T CELL RESPONSES TO ISLET ANTIGENS IMPROVES DETECTION OF AUTOIMMUNE DIABETES AND IDENTIFIES PATIENTS WITH MORE SEVERE BETA CELL LESION IN PHENOTYPIC TYPE 2 DIABETES.

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Abstract

Latent autoimmune diabetes of adults (LADA) or type1.5 diabetes is considered to be a T cell mediated autoimmune disease. However, identification of patients is based commonly on autoantibody detection. To determine if measuring T cell (T) reactivity to islet proteins compared to measuring autoantibodies (Ab) improves detection of autoimmune diabetes and how β-cell function correlates with T reactivity compared with Ab positivity, we assessed the T cell proliferative responses and Ab responses (ICA/ IAA/ IA-2Ab/ GAD-Ab) to islet proteins of 36 phenotypic type 2 patients. To be considered Ab+ or T+ , patients were required to be positive for a minimum of two consecutive time points. β-cell function was measured with fasting and glucagon stimulated C-peptide. Independent of T reactivity, Ab (+) and Ab (-) patients had comparable fasting and glucagon stimulated C-peptide. Independent of Ab status, T (+) patients demonstrated significantly lower glucagon stimulated ($p<0.003$) C-peptide compared to T (-) patients. This data suggests that measuring T cell responses to multiple islet proteins, in phenotypic type 2 diabetes patients, improves identification of patients with autoimmune diabetes and delineates those who have a more severe β-cell lesion compared to autoantibody assessment alone.

1. Introduction

Diabetes mellitus in humans is classified clinically into two main types, type 1 and type 2 diabetes with different underlying pathophysiology. The disease process in type 1 diabetes is T cell mediated autoimmune destruction of the pancreatic beta cells (1). In contrast, the disease process in classic type 2 diabetes is not autoimmune. Decreased sensitivity to insulin action (2) is central to the disease process and a non-immune mediated beta cell lesion occurs which diminishes insulin secretion (3). The diagnosis of type 1 versus type 2 diabetes is usually made using clinical criteria but clinical distinction of type 1 and type 2 is recognized to be imperfect.

There is also a third group of patients, who clinically are phenotypically similar to type 2 diabetes but also are positive for one or more of the autoantibodies (ICA, GAD-Ab, IAA, IA-2Ab) commonly seen in type 1 disease. This autoantibody positive phenotypic type 2 diabetes has been termed LADA (4-5), latent autoimmune disease in adults or type 1.5 diabetes (6-7) or type 11/2 diabetes (8). Type 1.5 diabetes is usually diagnosed after 35 years of age, one or more islet autoantibodies are present, there is not an immediate need for insulin, and comprises ~10% of caucasian adult phenotypic type 2 diabetic patients.
Like type 1 diabetes, type 1.5 diabetes is also considered an autoimmune disease, mediated by autoreactive T cells. T cells reacting to various islet proteins in peripheral blood have been demonstrated in both type 1 and LADA patients (10, 11). Recently we investigated T cell responses to islet proteins and autoantibody responses using multiple assays and discovered that there appear to be antigenic differences in the proteins recognized by the T cells and autoantibodies of the type 1.5 diabetes patients versus classic type 1 patients (9). Therefore, even though there are similarities between type 1 and type 1.5 diabetes patients, the pathogenesis appears to have some differences.

Although, type 1.5 diabetes is believed to be T cell mediated, studies identifying type 1.5 patients rely mainly on autoantibodies (15-27). Therefore, in this study, we asked if beta cell dysfunction was more associated with autoantibodies or T cell reactivity to islet antigens in patients with type 1.5 diabetes. We categorized patients as to autoantibody + (Ab+) or Ab- and T cell + (T+) or T -. We then asked whether antibody positivity or T cell positivity would better identify patients with a more severe β-cell lesion (i.e. lower C-peptide).

