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Identification of Unique Antigenic Determinants in the Amino Terminus of IA-2 (ICA512) in Childhood and Adult Autoimmune Diabetes: New Biomarker Development

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OBJECTIVE

The characterization of diverse subtypes of diabetes is a dynamic field of clinical research and an active area of discussion. The objective of this study was to identify new antigenic determinants in the neuroendocrine autoantigen IA-2 (ICA512) and assess whether circulating autoantibodies directed to new IA-2 epitopes identify autoimmune diabetes in young and adult populations with diabetes.

RESEARCH DESIGN AND METHODS

Clinically diagnosed patients with type 2 diabetes (n = 258; diabetes duration: 0.01–31 years) were evaluated using a new biomarker detecting autoantibodies directed to the extracellular domain of the neuroendocrine autoantigen IA-2 (IA-2ec). The proportion of IA-2ec autoantibodies was also evaluated in newly diagnosed patients with type 1 diabetes (n = 150; diabetes duration: 0.04–0.49 years). In addition, IA-2 (intracellular domain), GAD65, and zinc transporter 8 autoantibodies were assayed.

RESULTS

IA-2ec autoantibodies were detected in patients with type 1 diabetes and, surprisingly, in 5% of patients with type 2 diabetes without serologic responses to other IA-2 antigenic epitopes or other islet autoantigens. We also assessed the ability of IA-2ec–derived peptides to elicit CD4^{+} T-cell responses by stimulating peripheral blood mononuclear cells from patients with type 1 diabetes (n = 18) and HLA-matched healthy subjects (n = 13) with peptides and staining with the peptide/DQ8-specific tetramers, observing disease-associated responses to previously unreported epitopes within IA-2ec.

CONCLUSIONS

We developed a new antibody biomarker identifying novel antigenic determinants within the N terminus of IA-2. IA-2ec autoantibodies can be detected in patients with type 1 diabetes and in a subgroup of adult autoimmune patients with type 2 diabetes phenotype negative for conventional islet autoantibody testing. These observations suggest that islet autoimmunity may be more common in clinically diagnosed type 2 diabetes than previously observed.

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Autoimmune diabetes is considered to be the end result of an immune-mediated injury of the β-cells within the islets of Langerhans (1,2). Circulating autoantibodies and T-cell responses to islet autoantigens have allowed the development of diagnostic biomarkers that aid in the identification of subjects at risk for type 1 diabetes and of a subset of type 2 diabetes with evidence for islet autoimmunity (3–6). The latter condition is often termed latent autoimmune diabetes in adults (LADA) (7,8).

In autoimmune diabetes, several elements of the secretory pathway of pancreatic β-cells, such as insulin and protein islet tyrosine phosphatase-like protein (IA-2), are targeted by autoantibody and T-cell responses. In type 1 diabetes, the neuroendocrine molecule IA-2 is one of major targets of immune-mediated responses (9–12). IA-2 is a transmembrane glycoprotein of the tyrosine phosphatase-like protein family, which is localized in the insulin-secretory granules of the pancreatic β-cell. This molecule contains three domains: the N-terminal extracellular (or luminal) domain (amino acids 1–556), the transmembrane domain (amino acids 557–600), and the C terminus intracellular (or cytoplasmic) domain (amino acids 601–979) containing a juxtamembrane domain (amino acids 601–686) and an inactive protein tyrosine phosphatase domain (amino acids 687–979). IA-2 is a pseudophosphatase that plays a number of roles within the pancreatic islet such as contributing in β-cell proliferation, aiding in regulating insulin exocytosis, and acting to tether secretory granules to the cytoskeleton. During insulin secretion, the cytoplasmic domain of IA-2/ICA512 is cleaved and traffics to the nucleus, whereby it stimulates the transcription of the insulin gene. Albeit the biological role of IA-2 extracellular domain (IA-2ec) has not been entirely elucidated, stability of pro-ICA512/IA-2 and its targeting to insulin secretory granules require β4-sheet–mediated dimerization of its ectodomain in the endoplasmic reticulum (13).

