Impact of Islet Autoimmunity on the Progressive β-Cell Functional Decline in Type 2 Diabetes

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OBJECTIVE

Cross-sectional studies have suggested that islet autoimmunity may be more prevalent in type 2 diabetes (T2D) than previously appreciated and may contribute to the progressive decline in β-cell function. In this study, we longitudinally evaluated the effect of islet autoimmune development on the progressive β-cell dysfunction in T2D patients.

RESEARCH DESIGN AND METHODS

Twenty-three T2D patients negative for islet autoantibodies (GAD antibody and insulinoma-associated protein 2) and islet-specific T cells were evaluated prospectively for up to 36 months. We investigated the percentage of patients who developed islet autoantibodies (Ab+) and/or islet-reactive T cells (T+) and the effect of the islet autoimmunity on fasting and glucagon-stimulated C-peptide responses. We defined positive islet autoimmunity as Ab+ and/or T+ for at least two study visits.

RESULTS

Of the 23 patients, 6 (26%) remained negative for islet autoimmunity (Ab−T−), 14 (61%) developed Ab+ and/or T+, and 3 (13%) were unclassifiable because they developed islet autoimmunity at only one study visit. Islet Ab+ was observed to be less stable than islet-specific T-cell responses. Development of islet autoimmunity was significantly associated with a more rapid decline in fasting ($P < 0.0001$) and glucagon-stimulated ($P < 0.05$) C-peptide responses.

CONCLUSIONS

These pilot data suggest that the development of islet autoimmunity in T2D is associated with a significantly more rapid β-cell functional decline.

Historically, type 2 diabetes (T2D) has not been considered to be immune mediated. However, many notable discoveries in recent years have provided evidence to support the concept of immune system involvement in T2D pathophysiology (1–5). Immune cells have been identified in the pancreases of phenotypic T2D patients (3–5). Moreover, treatment with interleukin-1 receptor agonist improves β-cell function in T2D patients (6–8). These studies suggest that β-cell damage/destruction mediated by the immune system may be a component of T2D pathophysiology.

Although the β-cell damage and destruction in autoimmune diabetes is most likely T-cell mediated (T), immune markers of autoimmune diabetes have primarily centered on the presence of circulating autoantibodies (Abs) to various islet antigens (9–15). Abs commonly positive in type 1 diabetes (T1D), especially GAD

antibody (GADA) and islet cell Abs (ICA), have been shown to be more common in patients with T2D than in nondiabetic control populations, and the presence of multiple islet Abs, such as GADA, ICA, and tyrosine phosphatase-2 (insulinoma-associated protein 2 [IA-2]), have been demonstrated to be associated with an earlier need for insulin treatment in adult T2D patients (14,16–20).

In 1996, our laboratory developed a T-cell assay, cellular immunoblotting (CI), with excellent sensitivity and specificity for measuring islet-specific T-cell responses in T1D (21,22). We have used CI to measure islet-reactive T cells in type 1 (T1D) patients (23–26) and in phenotypic T2D patients with and without islet Abs (26–30). In our previous cross-sectional studies, we demonstrated that T-cell reactivity to islet proteins in phenotypic T2D patients more strongly correlated with impaired β-cell function compared with Ab positivity (27). Furthermore, we also demonstrated that attenuation of islet-reactive T-cell responses was associated with improvement in β-cell function in T2D patients (29). Longitudinal data are still lacking on the percentage of nonautoimmune T2D patients (Ab−T−) who develop Ab or T-cell positivity over time and how this development of islet autoimmunity affects the progressive β-cell dysfunction associated with T2D disease.

In this pilot study, 23 phenotypic Ab−T− T2D patients were evaluated prospectively for up to 36 months and tested every 3 months for the development of islet autoimmunity indicated by islet Abs (GADA and IA-2) and/or islet-specific T cells. Fasting C-peptide (FCP) levels and glucagon-stimulated C-peptide (SCP) responses were assessed to evaluate the interrelationship between islet autoimmune development in T2D and β-cell functional status.