2. Methods

2.1 Subjects

Thirty- six phenotypic type 2 diabetes patients within five years of diagnosis were studied. The patients were obtained from a prospective study comparing the effect of rosiglitazone and glyburide on beta cell function. A type 2 diabetic phenotype required the onset of diabetes between ages 35 and 70 years, no history of ketonuria or ketoacidosis, and initially not requiring insulin for glycemic control. Written informed consent was obtained from each patient prior to study enrollment. Blood was drawn from patients after a 2 week washout period off all medication. All patients were required to have a hemoglobin A1c between 6% and 10% and fasting C-peptide ≥0.80ng/ml. The cut point of 0.8 ng/ml was chosen because we wanted patients in the drug study who, at least in the beginning of the study, could be treated with oral agents and not require insulin therapy. Exclusion criteria included:

history of chronic pancreatitis or other secondary cause of diabetes, treatment with systemic corticosteroids, concurrent severe systemic illness, renal insufficiency or hepatic dysfunction. Diabetes associated autoantibodies and T cell proliferative responses to islet antigens were assessed to classify the patients. The autoantibodies measured were glutamic acid decarboxylase autoantibodies (GAD-Ab), autoantibodies to the tyrosine phosphatase IA-2 (IA-2Ab), islet cell autoantibodies (ICA), and insulin autoantibodies (IAA). T cell responses were measured using cellular immunoblotting. The patients are enrolled in a drug study, are seen every 3 months and blood samples taken. The definition for positivity or negativity is "for 2 consecutive time points". However, the C-peptide data presented in this manuscript is from the baseline visit of the patients. At the baseline visit patients were on no diabetes drugs (2 week washout period) and C-peptide at this time point was used because the drugs the patients were randomized to could differentially affect their C-peptide results. The study is still ongoing and follow-up C-peptide data on these patients will be analyzed when the study is completed. Beta cell function was measured using fasting and glucagon stimulated C-peptide. Demographic information like age, weight, height, and duration of diabetes were also obtained.

2.2 Islet cell autoantibody assay. This assay was performed as previously described (9-10). All sera with detectable ICA were end-point titered. The lower detection limit of our assay was 1 JDF and the 95th percentile positivity threshold was established at 6 JDF units based on approximately 4,000 normal school children (28). Our laboratory had participated in a total of 8 Immunology of Diabetes Society Workshops (IDW) and Immunology of Diabetes Society (IDS)-sponsored proficiency programs for ICA with an average sensitivity of 80% and a specificity of 100% (in identification of patient versus control sera). In the IDS sponsored Combined Antibody Workshop (29), our ICA assay had a specificity of 98% and a sensitivity of 76%. Our ICA assay had been validated in a serum exchange with the Diabetes Prevention Trial –Type 1 Diabetes (DPT-1) ICA core lab. In this exchange, the sensitivity of our assay was 85% with a specificity of 100%.

2.3 GAD Autoantibody Assay. The determination of GAD-Ab autoantibody levels was performed at the Immunoassay Core of the Diabetes Endocrinology Research Center, University of Washington. GAD-Ab was measured in a radiobinding immunoassay on coded serum samples as described (30). The levels of GAD-Ab were expressed as a relative index (GAD index) using one positive serum (JDF World Standard for ICA) and three negative standard sera from healthy subjects. GAD index was calculated and a positive was considered at $\geq$0.085 which is the 99th percentile based on 200 normal controls. Positive and negative controls are run in duplicate in each assay. The performance of the assays is monitored by a set of quality controls and participation in external laboratory proficiency comparisons. The laboratory has participated in the Diabetes Antibody Standardization Program (DASP) and scored 74% sensitivity and 97% specificity in 2005 (in identification of patient versus control sera). In the IDS-sponsored GAD-Ab serum exchange and the Combined Antibody Workshop, our laboratory scored 100% for both sensitivity and specificity (31).

2.4 Insulinoma associated protein-2 autoantibody (IA-2Ab) Assay. Autoantibodies to IA-2 were measured under identical conditions as described for GAD-Ab (30) using the plasmid containing the cDNA coding for the cytoplasmic portion of IA-2. The IA-2 autoantibody index for each sample was calculated using the same JDF standard serum and control sera that were used in the GAD-Ab assay. An IA-2

index $\geq$ 0.017, the 99th percentile based on 200 normal controls, was the cut off for positivity. The performance of the assays is monitored by a set of quality controls and participation in external laboratory proficiency comparisons. The laboratory has participated in DASP. In the 2001 evaluation, the laboratory scored 54% sensitivity and 96% specificity (31)(in identification of patient versus control sera). In the 2005 evaluation, the laboratory scored 62% sensitivity and 95% specificity.

2.5 Insulin autoantibody assay. The IAA assay was performed as previously described (10, 11). The threshold for positivity was the 99th percentile of values of approximately 100 normal controls. IAAs were evaluated before the start of insulin therapy.

