Relationship between T and B Cell Responses to Proinsulin in Human Type 1 Diabetes

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ABSTRACT: In type 1 diabetes, humoral and cell-mediated responses to insulin and proinsulin are detectable. Autoantibodies to insulin are associated with impending disease in young individuals and are used as predictive markers to determine disease risk. The aim of this study was to investigate whether different cytokine patterns of cellular reactivity to insulin might serve as additional specific markers of disease maturation and might improve disease prediction in individuals at risk. We correlated T and B cell responses to insulin in subjects with increased genetic risk (HLA-DRB1*04, DQB1*0302) for diabetes with or without islet autoantibodies (Ab+ subjects and controls, respectively) and HLA-matched patients. Peripheral blood mononuclear cells were stimulated with 15 overlapping proinsulin peptides (16-mer), and proinflammatory Th1 (IFNγ) and anti-inflammatory Th2 (IL-4) cytokines were analyzed. We observed a simultaneous increase in IL-4 and IFNγ secretion in early islet autoimmunity of Ab+ subjects, but not in insulin-treated T1D patients. Furthermore, the increase in IL-4 secretion in Ab+ subjects was associated with insulin autoantibody responses. There was no correlation of either IFNγ or IL-4 secretion with insulin antibody responses in patients already treated with exogenous insulin. In conclusion, our findings reveal that quantification of cytokine responses to proinsulin in peripheral blood may prove to be a promising specific marker of diabetes progression and could, in addition to insulin autoantibodies, be used in the prediction of type 1 diabetes.

KEYWORDS: proinsulin; insulin; T cells; autoantibodies; cytokines

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Ann. N.Y. Acad. Sci. 1005: 288–294 (2003).
doi: 10.1196/annals.1288.045

INTRODUCTION

Type 1 diabetes (T1D) is considered to be a T cell–mediated autoimmune disease resulting from a disturbed cellular immunoregulation. Insulin and proinsulin are target antigens of β cell destruction. The effects of insulin to prevent T1D has currently been investigated in a large clinical trial.[1] In experimental models of T1D, insulin therapy has been shown to delay diabetes onset, while more recently disease induction was observed after preproinsulin treatment in NOD and RIP-B7.1 mice.[2]

T cells specific for islet β cell proteins also exist in healthy individuals, but are restrained by regulatory mechanisms.[3,4] In T1D, if regulatory T cell responses fail, it has been postulated that autoreactive T helper cells specific for β cell antigens become activated and clonally expand.[5–7] They provide help to B cells that secrete autoantibodies specific for the same autoantigens.[8,9] However, the pathogenesis of T1D is considered to be cell-mediated because T cells can transfer disease in animal models and in human T1D, while autoantibodies do not have pathogenic properties.[10–12]

Insulin and proinsulin autoantibodies develop spontaneously prior to the onset of clinical disease and correlate closely.[13–15] Insulin autoantibodies are also early sensitive markers of the impending disease onset in the young[16] and can precede other islet autoantibodies.[9,17] Moreover, in young subjects, increased T cell proliferation to insulin and proinsulin is associated with an inductive phase of the autoimmune response;[4,18] in contrast, in adult subjects, responses to insulin or proinsulin are generally weak.[3,6,19–21]

The intriguing question of whether Th1 and Th2 autoimmunity in human T1D could be associated with different stages of diabetes development or disease risk has still not been addressed. Autoantibodies and T cells to insulin appear predominantly in younger individuals[17,18,20,22] and more frequently in subjects with high genetic risk of T1D (HLA-DRB1*04).[4,23,24]

The aim of this study was to determine whether T cell cytokine phenotype of *in vivo* primed autoimmune response to proinsulin correlates with different stages of disease progression in individuals with genetic HLA-DRB1*04, DQB1*0302 risk for T1D. In addition, it was analyzed whether specific patterns of cytokine secretion correlate with the level of insulin autoantibodies.

METHODS

Subjects

A total of 35 HLA-DRB1*04, DQB1*0302–positive individuals were analyzed: 12 patients with T1D (median age, 26 years; range, 2–56 years; median duration of insulin treatment, 6 months; range, 1–12 months), 12 autoantibody-positive (Ab+) schoolchildren without family history of T1D from the Karlsburg Type 1 Diabetes Risk Study[25] (median age, 20 years; range, 8–24 years), and 11 healthy control subjects without family history of T1D (median age, 22 years; range, 2–43 years). Out of 12 Ab+ individuals, 9 were classified as "high risk" subjects since they were positive for more than one additional antibody specificity, that is, insulin autoantibodies (IAA), antibodies against glutamic acid decarboxylase (GADA) or islet tyrosine phosphatase (IA-2A), and/or with a high titer of cytoplasmic islet cell antibodies

(ICA > 20 JDF-U).[8,26] Informed consent was obtained from all individuals prior to analysis, and studies were performed in accordance to the Declaration of Helsinki.