RESEARCH DESIGN AND METHODS
Subjects
T2D patients were diagnosed by physicians in accordance with the American Diabetes Association standards of medical care in diabetes (31). T2D patients were recruited from the general population through advertisements. Two blood samples, within 3 months, were obtained from consecutive T2D patients to confirm autoimmune status. Inclusion criteria

for T2D patients for this study were a BMI >25 kg/m^2, no history of ketonuria or ketoacidosis, onset of T2D between ages 35 and 70 years, duration of diabetes ≤5 years, HbA$_{1c}$ levels between 7 and 10%, FCP level ≥0.8 ng/mL, negative for Abs to GADA and IA-2, and negative for islet-reactive T cells. Demographics, islet Ab and islet-reactive T-cell status, length of follow-up, baseline C-peptide data, and duration of diabetes are reported in Table 1. T2D patients were not requiring insulin treatment at diagnosis and not treated with insulin or a thiazolidinedione during follow-up. Prescribed medications for the enrolled patients are listed in Table 2. Study visits were scheduled at 3-month intervals for up to 36 months. Written informed consent was obtained from each patient before participation. Type 1 associated HLA data for the T2D patients are reported in Table 3. This study was approved by the University of Washington and the VA Puget Sound Health Care System Institutional Review Boards.

CI T-Cell Assay
CI was performed on freshly isolated peripheral blood mononuclear cells from T2D patients, as previously described, to test for the presence of islet-specific T cells (21–30). Briefly, normal human islet cell preparations underwent preparative one-dimensional 10% SDS-PAGE, electroblotted onto nitrocellulose, and the nitrocellulose particles containing the islet proteins were prepared using carbonate/bicarbonate buffers. The nitrocellulose particles were then added to freshly isolated peripheral blood mononuclear cells from patients, and the cultures were incubated for 5 days, pulsed with tritiated thymidine for 18 h, and thymidine incorporation was assessed using a β counter. Human pancreatic islets were obtained from the National Institutes of Health–supported Islet Cell Resource Centers Administrative and Bioinformatics Coordinating Center. The specificity of the T-cell responses to the islet proteins was demonstrated previously

Table 1—Characteristics of patients divided by islet Ab and islet-reactive T-cell positivity

	Patient category (N = 23)				
	Ab−T−	Ab+T−	Ab−T+	Ab+T+	Unknown*
Patients, n (%)	6 (26)	2 (9)	7 (30)	5 (22)	3 (13)
Age (years) (median ± SE)	53 ± 4	60 ± 1	57 ± 5	58 ± 3	49 ± 4
Sex (%)					
Males	17	100	71	80	67
Females	83	0	29	20	33
BMI (kg/m^2) (median ± SE)					
Baseline	43 ± 2	34 ± 2	36 ± 2	27 ± 1	32 ± 2
Study end	43 ± 2	34 ± 1	34 ± 2	29 ± 2	31 ± 2
Length of follow-up (months) (median ± SE)	36 ± 4	23 ± 14	15 ± 4	36 ± 4	33 ± 6
Baseline FCP (ng/mL) (median ± SE)	5 ± 0.4	5 ± 0.2	3 ± 0.4	3 ± 0.2	4 ± 1
Baseline glucagon-SCP (ng/mL) (median ± SE)	9 ± 1	9 ± 2	5 ± 1	5 ± 0.4	7 ± 2
Race (%)					
Caucasian	83	50	100	60	67
African American	0	0	0	20	0
Asian	17	50	0	20	33
T2D disease duration (%)					
<1 year	17	100	14	20	0
1–2 years	33	0	43	40	67
>2 years	50	0	43	40	33
Mean ± SD (years)	2 ± 2	0.3 ± 0.1	3 ± 2	2 ± 2	3 ± 2
Median ± SE (years)	3 ± 1	0.3 ± 0.1	2 ± 1	2 ± 1	2 ± 1
Positivity (%)					
GADA+	0	50	0	100	33
IA-2+	0	100	0	20	33
Islet T cells+	0	0	100	100	67

*Autoimmune positive 1 time point only.