2.6 C-Peptide assay. Fasting and glucagon - stimulated C-peptide was used as a measure of endogenous beta cell function in all 36 patients at entry into the study. Stimulated C-peptide was measured 6 minutes after the IVinjection of 1 mg glucagon. The C-peptide assay which is a two site immunoenzymometric assay, performed using a Tosoh 600 II auto-analyzer (Tosoh Bioscience Inc., South San Francisco CA), was performed in the Immunoassay core of the Diabetes Endocrinology Research Center (DERC) at the University of Washington. Briefly, C-peptide present in the sample is bound with a monoclonal antibody immobilized on a magnetic solid phase and an enzyme labeled monoclonal antibody is added. The magnetic beads are then washed to remove unbound enzyme labeled monoclonal antibody and then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled monoclonal antibody that binds to the beads is directly proportional to the C-peptide concentration in the patient sample. The inter-assay and intra-assay precision analysis show a coefficient of variation (CV) less than 10%. The assay has a sensitivity level of 0.04 ng/ml.

2.7 Cellular Immunoblotting. Cellular immunoblotting was performed as previously described for islets (11), and non-islet proteins (32, 33). Human pancreata were obtained from the NIH Islet Consortium. Islet cells were subjected to preparative 10% SDS-PAGE (34). Following electrophoresis, the gels were electroblotted onto nitrocellulose (BioRad, Richmond, CA) at 30 mA overnight, nitrocellulose particles prepared, and the nitrocellulose particles used to stimulate PBMCs in vitro.

A stimulation index (SI) for each molecular weight section was calculated as follows:

$$\text{Statistical Index (SI)} = \frac{\text{Mean CPM experimental wells}}{\text{Mean CPM control wells}}$$

Control wells contained nitrocellulose particles without antigen. Positive proliferation was considered to be a SI > 2.0 which corresponds to greater than the mean + 3 SD of control values (11). Antigen doses and specificity of PBMC responses of type 1 diabetes patients to the islet protein preparations and known islet autoantigens using cellular immunoblotting have been previously described (11). PBMC responses to tetanus toxoid were used as a control antigen along with the PBMC responses to mitogens, which were included to test for viability of the cultures. PBMC responses of type 1 diabetes patients, autoantibody positive type 2 diabetes patients, autoantibody negative type 2 diabetes patients, and controls to tetanus toxoid have been shown to be similar (10, 11). Based upon results in over 60 controls and 60 patients with type 1 diabetes, cellular immunoblotting is considered positive when > 3 blot sections have a SI > 2.0 (35). This assay has been validated by the Immune Tolerance Network where it was able to distinguish type 1 diabetics from control

subjects with a specificity of 83% and sensitivity of 91% (36).

2.8 Glucose assay. Analysis was performed enzymatically on a Hitachi 917 Clinical Chemistry autoanalyser utilizing the hexokinase method described by Peterson and young (37).

*2.9 Statistical analysis***.** Data were analyzed using unpaired t-test and the Mann-Whitney nonparametric test (StatView 4.0, Abacus Concepts, Inc.)

3. Results

3.1 General demographics

Among the 36 diabetic patients, 28 were male and 8 were female. Twenty-eight (77.8%) were Caucasians, 4 (11.1%) were African-Americans and, 4 (11.1%) were Asians or Native Americans. The mean duration of diabetes at the time of entry was 30.2 months (SEM±3.202, range 1-59 months).

3.2 Diabetes autoantibodies

Seventeen of the 36 patients were positive for autoantibodies. Of the 17 patients, 9 (52.9%) were positive for GAD-Ab, 8 (47.1%) were ICA positive, 5 (29.4%) were IA-2Ab positive, and 1 (5.9%) was positive for IAA (fig-1).

Five (29.4%) patients were positive for GAD-Ab alone, 4 (23%) were positive for ICA alone, 3 (17.6%) were positive for IA-2Ab alone, and 1 (5.8%) was positive for IAA only.

Four (23.5%) were positive for ICA and GAD-Ab and two of these patients were also positive for IA-2Ab.

To summarize, thirteen (76.4%) were positive for one autoantibody, 2 (11.7%) were positive for two autoantibodies and, 2 (11.7%) were positive for three autoantibodies. None of patients were positive for all four antibodies.

3.3 T cell proliferative responses

Of the 36 patients, 23 patients demonstrated positive PBMC responses to islet proteins (T+), and 13 were negative for PBMC responses (T-) (fig-2).

3.4 Beta cell function

Irrespective of T cell proliferative responses, no statistical difference was observed in fasting and glucagon stimulated C-peptide levels between the antibody negative and antibody positive groups (fig-3).