We previously found indirect evidence for autoantibodies binding to IA-2ec. The presence of these autoantibodies was associated with a high risk of progression of type 1 diabetes (14). We hypothesized that antigenic determinants are present within IA-2ec and identified both autoantibody and T-cell responses specifically directed to the NH$_2$ terminus of IA-2. We provide evidence for humoral responses directed to IA-2ec in patients with type 1 diabetes and, surprisingly, in a subgroup of patients with clinically diagnosed type 2 diabetes. Finally, we demonstrated cell-mediated immunity directed against posttranslationally modified epitopes of the extracellular domain of IA-2 in patients with type 1 diabetes, suggesting that IA-2ec may play a role in the pathogenesis of autoimmune diabetes.

RESEARCH DESIGN AND METHODS
Subjects
The study population consists of 150 patients with type 1 diabetes (74 male and 76 female; mean age 13.40 ± 10.35 years) and 258 patients with type 2 diabetes (135 male and 123 female; mean age 52.67 ± 9.14 years). The sex distribution in the two groups was not statistically significantly different ($P = 0.6078$). Patients with type 2 diabetes were significantly older than patients with type 1 diabetes ($P < 0.0001$).

We assayed sera from 150 patients with type 1 diabetes from the Barbara Davis Center for Childhood Diabetes, University of Colorado School of Medicine; 100 patients with clinically diagnosed type 2 diabetes from the Division of Metabolism, Endocrinology and Diabetes, University of Michigan Health System; and 158 patients with clinically diagnosed type 2 diabetes from the Diabetes Clinic, Azienda Ospedaliera G. Brotzu, Cagliari, Italy. The study was approved by the respective Institutional Review Boards.

The percentage of Caucasians was 87 and 92% in the patients with type 1 and type 2 diabetes, respectively. Diabetes was diagnosed according to standard American Diabetes Association criteria (15). Serum samples from 178 healthy control subjects (72 male and 106 female; mean age 34.62 ± 10.15 years; and 76% Caucasian) were assayed for IA-2ec autoantibodies.

Subjects with type 2 diabetes evaluated in this study were treated with diet, oral hypoglycemic agents, or insulin therapy. All participants were assayed for GAD65, IA-2ic, IA-2ec, and zinc transporter 8 (ZnT8) autoantibodies.

IA-2 Autoantibody Radiobinding Assays
The IA-2ec construct (amino acids 26–577) contains the entire extracellular domain minus the signal peptide (14,16). IA-2ec was in vitro transcribed/translated in the presence of [^{35}S]methionine (PerkinElmer) using the TNT-coupled rabbit reticulocyte system (Promega, Madison, WI) with T7 RNA polymerase. IA-2ec autoantibodies were detected by radiobinding assay using 50% protein A–Sepharose to separate free [^{35}S]methionine from antibody-bound labeled products. The assay was run in triplicate, and the results were expressed as an index calculated as follows: index = (serum sample counts per minute [cpm] − negative control cpm)/(positive control cpm − negative control cpm). We used the protein tyrosine phosphatase IA-2 Q-20 antibody (sc-54749; Santa Cruz Biotechnology, Santa Cruz, CA) as positive control and serum from individuals without diabetes as negative controls. The cutoff point was established as the 99th percentile of values from serum samples obtained from 178 healthy volunteers. The interassay coefficient of variation (CV) was 13.4% ($n = 10$), and the intra-assay CV was 5.45% ($n = 15$). The IA-2ec autoantibody assay achieved ratings of 4% sensitivity and 99% specificity at the Islet Autoantibody Standardization Program 2012.

The intracellular domain of IA-2 (IA-2ic) construct (amino acid residues 605–979) was provided by Dr. E. Bonifacio (DFG-Center for Regenerative Therapies Dresden, TU Dresden, Dresden, Germany). The IA-2ic autoantibody radioimmunoassay has a similar assay format as that to detect IA-2ec autoantibodies (14). The interassay CV was 9.9%, and the intra-assay CV was 4.8%. IA-2ic autoantibodies achieved ratings of 72% sensitivity and 99% specificity at the 2007 4th assay proficiency evaluation of the Diabetes Autoantibody Standardization Program.

IA-2ec Antibody Specificity
To demonstrate specificity of IA-2ec autoantibodies, we performed inhibition of autoantibody binding studies. Unlabeled IA-2ec (amino acid residues 26–577) and IA-2ic (amino acid residues 605–979) were expressed in the reticulocyte lysate system with amino acid mixture containing unlabeled methionine (Fig. 3). For competitive binding studies, unlabeled antigens were each incubated separately with test human serum sample for 16 h at 4°C; after centrifugation at 12,000 × g for 20 min at 4°C, the supernatants were then incubated with 20,000 cpm of [^{35}S]methionine-labeled or unlabeled antigen (IA-2ec or

IA-2ic) for 16 h at 4°C. Immunoprecipitates were processed, analyzed by scintillation counting, and expressed as an index. All serum samples were assayed in triplicate. The results were presented as the means of three different experiments. Unabsorbed serum was used as a positive control to provide a baseline total cpm for the assay.