Table 2—Percentage of patients in each group receiving specified classes of drugs throughout follow-up

	Patient category				
	Ab−T− n = 6	Ab+T− n = 2	Ab−T+ n = 7	Ab+T+ n = 5	Unknown* n = 3
Diabetes medications†					
Sulfonylureas	83	50	57	80	67
Biguanides	83	100	86	80	100
Meglitinides	17	0	0	0	0
α-Glucosidase inhibitors	33	0	14	0	33
Cholesterol medications					
Statins	33	100	29	60	67
Fibrates	0	0	14	40	33
Niacin	0	50	0	0	33
Blood pressure medication					
ACE inhibitors	50	100	14	20	67
Angiotensin receptor blocker	0	0	14	0	0
β-Blockers	50	50	29	20	100
Calcium channel blockers	0	0	43	0	100
Diuretics	33	0	43	0	67
α-Blockers	17	0	43	60	0
Other medications					
Levothyroxine sodium	33	0	0	0	0
Aspirin	17	50	14	40	67
Antiepileptic agent	17	0	14	40	0
Serotonin reuptake inhibitor	0	0	29	0	33
β₂-Adrenergic agonist	0	0	14	20	0

*Autoimmune positive 1 time point only. †No patients were treated with thiazolidinediones, dipeptidyl peptidase-4 inhibitors, or sodium-glucose linked transporter 2 inhibitors.

(23). T-cell responses from T1D patients were not hyperresponsive to control antigens nor stimulated by proteins from other control tissues (23).

CI has been evaluated in two distinct T-cell validation workshops. One validation workshop was sponsored by the Immune Tolerance Network (ITN) and the other by TrialNet (21,22). The workshops were designed to test the ability of several different assays, including CI performed in our laboratory, to distinguish T-cell responses to islet proteins of T1D patients from control subjects. Both workshops used masked specimens, and CI was identified as an assay capable of distinguishing T1D patients from control subjects with excellent sensitivity and specificity. A cutoff stimulation index ≥2.0 was used to determine positive responses to each blot section. This cutoff corresponds to 3 SDs above the mean response of normal control subjects to islet proteins (23). Overall T-cell positivity at each time point was set at four or more blots, the same cutoff used for T1D with this assay (21,22,24). Patients were considered positive for islet-reactive T cells if they were positive for four or more blots sections during at least two study visits.

C-Peptide Assays
FCP levels and glucagon-SCP responses were used as measures of endogenous β-cell function. SCP was measured 6 min after the intravenous injection of 1 mg glucagon. The C-peptide assay is a two-site immune-enzymatic assay performed using a Tosoh 600 II autoanalyzer (Tosoh Bioscience Inc., South San Francisco, CA) at the Northwest Lipid Metabolism and Diabetes Research Laboratories (NWLMDRL), Seattle, WA (27). The interassay and intra-assay precision analysis showed a coefficient of variation of less than 10%. The assay has a sensitivity level of 0.04 ng/mL.

GAD-65 Ab Assay
GADA levels were measured at NWLMDRL in a radiobinding immunoassay on coded serum samples, as previously described (32). The levels of GADA were expressed as a relative index (GADA index) using one positive serum (JDF World Standard for ICA) and three negative standard sera from healthy subjects. Positivity was set at a GADA index ≥0.085, the 99th percentile based on 200 normal control subjects. Positive and negative controls are run in duplicate in each assay. NWLMDRL has participated in IDS-sponsored Diabetes Antibody Standardization Programs (DASP). In the 2010 DASP workshop, the sensitivity of the GADA assay was 82% and specificity was 93.3% (33). NWLMDRL is actively participating in the National Institutes of Health–sponsored Ab harmonization program. Patients were considered positive for GADA if they were positive during at least two study visits.