Irrespective of autoantibody status, T cell positive (T+) patients had significantly lower glucagon-stimulated -C-peptide compared to T cell negative (T-) patients though no significant difference was observed in fasting C-peptide secretion between the two groups (fig-4). Data is summarized in Table 1.

We further divided the T+ patients into 3 categories (4-6 blots positive, 7-11 blots positive, and 14-18 blots positive); there were no significant differences in fasting or glucagon stimulated C-peptide levels in the patients within the 3 groups. If we divided the subjects into low (4-6 blots positive) or high (7-18 blots positive), again there was no significant differences in the fasting or glucagon stimulated C-peptide responses between the two groups.

3.4 Clinical characteristics

No statistical difference (t-test or Mann-whitney non-parametric test) was observed in fasting glucose, glycemic control (HbA1c%), BMI, age and, duration of diabetes between Ab(-) and Ab(+) groups and T cell (-) and T cell (+) groups (table 2).

We also investigated insulin resistance using the HOMA model (38, 39) and did not find any significant differences between T cell positive and T cell negative subjects.

4. Discussion

Type 1.5 diabetes, like type 1, is considered an autoimmune disease characterized by the presence of autoantibodies and autoreactive T cells to islet proteins. Previously, using cellular immunoblotting, we demonstrated that T cells in T1DM patients are responsive to a wide spectrum of islet proteins. This particular T cell assay was developed in our laboratory for type 1 diabetes patients. This assay has been validated by the Immune Tolerance Network where it was able to distinguish type 1 diabetics from control subjects with a specificity of 83% and sensitivity of 91% (36). Using the same assay, we demonstrated that type 1.5 diabetic patients also show T cell reactivity to islet proteins (10). In the study reported here, we asked how beta cell function in type 1.5 diabetes correlates with T cell reactivity to islet proteins and autoantibody status.

In our study, T cell reactivity and autoantibodies were used to identify the type 1.5 diabetes patients. First, we identified type 1.5 diabetes based on the presence of autoantibodies. Out of 36 phenotypic T2DM patients, 19 patients were negative and 17 were positive for autoantibodies (ICA/GAD-Ab/IAA/IA-2Ab). No significant difference was observed in fasting and glucagon stimulated C-peptide at baseline between the two groups. This is in agreement with some previous studies that also did not find a difference in C-peptide levels between type 1.5 diabetes and T2DM (23-27).

However, there are other studies that have reported differences in fasting C-peptide levels between T1.5DM and T2DM. These studies differ greatly from one another and from our study. Minimum age criteria for patients in the studies vary. For example, the studies by Gottsater, et al (15) and Torn, et al (16) included patients ≥15 years of age. The study by Borg, et al (17) had patients >20 years old, whereas Turner, et al (18) and Hosaazufalusi, et al (19) included patients with age >25 years old. The study by Monge, et al (20) included patients >50 years. Due to the young age of the patients in some of the studies, it is possible that T1DM patients who are usually autoantibody positive with lower beta cell function were included. In contrast, in our study, we included only phenotypic type 2 diabetes patients who were between 35-70 years of age to assess beta cell function in relation to autoantibody and T-cell reactivity status.

In our study we also included 4 autoantibodies to determine whether patients were autoantibody positive or negative. However, the autoantibodies (ICA/GAD/IA-2/IAA) were originally discovered in type 1 diabetes patients. We have reported differences in both autoantibody and T cell reactivity to islet proteins between type 1 and type 1.5 diabetes patients (9). Therefore, there is still a possibility in our current study that patients identified as autoantibody negative may indeed demonstrate autoantibodies to unknown islet antigens. Though Ab reactivity to unidentified islet proteins is a definite possibility, the inclusion of ICA helps address this issue. Sera positive for ICA are recognized to frequently contain autoantibodies to antigens other than GAD, IA-2 and insulin. Therefore, we believe that assaying patients using all 4 autoantibodies is the best attempt available at this time for identifying a patient's autoantibody status.

Furthermore, many of the patients in other studies (15,17,19,21, 22) were on insulin at the time of diagnosis or required insulin within a few years of diagnosis. Again, this could lead to skewing of results since the majority of insulin treated subjects were in the autoantibody positive group and

probably had lower beta function that required treatment with insulin. In contrast, we excluded any patient who required insulin for glycemic control or patients with a history of ketoacidosis or ketonuria to avoid including any adult type 1 diabetics.