GAD65 and ZnT8 Autoantibody Radiobinding Assay

All serum samples including controls were analyzed in triplicate for autoantibodies targeted against GAD65 and ZnT8 using in vitro transcribed/translated [^{35}S]methionine-labeled recombinant human GAD65 and [^{35}S]methionine-labeled recombinant human ZnT8, as previously reported (17). The GAD65 construct was donated by Dr. Å. Lernmark (Lund University/CRC, Department of Clinical Sciences Diabetes & Celiac Disease Unit, Malmö, Sweden), whereas the ZnT8 construct was donated by Dr. J. Hutton (Barbara Davis Center for Childhood Diabetes, University of Colorado School of Medicine). The results were expressed as an index as previously reported (17,18). The cutoff points for these autoantibodies were based on the 99th percentiles of values from serum samples obtained from healthy volunteers (17,18).

Insulin Autoantibody Radioimmunoassay

Insulin autoantibody testing was performed at the Barbara Davis Center for Childhood Diabetes according to previously published protocols (19). This assay is currently used for protocols supported by TrialNet and The Environmental Determinants of Diabetes in the Young studies.

Tetramer Staining of In Vitro Expanded CD4$^+$ T Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll underlay, resuspended in T-cell media (RPMI, 10% human serum, 1% penicillin-streptomycin, and 1% L-glutamine) at 4×10^6 cells/mL, and stimulated with IA-2 peptides (20 μg/mL total) in 48-well plates for 14 days, adding medium and IL-2 as needed starting on day 7. Four peptide sequences that were recently shown to be epitopes were used for stimulation: IA-2 198–216 (SLSYEPALLEPYLFHEFGS), IA-2 467–482 (AAEEYGYIVTDEKPLS), IA-2 523–536 (QNLSLADVTEEAGL), and IA-2

545–562 (TGLEILETGVGEREEAAA). Each of these peptide sequences was modified to contain deamidated glutamate residues (at positions 207, 213, 478, 532, 533, 548, 551, and 556, respectively), as such modifications are required for presentation and recognition. To visualize responses, cells were stained with the corresponding DQ8 tetramers (or a negative control tetramer loaded with a nonstimulatory chromogranin A peptide) for 60 min at 37°C and then with CD4-PerCP (BD Biosciences), CD3-allophycocyanin (eBioscience), and CD25-FITC (BioLegend) for 15 min at 4°C and analyzed on an FACSCalibur (BD Biosciences) and using FlowJo software (Tree Star). As a positive control, PBMCs were stimulated with a well-characterized influenza peptide (matrix protein 97–116 VKLYRKLKREITFHGAKEIS) and stained with the corresponding DRB1*0401 tetramer. Matrix protein 97–116 (MP-97) is a conserved epitope essentially present in any seasonal influenza vaccine. Positive responses were gated based on the negative control tetramer. Each in vitro expansion was standardized as follows: we added IL-2 on day 7 of the culture. Subsequently, the cells were given fresh medium and IL-2 every 2 days, splitting the cells into new wells as needed to keep each well between 50 and 100% confluence.

Statistical Analysis
Data were analyzed using Prism 6 (GraphPad Software, La Jolla, CA) and SPSS 23.0 (SPSS, Chicago, IL). The χ^2 and Fisher exact tests were used to compare proportions and evaluate statistically significant associations between two categorical variables. The t test for independent samples was used to compare continuous variables between two groups. Venn diagrams were used to show the autoantibody frequencies. Bonferroni correction was used for multiple comparisons. Differences between T-cell responses to IA-2ec in patients with type 1 diabetes and control subjects were evaluated using an unpaired t test (two-tailed) with Welch's correction. A P value <0.05 was considered significant.