IA-2 Ab Assay
IA-2 levels were measured at NWLMDRL under conditions identical to those described for the GADA assay, using the plasmid containing the cDNA coding for the cytoplasmic portion of IA-2. Positivity was set at an IA-2 index of ≥0.017, the 99th percentile based on a set of 200 normal control subjects. In the 2010 DASP workshop, this assay scored a sensitivity of 62% and specificity of 100% for its ability to distinguish T1D patients from normal

Table 3—Percentage of T1D-associated HLA class II alleles present in the T2D patients

	Patient category				
	Ab−T− n = 6	Ab+T− n = 2	Ab−T+ n = 7	Ab+T+ n = 5	Unknown* n = 3
HLA class II (%)					
Susceptibility alleles (DRB1*03xx/04xx/*0101; DQB1*0302/*0301/*0501)	50 (3/6)	0	86 (6/7)	40 (2/5)	67 (2/3)
Protective alleles (DRB1*1501; DQB1*0602)	0	0	14 (1/7)	60 (3/5)	0
Other alleles	50 (3/6)	100 (2/2)	0	0	33 (1/3)

*Autoimmune positive 1 time point.

control subjects (33). Patients were considered positive for IA-2 if they were positive during at least two study visits.

Statistics

Data were analyzed with nonparametric tests for comparison of medians and means, χ^2 and Fisher exact tests for comparison of percentages, and multivariable linear regression. The median percentage change was calculated as described by Sosenko et al. (34). The median percentage change was compared among patients before and after development of autoimmunity and patients who did not develop autoimmunity using ANOVA for three-group comparisons and the Mann-Whitney U test for two-group comparisons. Two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Patient Categories and Demographics

We identified 6 of 23 patients (26%) who remained nonautoimmune (Ab−T−) during follow-up. In contrast, 14 (61%) demonstrated positivity for islet Abs (GADA and/or IA-2) and/or islet-specific T cells. We categorized the patients into the following groups by their autoimmune status: Ab−T− (n = 6), Ab+T− (n = 2), Ab−T+ (n = 7), and Ab+T+ (n = 5). We also observed three patients (13%) who were positive for Abs and/or T cells on only one study visit. The

responses of these 3 patients were kept separate from the other nonautoimmune patients and placed in a separate group labeled "unknown." The "unknown" patients were not used in further analysis.

Tables 1 and 2 report characteristics of subjects, including age, race, sex, BMI, diabetes features, and medication use by autoimmune (Ab+T−, Ab−T+, and Ab+T+) and nonautoimmune (Ab−T−) T2D status. HLA alleles protective for T1D (DR*1501; DQB1*0602) were more frequent in the Ab+T+ than in any other group, whereas the T1D susceptibility alleles were more frequent in the Ab−T+ patients (Table 3).

Autoimmune Responses to Islet Proteins

Six of the 23 T2D patients (26%) remained negative for islet Abs and islet-reactive T cells (Ab−T−), whereas 7 patients (30%) developed islet-reactive T cells but remained Ab−T+. Figure 1 illustrates the longitudinal T-cell responses for the Ab−T− (Fig. 1A) and Ab−T+ (Fig. 1B) T2D patients. Figure 2 shows the longitudinal islet Ab responses for the two Ab+T− T2D patients. Both patients were positive for IA-2 (Fig. 2A) and one patient was also positive for GADA (Fig. 2B). The negative islet-specific T-cell responses for these patients (Ab+T−) are not shown.

Islet Abs and islet-reactive T cells (Ab+T+) developed in 5 of the 23 patients (22%).

All five patients were positive for GADA, and one was positive for both GADA and IA-2. The T-cell results for the Ab+T+ patients are shown in Fig. 3A, and the Ab data are shown in Fig. 3B (GADA) and Fig. 3C (IA-2). The patient who was positive for IA-2, developed IA-2+ and T+ positivity at 3 months, became IA2− at 6 months, but remained T+ throughout the study (36 months). This patient also became GADA+ at 9 months, GADA− at 15 months, then GADA+ again from 18 to 36 months. Another patient who developed T-cell positivity at 33–36 months was GADA+ between 6 and 24 months and then GADA− negative from 27 to 36 months. This patient was IA-2− throughout follow-up.

Three patients who developed positivity for either islet Abs (GADA or IA-2) or islet-reactive T cells on only one study visit were classified as "unknown." These patients were observed to have a high percentage of HLA T1D susceptibility alleles (Table 3). Even though these patients were negative as defined for this study, they appeared to be potentially different from the Ab−T− patients. Therefore, we did not include these patients in any further C-peptide analysis.

