



***In Vitro* Secretion of Interleukin 2 and Expression of IL-2 Receptor in Peripheral Blood Lymphocytes in High Risk of Insulin-Dependent Diabetes Mellitus Subjects**

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Abstract. Interleukin 2 (IL-2) – a Th1 lymphocyte-derived cytokine is at present considered to play an important role in the etiopathogenesis of insulin-dependent diabetes mellitus. In the previous studies increased, decreased and unchanged IL-2 levels in patients with recent onset of insulin-dependent diabetes mellitus (IDDM) were found. These differences could be a result of different metabolic status or/and a different stage of the autoimmune process. The aim of our study was to estimate *in vitro* secretion of IL-2 and CD25 antigen expression by the peripheral blood T lymphocytes in subjects at the preclinical stage of IDDM (prediabetes), but still without metabolic disturbances. In 27 first degree relatives of IDDM patients with antibodies against different pancreatic islet cell antigens (ICA, GADA, IAA, IA-2) CD25 antigen expression on peripheral blood lymphocytes T was measured by flow cytometry and IL-2 concentration in supernatants of 48 and 72 h cultures of peripheral whole blood with 10 µg/ml PHA was estimated by ELISA. The control group was comprised of 34 age and sex-matched healthy volunteers. In the studied high risk IDDM subjects the decreased CD25 expression in peripheral CD4⁺ lymphocytes T and a negative correlation between the percentage of CD25⁺ cells and islet cell antibodies (ICA) titres was observed. No differences in IL-2 levels in supernatants of 48 h and 72 h blood cultures was found in subjects with single antibody (ICA⁺) in comparison to healthy controls. A significant increase of IL-2 secretion at 72 h of PHA stimulation was shown in first degree relatives of IDDM patients with a combination of 3 or more antipancreatic-B cell antibodies. There were also a significant negative correlation between glutamic acid decarboxylase antibodies (GADA) titres and IL-2 levels in 72 h of culture. The present study suggests the involvement of IL-2 in the pathogenesis of IDDM. The estimation of CD25 antigen expression in the peripheral blood lymphocytes could be an additional immunological marker of identification of subjects in prediabetes.

Key words: interleukin 2; CD25 antigen; diabetes mellitus type 1; etiopathogenesis.

Abbreviations used: IDDM – insulin-dependent diabetes mellitus, IL-2 interleukin 2, OGTT – oral glucose tolerance test, GADA – glutamic acid decarboxylase antibodies, ICA – islet cell antibodies, IAA – insulin auto-antibodies, FPIR – first phase of insulin release.

Introduction

It was recently suggested that the IL-2/IL-2 receptor system plays a central role in the mediation of the immune response, especially in the development of autoimmune disorders^{11, 12}.

Interleukin 2 is a cytokine secreted mainly by activated T helper lymphocytes after stimulation with mitogens or after the interaction with antigen/MHC complexes on the surfaces of antigen-presenting cells^{11, 20}. Interleukin 2 receptors (CD25) are present on the surface of T lymphocytes and mediate the action of IL-2. Since the continuous stimulation of T lymphocytes induces CD25 synthesis, it has been proposed for IL-2 receptors to be a marker of T cell activation^{4, 13}.

The involvement of IL-2 in the internal network of hormonal factors leading to animal diabetes was shown in diabetes-prone BB rats²¹, but its role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) is not well defined in humans. There were, however, few investigations on IL-2 levels and lymphocytes IL-2 receptor expression in patients with a recent onset of IDDM, but their results have been conflicting^{7, 13, 19}.

The aim of our study was to estimate the role of IL-2 alterations in the pathogenesis of IDDM. We also wanted to determine, if the expression of CD25 antigen on the peripheral lymphocytes could serve as an early, additional marker for the prediabetic period. The study was performed in non-diabetic, first degree relatives of IDDM patients with present humoral autoimmune disturbances – the combinations of antibodies against different pancreatic islet cell antigens. These subjects have on 80–100% probability of developing clinical symptoms of insulin-dependent diabetes in the next 5 years⁵.

Materials and Methods

The study was carried out in 27 non-diabetic (according to WHO criteria in OGTT) first degree relatives of patients with type 1 diabetes with persistent islet cell antibodies (ICA $\geq$ 10 JDF; prediabetics; M/W: 14/13, mean age 21.4 $\pm$ 9.3 years, range 11–40 years). Sixteen of the studied subjects were also GADA positive ($\geq$ 8 U/ml), 5 had IA-2 antibodies (IA-2A $\geq$ 99th percentile) and 4 of them had insulin auto-antibodies (IAA $\geq$ 99th percentile). The control group comprised 34 age and sex-matched healthy volunteers (21 men and 13 women; aged 22.4 $\pm$ 7.9 years, range 12–38 years) who had no family history of insulin-dependent diabetes or other autoimmune diseases. Acute infections or illnesses were not present during the 3 weeks

prior to the study. Informed consent was obtained from adults and the children's parents.

Between 8 and 8³⁰ a. m. fasting venous blood was collected in sterile Vacutainer preservative-free heparin tubes (Becton Dickinson, USA) for cell culture and in EDTA tube for morphology parameters, HbA1C levels and CD phenotyping.