RESULTS

IA-2ec Autoantibodies Are Present in Patients With Both Type 1 and Type 2 Diabetes

In an effort to identify novel humoral epitopes within the neuroendocrine autoantigen IA-2, we initially performed immunoprecipitation studies using recombinant IA-2ec (amino acid residues 26–577), sera from patients with type 1 diabetes, patients with type 2 diabetes, and healthy volunteers. Immunoprecipitation of the IA-2ec protein can also be visualized by autoradiography following incubation with serum from patients with type 1 and type 2 diabetes with no detectable IA-2ic autoantibodies (Fig. 1A, lanes 1 and 2). In contrast, serum from a healthy control subject did not immunoprecipitate the IA-2ec protein (Fig. 1A, lane 3).
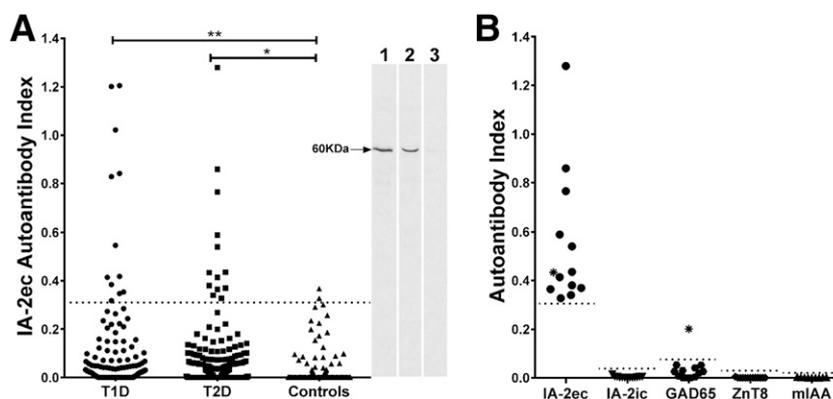
Figure 1—A: Autoantibodies directed to IA-2ec (amino acids 26–577) can be detected in patients with type 1 and 2 diabetes. Dashed line represents the cutoff point for the assays. Eight percent of patients with type 1 diabetes and 5% of patients with type 2 diabetes exhibited antibody responses to IA-2ec. *P = 0.0371; **P = 0.0023. Two-dimensional SDS-PAGE fractionation of IA-2ec immunoprecipitates followed by autoradiography illustrates that serum from one patient with type 1 diabetes and from another patient with type 2 diabetes (blot, lanes 1 and 2, respectively) strongly reacted with a 60-kDa band (IA-2ec), unlike serum from a healthy volunteer (blot, lane 3). B: The majority of patients with clinically diagnosed type 2 diabetes carrying IA-2ec autoantibodies tested negative for IA-2ic, GAD65, ZnT8, and mIAA. *Positive for both IA-2ec and GAD65 autoantibodies.

We then found that the proportion of autoantibodies against IA-2ec was higher in patients with type 1 as well as type 2 diabetes as compared with that of healthy control subjects (8% [12 out of 150] vs. 1% [2 out of 178], P = 0.0023; 5% [13 out of 258] vs. 1% [2 out of 178], P = 0.0317) (Fig. 1A). Interestingly, the majority of patients with type 2 diabetes who tested positive for IA-2ec autoantibodies were negative for all traditional islet autoantibodies. In the latter group, only one subject tested positive for GAD65 autoantibodies (Fig. 1B). Four of those patients with type 2 diabetes were on insulin therapy.

Surprisingly, 13 patients with type 2 diabetes tested positive for IA-2ec autoantibodies and negative for IA-2ic autoantibodies (Fig. 2B), whereas 1 out of 12 patients with type 1 diabetes tested positive for both IA-2ec and IA-2ic autoantibodies (Fig. 2A). The majority of clinically diagnosed patients with type 2 diabetes carrying IA-2ec autoantibodies

tested negative for IA-2ic, GAD65, ZnT8, and micro-insulin autoantibodies (mIAA) (Fig. 1B). IA-2ec antibody index was higher in patients with type 1 diabetes compared with that of patients with type 2 diabetes (P = 0.036) and control subjects (P < 0.0001). The Venn diagram shows the frequency of islet autoantibodies (alone or in combination) in both patients with type 1 (Fig. 2A) and type 2 diabetes (Fig. 2B). There was no statistically significant difference with respect to age or ethnicity in IA-2ec antibody–positive compared with IA-2ec antibody–negative patients with type 2 diabetes.

Of 178 healthy volunteers, 0.6%, 1.1%, and 1.7% exceeded the thresholds for IA-2ic, GAD65, and ZnT8 antibodies, respectively.