Autoantibodies measured for classification of type 1.5 diabetes in previous studies were also not the same. Most studies measured GAD-Ab and sometimes ICA or IA-2Ab. The studies by Arikan, et al (22), Yang, et al (27) and Brikeland, at el (24) measured only GAD-Ab. The UKDPS study (18) and the studies by Gottsater, et al (15), Monge, et al (20) measured GAD-Ab and ICA. The studies by Borg, et al (17), Hosaazufalusi, et al (19), and Tuomi, et al (21) measured 3 autoantibodies – GAD-Ab/ICA/IA-2Ab. None of the studies measured all four autoantibodies. In all of the studies, classification of type 1.5 diabetes was based on measurement of autoantibodies at only one point in time. In contrast, in our study, we checked patients for all four autoantibodies (GAD-Ab, IA-2Ab, ICA, and IAA) to decrease misclassification of patients as type 1.5 diabetes versus T2DM. We considered a patient autoantibody positive if he or she had positive titers for the same antibody for two consecutive time points. We believe this to be a better way to ensure consistent autoantibody positivity in patients because we have seen that antibody titers can fluctuate especially if they are borderline. We also restricted the duration of diabetes to five years in an attempt to maximize the sensitivity of antibody screening as a marker of type 1.5 diabetes. Restricting the analysis to more recently diagnosed diabetic patients lessens the possibility of a misclassification of type 1.5 diabetes as T2DM because of reversion to an autoantibody negative status with increasing duration of diabetes. Such a reversion has been observed in type 1.5 diabetes over time (17).

Another confounding factor is the time from diagnosis of hyperglycemia. Beta cell function decreases with time and may decline relatively faster in autoantibody positive patients compared to autoantibody negative patients. One large population based study involving 1,122 T2DM patients by Tuomi et al (21) showed that GAD-Ab+ patients had significantly lower fasting C-peptide compared to GAD-Ab- T2DM patients. The duration of diabetes was 81.6 and 68.4 months respectively (21). This difference in duration of diabetes may account for at least some of the lower C-peptide in the GAD-Ab+ patients. In our study, all patients were required to have a hemoglobin A1c between 6% and 10% and fasting C-peptide ≥0.80ng/ml. The cut point of 0.8 ng/ml was chosen because we wanted patients in the drug study who, at least in the beginning of the study, could be treated with oral agents and not require insulin therapy. Moreover, the mean duration of diabetes was 30.0 and 30.2 months respectively in our patients. Therefore, the patients in this study are hypothesized to be earlier in their disease progression based on higher hemoglobin A1c and C-peptide inclusion criteria. If this is the case, then our results would demonstrate that the T cell responses can identify patients with a more severe β-cell lesion prior to detection by autoantibodies. Since type 1.5 diabetes, like type 1, is believed to be cell mediated, the fact that T cells can identify patients with a more severe β-cell lesion earlier would be reasonable. Whether the C-peptide differences are detected by T cells earlier than detected by autoantibodies or irrespective of autoantibodies remains to be decided. Future studies should help answer this question. However, the results in this study demonstrate that T cells are able to identify patients with more a severe β-cell lesion irrespective of autoantibodies.

In our study, we focused on the presence or absence of T cell and autoantibody reactivity (positive for at least 2 consecutive time points) and the relationship to fasting and stimulated C-peptide. We found significantly lower ($p<0.0035$) stimulated C-peptide correlated with T cell positivity to islet proteins and not autoantibody positivity alone. We further investigated the relationship between the magitude of T cell response and C-peptide response by separating the T cell positive responses into those patients with either low, medium or high numbers of blot sections positive, We observed that neither fasting or glucagon stimulated C-peptide correlated with the magnitude of T cell responses but rather the presence or absence of T cell reactivity. Morevover, we further separated the patients into 4 groups based on both their autoantibody status and T cell status (Ab-T-/Ab+T-/Ab-T+/Ab+T+), 8 patients were Ab-T-, 5 patients were Ab+T-, 11 patients Ab-T+, and 12 patients Ab+T+. The stimulated C-peptide responses demonstrated significant differences between the Ab+T+ (mean 5.1ng/ml) and Ab+T- (mean 8.2 ng/ml) with $p<0.01$, and between Ab+T+ (mean 5.1 ng/ml) and Ab-T- (mean 7.3 ng/ml) with $p<0.03$. Stimulated C-peptide in Ab+T- and Ab-T- was not significantly different ($p=0.47$). Thus, just the presence of autoimmunity as observed by autoantibody positivity alone did not identify the patients with more severe β-cell lesion.