HLA Typing

HLA typing was performed using a locus-specific PCR amplification procedure as described elsewhere.[27]

Autoantibody Assays

Autoantibody assays used have been previously described in detail.[25,28] Insulin autoantibodies were determined by the microassay using the protein A/G method with and without the addition of unlabeled insulin. In the Second Diabetes Antibody Standardization Program (DASP-2) proficiency evaluation, this assay achieved a diagnostic sensitivity and specificity of 26% and 99%, respectively, at or above the 99th percentile calculated from 991 (537 m/454 f) healthy schoolchildren (204.6 μU/L). If the 97th percentile is used as threshold, this assay achieved a sensitivity and specificity of 52% and 98%, respectively, in the workshop.

Autoantigens and Peptides

Human proinsulin (Eli Lilly International, Indianapolis, IN) and insulin (Aventis, Frankfurt, Germany) were tested simultaneously with 15 proinsulin peptides (16 amino acids long and 12 amino acids overlapping), which were synthesized according to the primary preproinsulin structure (GenBank accession no. P01308). All antigens and peptides were highly purified and did not contain significant levels of endotoxin as determined by the Limulus lysate assay (<0.06 EU/mL at 10 μg peptide/mL).

Cell Separation and Stimulation Assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-paque (Pharmacia, Freiburg, Germany) density centrifugation, aliquoted, and cryopreserved in liquid nitrogen until use as described previously.[29] Microtiter plates were prepared by adding 50 μL of autoantigens (proinsulin and insulin, 10 μg/mL) or peptides (5 μg/mL) per well in triplicates, followed by addition of 150 μL of PBMC (15×10^4/well). On day 5 of incubation, supernatants of replicate cultures were pooled and stored at −80°C for cytokine analysis.

Cytokine Secretion Assay

IFNγ and IL-4 were analyzed according to the manufacturer's instructions using an antigen-capture ELISA from PharMingen (San Diego, CA). Detection limits were 34.2 pg/mL for IFNγ and 19.5 pg/mL for IL-4. Quantification of spontaneous cytokine release was performed by incubating the cells of each individual under the same conditions, but in the absence of antigens. Positive cytokine secretion was defined by subtracting spontaneous cytokine release + 2 SD from experimental values. The amount of secreted cytokines is expressed in pg/mL.

Statistical Analysis

Data were analyzed using the SPSS software package (SPSS GmbH Software, Munich, Germany). Correlation between autoantibody responses and cytokine responses was analyzed by Bravais-Pearson correlation analysis. The nonparametric Mann-Whitney *U* test was used for unpaired observations, with an appropriate adjustment to the significance level for multiple comparisons.

RESULTS AND DISCUSSION

In the present study, we investigated T and B cell responses to insulin and its precursor, proinsulin. We quantified cytokine responses in the supernatants of PBMC cultures stimulated with 15 overlapping peptides spanning the proinsulin molecule. These cytokine responses were correlated with antibody responses to insulin.

Two groups of subjects with islet autoimmunity, Ab+ subjects and insulin-treated T1D patients with median insulin therapy duration of 6 months, and nondiabetic controls were investigated. All three groups of subjects were strictly selected for the expression of DRB1*04, DQB1*0302 haplotype associated with high risk for T1D. Our aim was to investigate whether cytokine responses, in addition to antibody responses, may prove to be additional specific markers of disease progression in subjects with increased genetic risk for T1D.

In early islet cell autoimmunity of Ab+ subjects, an increase in the magnitude of both investigated cytokines, IL-4 ($p < 0.0001$) and IFNγ ($p < 0.007$), was seen compared to the other two groups, resulting in a Th0 phenotype of cytokine responses (FIG. 1). In Ab+ subjects, T cell responses characterized by increased levels of IL-4 secretion correlated well with increased levels of IAA ($r = 0.8$, $p < 0.01$) (FIG. 2a).

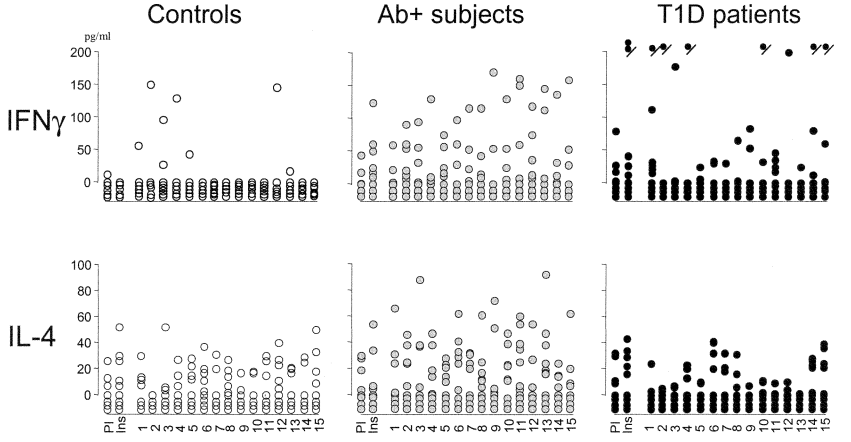

FIGURE 1. *In vitro* IFNγ and IL-4 secretion in response to proinsulin, insulin, and 15 overlapping proinsulin peptides in PBMC of nondiabetic control subjects, Ab+ subjects, and recent-onset T1D patients. PI = proinsulin, Ins = insulin, 1–15 = overlapping proinsulin peptides (16 amino acids long).