As expected, the vast majority of patients with type 1 diabetes had at least one islet autoantibody as compared with patients with type 2 diabetes. In particular, 146 out of 150 (97.3%)

patients with type 1 diabetes and 36 out of 258 (14%) patients with type 2 diabetes had at least one islet autoantibody (P = 0.0011). Four (2.7%) patients with type 1 diabetes and none of the patients with type 2 diabetes tested positive for all autoantibodies (P = 0.1958). Patients with type 1 diabetes were more frequently positive for single and multiple autoantibodies as compared with patients with type 2 diabetes.

Although the prevalence of GAD65, mIAA, ZnT8, and IA-2ic autoantibodies was significantly higher in patients with type 1 diabetes compared with that of patients with type 2 diabetes (74 vs. 6.9, 52.7 vs. 3.1, and 72 vs 3.1%, respectively; P < 0.0003), the prevalence of IA-2ec autoantibodies was higher in patients with type 1 diabetes as compared to that of patients with type 2 diabetes (8 vs. 5%).

Although the prevalence of IA-2ic autoantibodies alone was significantly higher in patients with type 1 diabetes than that of patients with type 2 diabetes (15 [10%] vs. 2 [0.8%]; P < 0.0011), the prevalence of IA-2ec autoantibodies alone was higher in patients with type 2 diabetes as compared with that of subjects with type 1 diabetes (12 [4.7%] vs. 1 [0.7%]). The most frequent single islet autoantibody in patients with type 1 diabetes was GAD65 autoantibody and IA-2ec autoantibody in patients with type 2 diabetes. Overall, the addition of the IA-2ec autoantibodies in patients with type 2 diabetes to islet autoantibodies, measured by routine assays, resulted in an increase in sensitivity from 9.3% (24 out of 258) to 14% (36 out of 258).

Table 1 shows the islet autoantibody status in patients with type 1 diabetes and clinically diagnosed patients with type 2 diabetes with diabetes duration <6 months. The vast majority of these patients was either on diet alone or oral hypoglycemic therapy. Interestingly, 1 out of 3 (33.3%) IA-2ec antibody–positive new-onset patients with type 2 diabetes was on insulin therapy (Table 1). In addition, we analyzed the data after matching patients with type 1 and type 2 diabetes for insulin use (Table 2). As expected, we found a much higher prevalence of islet autoantibodies in subjects with type 1 diabetes on insulin therapy compared with that of patients with type 2 diabetes on dietary or oral hypoglycemic therapy. Islet autoantibodies
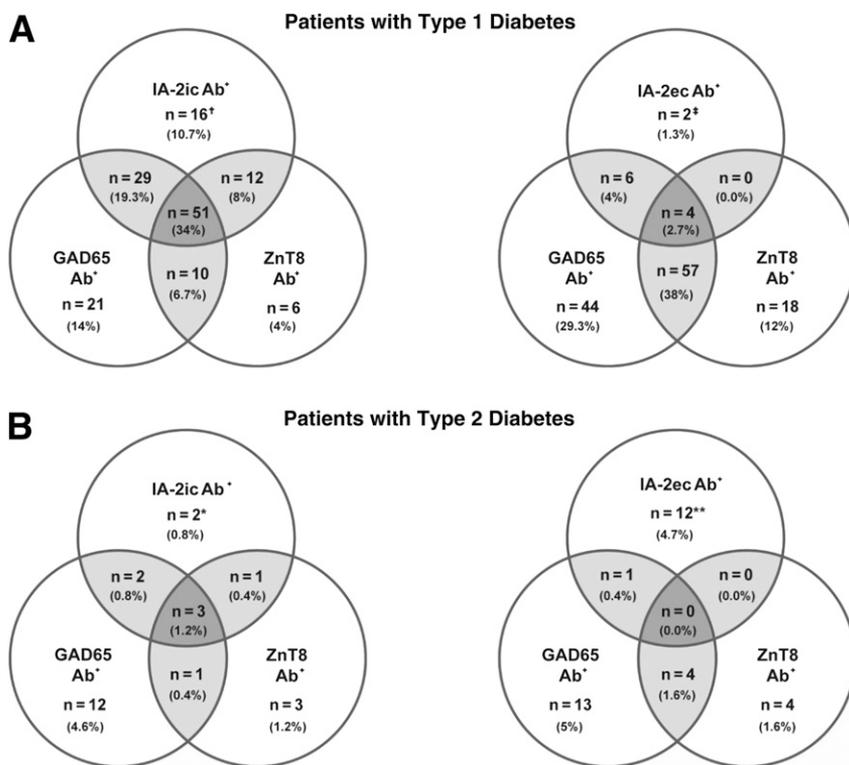
Figure 2—Venn diagrams of autoantibody combinations in autoantibody-positive patients with type 1 and 2 diabetes. The frequency of autoantibodies in patients with type 1 (A) and type 2 (B) diabetes (IA-2ec, IA-2ic, GAD65, and ZnT8 autoantibodies). Intersecting regions (lightly shaded) indicate the number of patients positive for different combinations of islet autoantibodies. Insulin autoantibodies were not included in this analysis because many patients were on insulin therapy. *Both patients with type 2 diabetes were negative for IA-2ec antibody (Ab) autoantibodies. **All patients with type 2 diabetes were negative for IA-2ic antibodies. †One out of 16 patients with type 1 diabetes was IA-2ec antibody positive. ‡One out of two patients with type 1 diabetes was IA-2ic antibody positive.