We also questioned whether differences in insulin resistance (assessed using the HOMA model) might also contribute to our results. However, there was no significant difference between T cell positive or T cell negative subjects in their insulin sensitivity. Thus, the more severe β-cell lesion appears to correlate with the presence or absence of T cell reactivity and cannot be explained in this study by differences in insulin sensitivity, or magnitude of the T cell response. We believe we have demonstrated that glucagon-stimulated C-peptide secretion is different in patients with and without autoimmunity as determined using T cell assays whereas this difference is not seen using only autoantibody assays. Therefore, we believe that cellular immunoblotting can identify patients with a more severe β-cell lesion irrespective of whether the patient is positive or negative for autoantibodies to islet proteins.

Consequently, we conclude that T cell reactivity may be a better marker than autoantibodies in detecting β- cell dysfunction in type 1.5 diabetes.

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REFERENCES

1. Lampeter EF, Homberg M, Quabeck K, Schaefer UW, Wernet P, Bertrams J, Grosse-Wilde H, Gries FA, Kolb H: Transfer of insulin dependent diabetes between HLA-identical siblings by bone marrow transplantation. *Lancet* 341:1243-1244, 1993.
2. DeFronzo RA: Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: A balanced overview. *Diabetologia* 35:389-397, 1992
3. Porte D Jr: Banting lecture 1990. Beta-cells in type II diabetes mellitus. *Diabetes* 40:166-180, 1991
4. Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR: Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. *Diabetes* 42:359-362, 1993
5. Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, Lang DA: Latent autoimmune diabetes mellitus in adults (LADA): The role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabet Med* 11:299-303, 1994
6. Editorial: Insulin-dependent? *Lancet* 2:809-810, 1985
7. Harris M, Zimmet P: Classification of diabetes mellitus and other categories of glucose intolerance. In *International Textbook of Diabetes Mellitus.* Alberti K, DeFronzo R, Keen H, Zimmet P, Eds. New York, NY, Wiley, 1992, p. 3-18
8. Juneja R, Palmer JP: Type 11/2 diabetes: Myth or reality? *Autoimmunity* 29: 65-83, 1994
9. Palmer JP, Hampe CS, Chiu H, Goel A, Brooks-Worrell BM: Is latent autoimmune diabetes in adults distinct from type 1 diabetes or just type 1 diabetes at an older age? Diabetes. 54 (2):S62-7, 2005
10. Brooks-Worrell BM, Juneja R, Minokadeh A, Greenbaum CJ, Palmer JP: Cellular immune responses to human islet proteins in antibody positive type 2 subjects. *Diabetes* 48:983-988, 1999
11. Brooks-Worrell BM, Stakebaum GA, Greenbaum CJ, Palmer JP: Peripheral blood mononuclear cells of insulin-dependent diabetic patients respond to multiple islet proteins. *J Immunol* 157:5668-5674, 1996
12. Anderson MS, Bluestone JA: The NOD mouse: A model of immune dysregulaton. *Annu. Rev. Immunology* 23:447-485, 2005
13. Boitard C, Yasunami R, Dardenne M, Bach JF: T cell-mediated inhibition of the transfer of autoimmune diabetes in NOD mice. *J Exp Med* 169:1669-1680, 1989
14. Palmer JP, Fleming GA, Greenbaum CJ, Herold KC, Jansa LD, Kolb H, Lachin JM, Polonsky KS, Pozzilli P, Skyler JS, Steffes MW: C-peptide is the appropriate outcome measure for type 1 diabetes clinical trails to preserve β-cell function: report of an ADA workshop,21-22 October 2001. *Diabetes* 53:250-264, 2004
15. Gottsater A, Landin-Olssan M, Lenmark A, Ferlund P, Sundkvist G, Hagopian WA: Glutamate decarboxylase antibody levels predict rate of beta cell decline in adult onset diabetes. *Diabetes Res Clin Pract* 27: 133-140, 1995