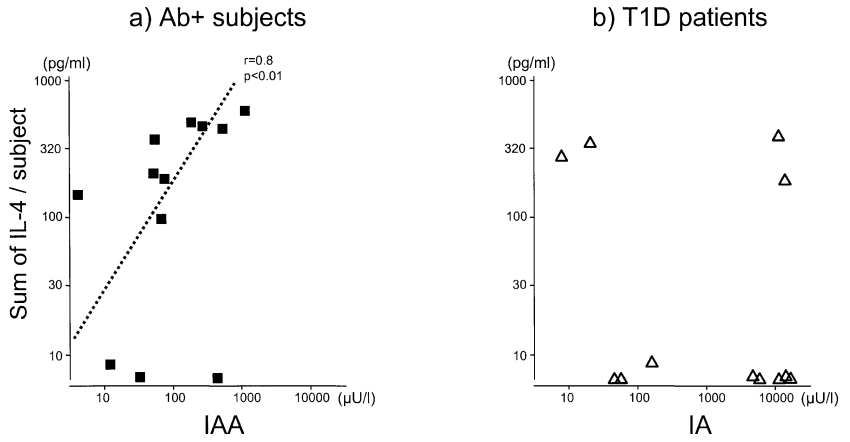

FIGURE 2. Positive correlation of T cell IL-4 secretion to proinsulin peptides with autoantibody response to insulin (IAA) in Ab+ subjects (*black squares and dotted line*) **(a)**. No correlation of IL-4 secretion to proinsulin peptides with antibody response to insulin (IA) in insulin-treated T1D patients (*open triangles*) **(b)**.

IAA develop in subjects at high risk for T1D prior to the onset of clinical disease and exposure to exogenous insulin.[16] In our study, 5 out of 12 Ab+ subjects were positive for IAA; and in 4 out of these 5, the sum of IL-4 responses to proinsulin peptides was higher than 300 pg/mL.

In contrast, in T1D patients (receiving insulin therapy for a median duration of 6 months), no correlation of cytokine responses with insulin antibody (IA) responses was observed (FIG. 2b). IA frequently develop in patients treated with exogenous insulin and reach higher levels in individuals who were IAA-positive before the diagnosis of T1D.[30] Eight out of 12 T1D patients in our study were positive for IA, and only 2 of these 8 had high IL-4 responses. The other patient had IFNγ responses higher than 300 pg/mL.

Different studies have analyzed the relationship between the proliferative T cell versus antibody responses to insulin with conflicting results. An inverse relation of IAA and insulin-reactive T cells was found in the study where recent-onset T1D patients were combined with nondiabetic subjects.[3] No relationship was observed between humoral and cellular responses to insulin in T1D patients, although both T cell responses and autoantibody titers were higher in younger subjects.[20] In our previous study where we analyzed young Ab+ subjects and T1D patients at diagnosis, a weak positive association of antibodies and T cell reactivity to insulin was observed.[31]

Number and level of circulating autoantibodies as indirect markers of the disease activity accompany active autoimmunity in T1D.[26] In this study, we postulated that T cells of subjects with islet autoimmunity (i.e., Ab+ subjects and T1D patients) have been primed *in vivo* during a spontaneous autoimmune response and, therefore, should exhibit a cytokine pattern of activated peripheral memory cells characterized by high IFNγ and IL-4 secretion.[32] By analyzing cytokine secretion patterns and Th1/Th2 differentiation state of these cells, we defined the cytokine specificity of the

proinsulin T cell response and correlated their cytokine profile with autoantibody response and with disease progression.

In conclusion, we propose that both autoreactive T cells and autoantibodies to proinsulin simultaneously coexist in T1D and preclinical T1D, and both may play a significant role in the pathogenesis of the disease. Autoantibody responses to insulin are in an early preclinical stage of T1D autoimmunity (Ab+ subjects) associated with Th2 (IL-4) phenotype of cytokine responses. Both in combination may become of increasing value in the prediction and diagnosis of preclinical T1D and in the monitoring of prevention trials.

ACKNOWLEDGMENTS

Wolfram Karges and Bernhard O. Boehm contributed equally to this paper.

This work was supported by grants from the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich, SFB 518 (to I. Durinovic-Belló, W. Karges, and B. O. Boehm); Eli Lilly Foundation International (to I. Durinovic-Belló); Deutsche Diabetes Stiftung, Stiftung "des Zuckerkranke Kind" (to I. Durinovic-Belló); and Deutsche Diabetes Gesellschaft (to I. Durinovic-Belló). We acknowledge H-J. Schreckling, B. Feldmann, and M. Kuhn-Halder for clinical care of the patients.

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