Two of the "unknown" patients developed T-cell positivity at one time point but then became negative at subsequent time points. In the remaining 12 subjects (52%) in the study who developed T-cell reactivity, the T-cell
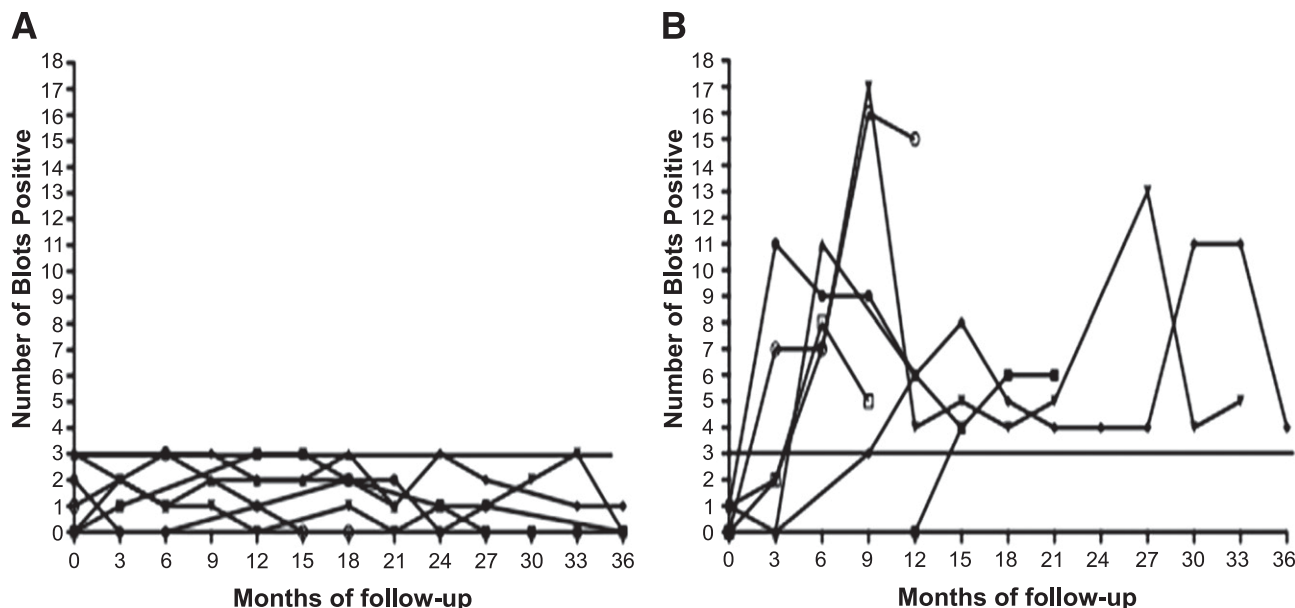
A

B

Figure 1—Longitudinal islet-specific T-cell responses for six Ab−T− T2D patients (A) and seven Ab−T+ T2D patients (B). The horizontal line at three blot sections represents the cutoff for T-cell positivity.
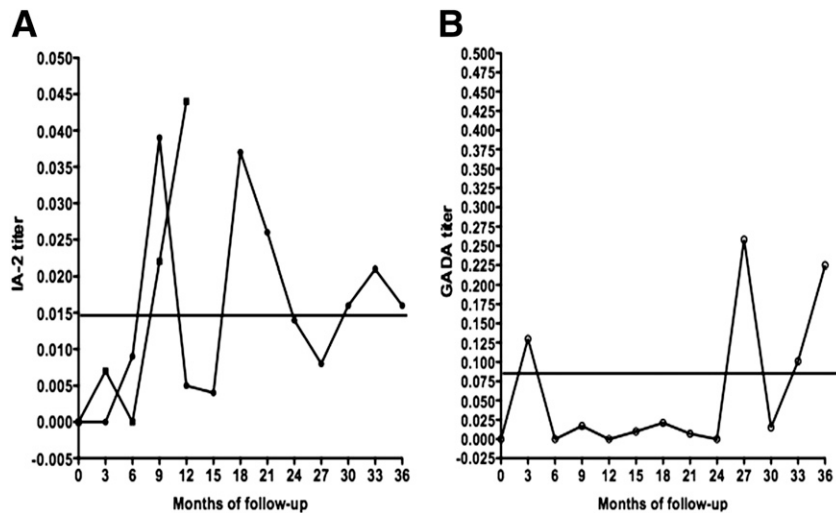
Figure 2—Longitudinal islet Ab responses for two Ab+T− T2D patients. *A*: Both patients developed IA-2 positivity, and one patient developed both IA-2 and GADA. *B*: GADA results. The horizontal lines show the cutoff for positive responses.