At the same time IVGTT was performed according to ICARUS protocol to estimate the first phase of insulin secretion (sum of insulin levels at 1 and 3 min after the end of the glucose infusion).

The absolute number of leukocytes and lymphocytes in peripheral blood was measured by Technicon RA-100 hematological counter.

An appropriate amount of whole blood was placed in 6-well plates (Corning, NY, USA) at a concentration of 2×10^3 lymphocytes/ml and cultured in RPMI-1640 medium supplemented with L-glutamine and 10% of Fetal Bovine Serum (Sigma, St. Louis, USA) in the presence of the 10 μ g/ml of phytohemagglutinin (PHA, Sigma-Aldrich) at 37°C in humidified 5% CO₂ atmosphere. Culture supernatants were collected after 48 and 72 h of incubation, centrifuged at 550 g for 5 min and stored at –70°C until further analysis. IL-2 levels in supernatants were quantified by ELISA (Endogen Interleukin 2 ELISA, Cambridge, USA).

Leukocyte preparation from whole blood was performed on the Q-Prep EPICS Immunology Workstation¹. The percentages of T cell subsets were measured using the combinations of conjugated with fluorescein isothiocyanate (FITC) or R-phycoerythrin (PE) monoclonal antibodies directed against CD3/CD4, CD25 lymphocytes surface antigens on Coulter EPICS XL Flow Cytometer⁹. A minimum of 5000 lymphocytes were analyzed for each sample.

ICA were performed by indirect immunofluorescence method with an antigen of human pancreas, GADA by RIA (Brahms Diagnostica, Berlin), IAA and IA-2 by RIA in the ENDIT (European Nicotinamide Intervention Trial) laboratory in London.

HbA1C was quantified by liquid chromatography technique (Bio-Rad), glucose concentration was measured by enzymatic method (Cormay).

The statistical significance between the groups was evaluated by the Mann-Whitney U test, regression analysis was performed using Spearman's correlation coefficient and CSS-Statistica program (StatSoft).

Results

Our cytometric analysis has shown a decreased CD25 expression in peripheral CD4⁺ lymphocytes of

prediabetics. The lowest percentage of CD25⁺ cells was observed in subjects with high ICA titres (> 40 JDF; Fig. 1) and in first-degree relatives with 3 or more antibodies against pancreatic B cells (Fig. 2). Negative

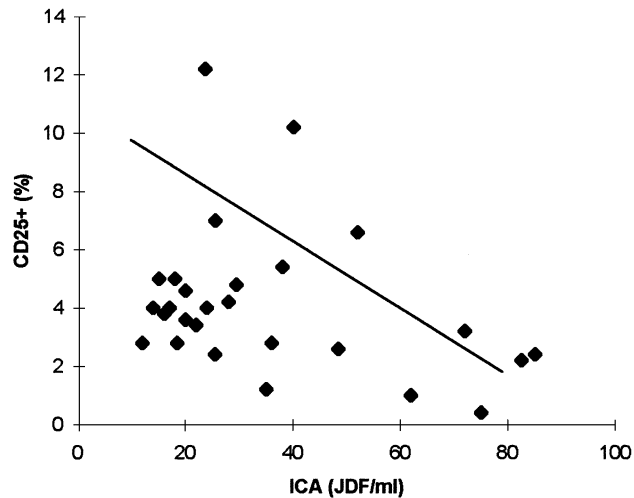


Fig. 1. The correlation between percentage of CD4⁺ T lymphocytes expressing CD25 antigen in peripheral blood and ICA titre in first degree relatives of IDDM subjects

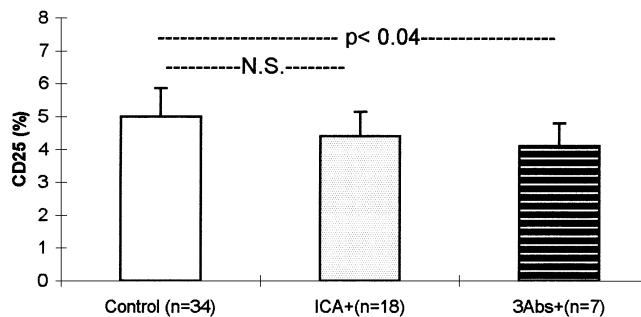
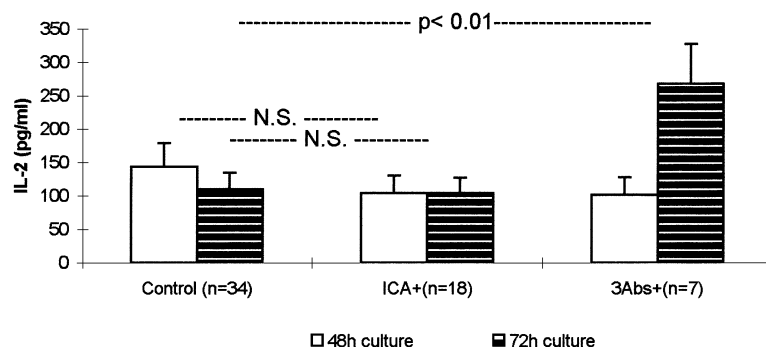