16. Torn C, Landin-Olsson, Lernmark A, et al. Prognostic factors for the course of beta cell function in autoimmune diabetes. *J Clin Endocrinol Metab* 85:4619-4623, 2000
17. Borg H, Gottsater A, Landin-Olsson M, Ferlund P, Sundkvist G: High levels of antigen specific islet antibodies predict future beta cell failure in patients with onset of diabetes in adult age. *J Clin Endocrinol Metab* 86: 3032-3038, 2001.
18. Turner R, Stratton I, Horton V,Manley S, Zimmet P, Mackay IR, Shattock M, Bottazzo GF, Holman R: UKPDS 25: Autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes*. Lancet* 350:1288-1293.
19. Hosszufalusi N, Vatay A, Rajczy K, Prohaszka Z, Pozsonyi E, Horvath L, Grosz A, Gero L, Madacsy L, Romics L, Karadi T, Fust G, Panczel P: Similar genetic features and different islet cell autoantibody pattern of latent autoimmune diabetes in adults (LADA) compared with adult-onset type 1 diabetes with rapid progression. *Diabetes care* 26:452-457, 2003
20. Monge L, Brunot G, Pinach S, Grassi G, Maghenzani G, Dani F, Paganot G: A clinically oriented approach increases the efficiency of screening for latent autoimmune diabetes in adults (LADA) in a large clinic-based cohort of patients with diabetes onset over 50 years. *Diabet Med* 21(5):456-9, 2004
21. Tuomi T, Carlsson A. Li H, Isomma B, Miettinen A, Nilsson A, Nissen M, Ehrnstrom BO, Forsen B, Snickars B, Lahti K, Forsblom C, Saloranta C, Tasknen MR, Groop LC: Clinical and genetic characteristic of type 2 diabetes with and without GAD antibodies. *Diabetes* 48: 150-157, 1999
22. Arikan E, Sabuncu T, Ozer Esref M, Hatemi H: The clinical characteristics of latent autoimmune diabetes in adults and its relation with chronic complications in metabolically poor controlled Turkish patients with type 2 diabetes mellitus. *J Diabetes Complications* 19(5):254-258, 2005
23. Schölin A, Björklund L, Borg H, Arnqvist H, Björk E, Blohmé G, Bolinder J, Erikssn JW, Gudbjörnsdottir S, Nyström L, Östman J, Karlsson AF, Sundkvist G: Islet antibodies and remaining B-cell function 8 years after diagnosis of diabetes in young adults: a prospective follow-up of the nationwide diabetes incidence study in Sweden. *J Intern Med* 255:384-391, 2004
24. Birkeland KI, Kilhovd B, Thorsby P, Torjesen PA, Ganss R, Vaaler S, Hanssen KF: Heterogeneity of non-insulin-dependent diabetes expresses variability in insulin sensitivity, beta cell function and cardiovascular risk profile. *Diabetic Med* 20:37-45, 2003
25. Kasuga A, Maruyama T, Nakamoto S, Ozawa Y, Suzuki Y, Saruta T: high- titer autoantbodies against glutamic acid decarboxylase plus autoantibodies against insulin and IA-2 predicts insulin requirement in adult diabetic patients. *J Autoimmun* 12:131-135, 1999
26. Maruyama T, Kasuga A, Ozawa Y, Nagata A, Abiko F, Suzuki Y, Saruta T: Glutamic acid decarboxylase 65 (GAD65) antibodies and insulin autoantibodies in Japanese patients with non-insulin-dependent diabetes mellitus. *Endocr J* 44(1):43-51, 1997

27. 27.Yang L, Zhou Z, Huang G, Ouyang L, Li X, Yan X: Six year follow-up of pancreatic B-cell function in adults with latent autoimmune diabetes. *World J Gastroenterol* 11(9):2900-2905, 2005
28. Rowe R, Leech N, Nepom G, McCulloch D: High genetic risk for IDDM in the pacific northwest: first report from the Washington State Diabetes Prediction Study. *Diabetes* 43:87-94, 1994
29. Verge CF, Stenger D, Bonifacio E, et al: Combined use of autoantibodies (IA-1 ab, GADab, IAA, ICA) in type 1 diabetes: Combinatorial islet autoantibody workshop. *Diabetes* 47: 1857-1866, 1998.
30. Falorni A, Ortqvist E, Persson B, Lernmark A: Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using ^{35}S or ^{3}H recombinant human ligands*. J Immunol Meth* 196:89-99, 1995
31. Bingley PJ, Bonifacio E, Mueller PW: Diabetes antibody standardization program: first assay proficiency evaluation. *Diabetes* 52:1128-1136, 2003
32. Feldmann M, Dayan C, Rapoport B, Londei M: T-cell activation and antigen presentation in human thyroid autoimmunity. *J Autoimmun* 5:115-121, 1992
33. 33 Freeman M, Weetman AP: T and B cell reactivity to adrenal antigens in autoimmune addison's disease. *Clin Exp Immunol* 88:275-279, 1992
34. Laemmli UK: Clevage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:6880-6885, 1970
35. Brooks-Worrell B, Gerusk VH, Greenbaum CJ, Palmer JP: Intermolecular antigen spreading occurs during the preclinical period of human type 1 diabetes. *J immunol* 166:5265-5270, 2001
36. Seyfert-Margolis V, Gisler TD, Asare AL, Wang RS, Dosch HM, Brooks-Worrell B, Eisenbarth GS, Palmer JP, Greenbaum CJ, Gitelman SE, Nepom GT, Bluestone JA, Herold KC. Analysis of T-cell assays to measure autoimmune responses in subjects with type 1 diabetes: results of a blinded controlled study. *Diabetes*. 2006 Sep;55(9):2588-94.
37. Peterson JI, Young DS: Evaluation of the hexokinase/glucose-6-phosphate dehydrogenase methods of determination of glucose in urine. *Anal Biochemistry* 23:301-316,1968
38. Levy J, Matthews D, Hermans M. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care* 1998;21: 2191-2192.
39. Matthews D, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R. Homeostasis model assessment. Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28: 412-491.