reactivity remained positive throughout follow-up. Islet Ab responses were observed to fluctuate between positivity and negativity in most patients, although GADA positivity appeared to be more stable than IA-2 positivity.
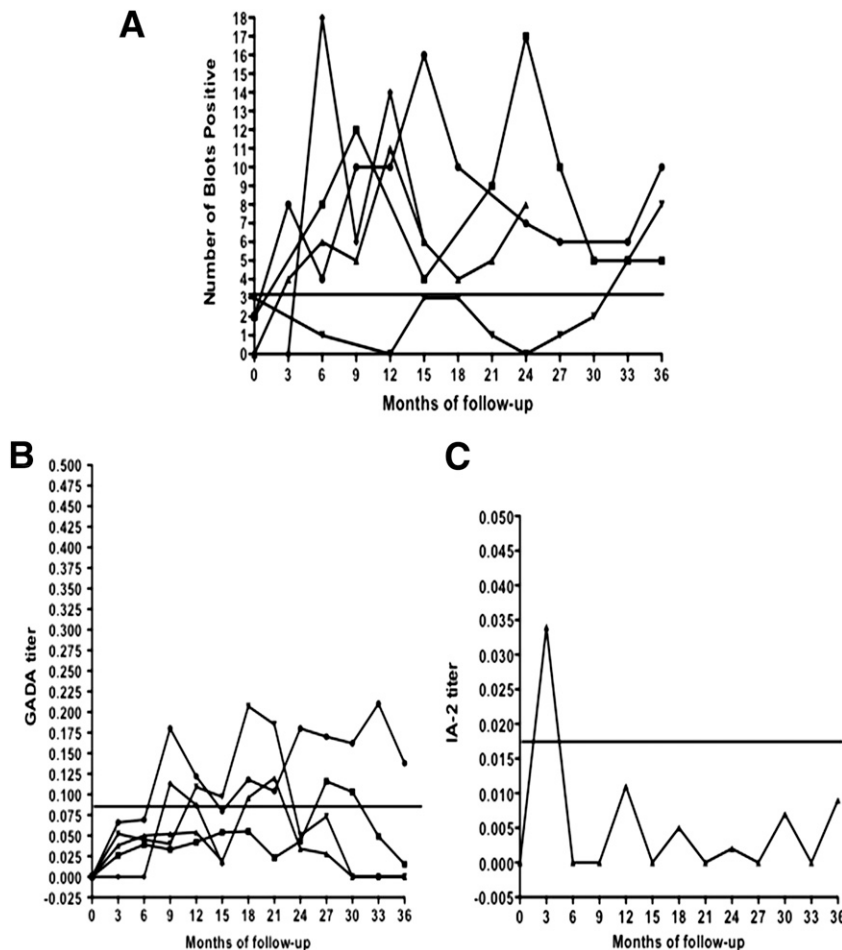

Figure 3—*A*: Five T2D patients developed both islet-specific T-cell reactivity and islet Abs during follow-up (Ab+T+). All five patients were GADA+ (*B*), and one patient was also IA-2+ (*C*). The horizontal lines show the cutoff for positive responses.

C-Peptide Responses

The median baseline FCP and glucagon-SCP responses for the five patient groups are reported in Table 1. The baseline glucagon-SCP was observed to be lower in T+ patients (Ab−T+ and Ab+T+) compared with the T− patients (Ab−T− and Ab+T−). These differences were present irrespective of age, BMI, diabetes duration, race, Abs, and sex.