Fig. 2. The mean percentages of T lymphocytes expressing CD25 antigen associated with the number of antibodies against pancreatic B cells in prediabetics and the control group



correlation was found between the ICA titres or HbA1C levels and the percentage of CD25 antigen expression in the studied group ($R=-0.28$, $p < 0.02$ and $R=-0.24$, $p < 0.05$; Fig. 1). *In vitro* IL-2 secretion, according to the number of antibodies against pancreatic B cells in prediabetic subjects and in the control group, is presented in Fig. 3. No differences in IL-2 levels in supernatants of 48 h and 72 h blood cultures was found in subjects with single antibody (ICA⁺) in comparison to healthy controls. A significant increase of IL-2 secretion in 72 h of PHA stimulation was shown in first-degree relatives of IDDM patients with a combination of 3 or more anti-pancreatic B cell antibodies (Fig. 3). There were also a significant negative correlation between GADA titres and IL-2 levels in 72 h of culture ($R=-0.54$, $p < 0.05$).

We did not observe any correlation between FPIR or glucose levels and IL-2 production or IL-2 receptor expression. There were no differences in total leukocytes, lymphocytes and fasting glucose levels between the studied and control groups. In both groups HbA1C levels were within normal range, but they were significantly higher in the prediabetics ($5.4 \pm 1.5\%$ vs $4.1 \pm 0.5\%$, $p < 0.001$).

Discussion

The role of IL-2 – a Th1-derived cytokine and IL-2 receptor on lymphocytes T is at present considered in the etiopathogenesis of IDDM^{12, 14}. In the previous studies increased but also decreased IL-2 levels in patients with recent onset of IDDM were found^{2, 6, 19, 22}. A dose- and time-dependent inhibition of *in vitro* production of IL-2 by elevated glucose concentration, observed by REINHOLD et al.¹⁵, provided a possible explanation that differences in previous studies could result from different metabolic status of examined subjects. The present study was performed in subjects with a high risk of IDDM (prediabetics) with circulating hu-

Fig. 3. The mean interleukin 2 (IL-2) levels in the supernatants of 48 and 72 h cultures of peripheral blood with PHA (10 µg/ml) in prediabetics and the control group

moral markers of autoimmune process, but still without metabolic disturbances.

In our cytometric analysis we found the decreased IL-2 receptor expression in peripheral CD4⁺ lymphocytes in prediabetics. Similar results of lower IL-2 receptor levels in patients with newly diagnosed diabetes type 1, in comparison to healthy controls, was shown by WAGNER et al.²⁰ Since in our previous study a decreased number of total CD4⁺ lymphocytes was found in subjects at high risk of IDDM⁸, it could be suggested that a decreased percentage of CD25⁺ cells is a result of the decreased number of these cells in the peripheral blood, caused, for example, by the accumulation of CD25⁺ lymphocytes T in the *insulitis* in the pancreas. The infiltration by significant number of activated lymphocytes expressing CD25⁺ antigen was found in pancreas of NOD mice and prediabetic humans using recombinant IL-2 labeled with I-123^{16, 17}. The decreased expression of IL-2 receptor observed after *in vitro* PHA stimulation of lymphocytes T by GIORDANO et al.³, suggests that immunoregulatory defect of T cells may be involved in this process. It cannot be excluded that it is a primary (genetically inherited) defect of cellular immune response, since the important role of genetic factor in IDDM pathogenesis is considered.

In the present study, in the subgroup of examined subjects with a very high risk of IDDM development (3 or more antibodies against pancreatic islets), IL-2 concentration in supernatants at 72 h of culture was significantly higher than at 48 h of culture and in comparison to the control group. The highest IL-2 levels at 24 h of culture, followed by a decrease of secretion in the next hours after *in vitro* PHA-stimulation of peripheral T lymphocytes, was shown in healthy subjects¹⁰. Our results of increased and prolonged *in vitro* production of IL-2 by activated immune cells of the peripheral blood could suggest the role of IL-2 in the pathogenesis of IDDM. The present findings is supported by the observation of insulin-dependent diabetes mellitus development in a terminally ill patient (with advanced colorectal cancer) treated with IL-2¹⁸. In opposition to our observations HUSSAIN et al.⁶ did not find any differences in serum IL-2 levels between twins remaining non-diabetic and prediabetic twins studied several months before the development of diabetes. In our opinion, the estimation of *in vitro* IL-2 production after mitogen stimulations better reflects the ability of a local production of IL-2 (e.g. in pancreas) than the levels of this cytokine in serum.

No significant differences of *in vitro* IL-2 secretion between first-degree relatives with only single islet cell antibodies and the control group, found in our study,

was also observed by other authors²⁰. These subjects, however, have a lower risk of developing IDDM or/and probably could represent different stages of the development of the autoimmune process.

In conclusion, the present study suggests the involvement of IL-2 in the pathogenesis of IDDM. The estimation of CD25 antigen expression in the peripheral blood lymphocytes could be an additional immunological marker of identification of subjects in prediabetes.

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