Table 1

Beta cell function in Ab+/Ab- and T cell+/T cell- groups.

C-peptide (ng/ml)	Ab- (n=19)	Ab+ (n=17)	p value (Ab+/Ab-)	T cell- (n=13)	T cell+ (n=23)	p value (T+/T-)
Fasting	3.4±1.1	3.3±1.3	0.9893	3.8±.1.5	3.1±0.9	0.1379
Glucagon stimulated	6.3±2.3	5.9±2.5	0.6754	7.6±2.1	5.3±2.1	0.0035

Data are means ± SD.

Table 2

Clinical characteristics of 36 phenotypic diabetes patients.

Characteristic	Ab- (n=19)	Ab+ (n=17)	T cell- (n=13)	T cell+ (n=23)
Fasting glucose (mmol/l)	152±53	181±53	156± 57	171± 53
HbA1c (%)	7.0± 0.9	7.7± 1.3	7.2± 0.9	7.4± 1.3
BMI	34.1± 5.8	30.9± 5.2	34.0± 6.1	31.7± 5.4
Age (in years)	55.5± 8.8	60.3± 6.8	55.6± 9.0	59.0± 7.5
Duration of diabetes (months)	34.9±18.0	24.3± 18.4	27.6± 17.3	31.9± 19.7

Data are means ± SD.

Figure 1 Different autoantibodies in 17 autoantibody positive patients.

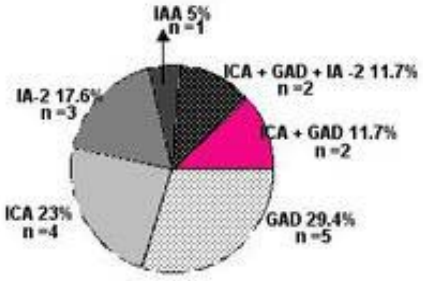

Figure 2 Blot section positivity in 36 phenotypic type 2 diabetes patients using cellular immunoblotting.

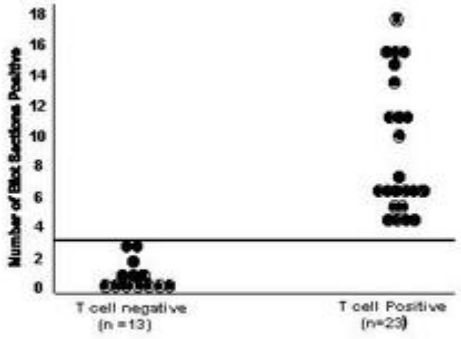

Figure 3 Fasting and glucagon stimulated plasma c-peptide in autoantibody negative (n=17) and autoantibody positive (n=19) patients independent of T cell reactivity. I represents mean ± SD.

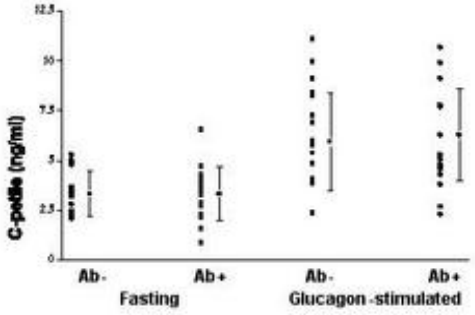

Figure 4 Fasting and glucagon stimulated plasma c-peptide in T cell negative (n=13) and T cell positive (n=23) patients independent of autoantibody status. I represents mean ± SD.