To investigate the effect of islet auto-immune development on C-peptide responses, we separated the responses for the groups developing islet auto-immunity (Ab+T−, Ab−T+, Ab+T+) corresponding to the time interval before or after autoimmune development. To account for the lower baseline FCP and SCP responses in the T+ patient groups, we compared the median percentage change from baseline in FCP and glucagon-SCP. We then compared these responses with the patients who did not develop islet autoimmunity (Ab−T−). No significant differences were observed in median percentage change for FCP levels between the nonautoimmune and the autoimmune patients before auto-immune development (Fig. 4*A*). However, after autoimmune development, there was a significant decline in FCP in the autoimmune patients compared with the FCP of the nonautoimmune patients ($P < 0.0001$) and compared with the FCP before autoimmune development ($P < 0.01$, Fig. 4*A*).

Significant declines were also observed for the glucagon-SCP responses between the autoimmune patients after autoimmune development and the non-autoimmune patients ($P < 0.05$) and also between the autoimmune patients before autoimmune development and after autoimmune development ($P < 0.001$, Fig. 4*B*). Interestingly, a significant difference ($P < 0.02$) was also noted in the SCP between the non-autoimmune and autoimmune patients before autoimmune development (Fig. 4*B*).

CONCLUSIONS

The analysis of T-cell responses to islet proteins using the CI assay has allowed us to identify, in cross-sectional studies, cellular islet autoimmunity in T1D (21–23,25), in subjects at risk for T1D (24), and in phenotypic T2D patients (26–30). The presence of islet-specific cellular autoimmunity in T2D patients was also
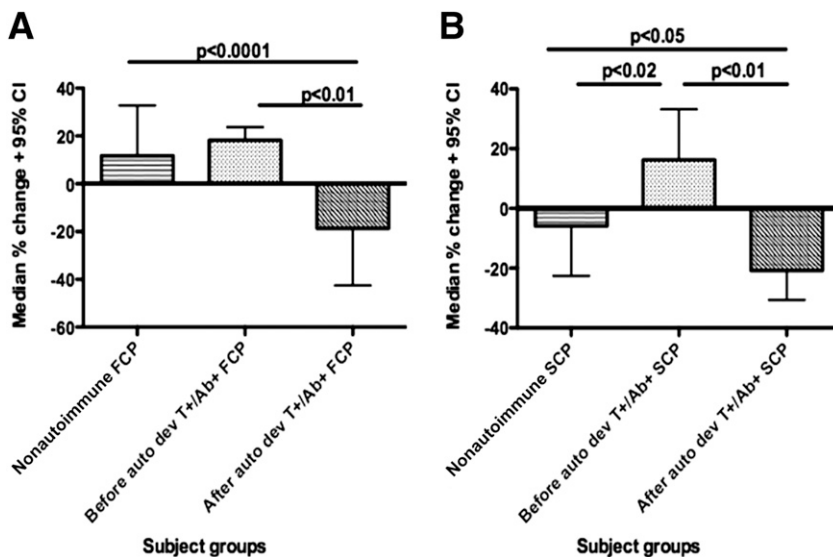
Figure 4—Median percentage change in FCP levels (*A*) and glucagon-SCP (*B*) responses for the nonautoimmune patients (Ab−T−) and patients developing islet autoimmunity (Ab+T−, Ab-T+, and Ab+T+) before and after autoimmune development.

recently confirmed by an independent laboratory (35). However, longitudinal studies investigating islet autoimmune development in T2D patients have been lacking.

In this study, we observed development of islet autoimmunity, measured by islet Abs and islet-specific T-cell responses, in 61% of the phenotypic T2D patients. We also observed a significant association between positive islet-reactive T-cell responses and a more rapid decline in β-cell function as assessed by FCP and glucagon-SCP responses. Moreover, the high percentage (30%) of Ab−T+ T2D patients observed in this study supports our previously published cross-sectional observations and demonstrates the importance of identifying islet autoimmunity by assaying both Abs and T cells (27,28,36). This result is of importance, noting that historically, islet autoimmunity in T2D had been determined based solely on islet Ab positivity (primarily GADA).

