA2 853–872 peptide was shown in the Report of the third immunology of diabetes society T-cell workshop [11]. We used PHA (10 $\mu g/10^6$ cells, Sigma) as a positive control. The negative controls were cultured in medium only. The cells were pre-incubated for 24 h and then transferred to an ELISPOT plate (10^5 cells/well) and cultivated for another 48 h (72 h in total).

Table 2
Concentrations of GAD65 and IA2 used for the PBMC stimulation.

Stimulation	Concentration (molarity)
GAD65-1	2.5 $\mu g/10^6$ cells ($3.8 \cdot 10^{-4}$ mol/l)
GAD65-2	5 $\mu g/10^6$ cells ($7.6 \cdot 10^{-4}$ mol/l)
GAD65-3	10 $\mu g/10^6$ cells ($1.5 \cdot 10^{-3}$ mol/l)
IA2-1	0.5 $\mu g/10^6$ cells ($2 \cdot 10^{-3}$ mol/l)
IA2-2	1 $\mu g/10^6$ cells ($4 \cdot 10^{-3}$ mol/l)
IA2-3	2 $\mu g/10^6$ cells ($8 \cdot 10^{-3}$ mol/l)

Table 1
Characteristics of the study groups. The T1D patients were divided into four groups according to their autoantibody profile: low levels of GAD65 and IA2 autoantibodies (LOW group), high levels of GAD65 autoantibodies (GAD group), high levels of IA2 autoantibodies (IA2 group) and high levels of both GAD65 and IA2 autoantibodies (DP double positive group).

Group	Inclusion criteria	Number of patients	Sex		Age		GAD65 autoantibodies		IA2 autoantibodies		HbA1C (mmol/mol; according to IFCC)		Duration of T1D	
					Median	Mean	Median	Mean	Median	Mean	Median	Mean	Median	Mean
LOW	GAD65 and IA2 <3 U/ml	10	M F	4 6	11.9	11.9	1.35	1.45	0.87	1.13	75.5	73.2	5.44	6.05
GAD	GAD65 > 3 U/ml	10	M F	2 8	9.5	9.0	10.1	29.83	0.5	0.59	67.4	65.8	3.67	4.17
IA2	IA2 > 3 U/ml	10	M F	4 6	12.0	12.0	0.45	0.85	5.68	13.12	71.5	74.9	6.44	5.18
DP	GAD65 and IA2 >3 U/ml	10	M F	5 5	12	12.9	11.77	29.94	14.1	18.88	66.5	64.2	2.08	2.89
HC	Healthy controls GAD65 and IA2 <1 U/ml	11	M F	5 6	8.0	8.0	<0.52	<0.52	0.49	0.55	NA	NA	NA	NA