In this study, we also observed that islet-reactive T-cell responses appeared to be more stable compared with the islet Ab responses in autoimmune T2D patients. With regard to the islet Abs, GADA responses were more consistently positive over time compared with the IA-2 responses. In our previous studies, we have observed very few T2D patients to be positive for insulin Abs (IAA) or zinc transporter Abs (ZnT8A).

Therefore, in our current study, we did not test our patients for IAA or ZnT8A. Though T1D has been hypothesized to be a "relapsing-remitting" disease, the stability of the positive islet-reactive T-cell responses we observed in this study do not support this concept in T2D autoimmune pathogenesis (37). The instability of the islet Abs may possibly suggest that islet Abs specific for T2D are different from those associated with T1D or reflect a different pathogenesis of islet autoimmunity in T2D versus T1D.

We also previously observed that T2D patients treated with rosiglitazone experienced attenuation of islet-reactive T cells and decreases in interferon-γ and interleukin-12 responses, with associated improvement in β-cell function (29). On the basis of our previous data, we excluded from our current study T2D patients who were treated with thiazolidinediones. Assessing the prescribed medications of the enrolled subjects in this study did not demonstrate any associations between their current medications and development or attenuation of islet autoimmunity (Table 2). We also did not evaluate cytokine response in this study, but this would be of interest in future work.

In the patients classified as Ab+T−, we observed a higher baseline C-peptide response compared with the T+ patients (Table 1). The higher baseline C-

peptide values resulted in the significant differences observed in SCP responses between Ab−T− and the autoimmune patients before autoimmune development may reflect the incorporation of the Ab+T−, who had higher baseline SCP, into the autoimmune patient group (Fig. 4*B*). We also observed a shorter T2D disease duration in the Ab+T−. These results may indicate that these patients are earlier in their disease pathogenesis and may be destined to develop islet-specific T-cell reactivity at a later date. Another hypothesis might be that the presence of IA-2 in T2D patients indicates a level of protection resulting in higher C-peptide preservation. Interestingly, these patients were found to lack both the HLA class II susceptibility and protective alleles associated with development of T1D (Table 3) and potentially islet autoimmunity in T2D. Follow-up of this group of patients and studies in larger numbers of patients will help to address these questions.

Differences were also observed between the baseline FCP and glucagon-SCP in the T+ T2D patients compared with the T− T2D patients. These results suggest that the appearance of T+ in the peripheral blood may be a biomarker for patients whose disease path includes islet autoimmune damage. The association of a more rapid decline in β-cell function in T+ patients indeed suggests that the disease path for these patients includes islet autoimmune damage within the pancreas. Interestingly, we also observed that 60% of our Ab+T+ patients and 15% of our Ab−T+, compared with 0% of our Ab−T− patients, were DR*1501 DQB1*0602 (Table 3). The results of our study may suggest that DR*1501 DQB1*0602 is associated with the development of islet-reactive T cells in adult T2D patients. If this is the case, DR*1501 DQB1*0602 may provide protection against development of islet autoimmunity and T1D in childhood but not against development of islet autoimmunity in adult T2D. If confirmed, our results would be in contrast to Williams et al. (38), who observed that HLA-DRB1*02 was protective for T2D in full-heritage Pima Indians and may indicate that racial or ethnic differences do exist with regards to development of islet autoimmunity in T2D patients.

The results of this pilot study led us to hypothesize that islet autoimmunity is

present or will develop in a large portion of phenotypic T2D patients and that the development of islet autoimmunity is associated with a more rapid decline in β-cell function. Moreover, the prevalence of islet autoimmunity in most previous studies is grossly underestimated because these studies have not tested for islet-reactive T cells in T2D patients but have based the presence of autoimmunity on antibody testing alone. Previously, the importance of quantifying islet autoimmunity through the measurement of the islet-reactive T cells in T1D patients was emphasized by the study of Wang et al. (36) demonstrating that ~20% of newly diagnosed autoimmune T1D patients were Ab−. The results of this pilot study suggest important changes to our understanding of T2D pathogenesis by demonstrating that the prevalence of islet autoimmune development is not only more prevalent in T2D patients than previously estimated but may also play an important role in β-cell dysfunction in the T2D disease process. The results of our pilot study await confirmation in future larger studies.

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