were present in ~21% of clinically diagnosed patients with type 2 diabetes on insulin therapy (Table 2).

Specific Autoantibody Binding to Novel Epitopes Within the IA-2ec

Competitive binding studies using unlabeled IA-2ec (amino acids 26–577), and IA-2ic (amino acids 605–979) proteins demonstrated specificity of IA-2ec autoantibodies (Fig. 3). Sera from patients with type 1 and 2 diabetes, positive for IA-2ec autoantibodies, were preincubated with unlabeled antigen (IA-2ec or IA-2ic). This resulted in complete inhibition of serum binding to [^{35}S]methionine-labeled IA-2ec only after preincubation with unlabeled IA-2ec and in no inhibition after preincubation with unlabeled IA-2ic (Fig. 3). These experiments suggest that autoantibody reactivity to IA-2ec is due to specific binding.

CD4^{+} T-Cell Responses to IA-2ec Peptides in Subjects With Type 1 Diabetes

It is well appreciated that T and B cells undergo cognate interactions and that antigen-specific CD4 T-cell interactions with B cells aid in the eventual production of antibodies. To evaluate IA-2ec–specific T-cell responses, we stimulated PBMCs from 18 patients with type 1 diabetes and 13 HLA-matched healthy subjects with epitopes from IA-2ec followed by staining with peptide/DQ8(DQB1*03:02) tetramers. We selected four IA-2ec peptides containing a posttranslational modification (each with at least one deamidated glutamate residue) for these experiments, as deamidated epitopes from other islet antigens have been reported in type 1 diabetes (20,21). Fig. 4A depicts representative positive tetramer staining for the negative control tetramer, each of the four IA-2 peptides, and a positive control tetramer. Each IA-2 peptide elicited positive responses in many subjects with type 1 diabetes (and more rarely in control subjects), but responses were more effectively visualized by combining all four tetramers. As shown in Fig. 4B, IA-2–specific T cells were present at significantly higher magnitudes in patients with type 1 diabetes than in control subjects ($P = 0.0017$). These results provide direct evidence that DQ8-restricted CD4^{+} T cells recognize deamidated epitopes derived from the IA-2ec and suggest that such T-cell responses are more commonly present in subjects with type 1 diabetes.

CONCLUSIONS

The specific characterization of diverse subtypes of diabetes has been a moving target and a major topic of debate (2,22). A well-documented example is the case of patients who are generally adults and present with a type 2 diabetic phenotype as well as circulating islet autoantibodies, generally GAD65 autoantibodies (23). These characteristics are defined as LADA and sometimes termed type 1.5 diabetes (24,25). The demonstration that autoimmune responses play a key role in the pathogenesis of type 1 diabetes led to the assumption that type 1 and 2 diabetes possess unique etiologies, disease courses, and, consequently, therapeutic regimens.

However, solid evidence indicates that overlap exists even among the most "typical" diabetes phenotypes (2,6,22,26). Thus, the current classification of diabetes poses challenges to the diagnosis and treatment of patients.

Autoantibodies are powerful tools in translational research, biomarker identification, and diagnostic testing for autoimmune disorders. Because of their robust measurements and ease of use, immunoassays are among the preferred methods for investigation of various biological and clinical questions. We optimized a new IA-2ec antibody biomarker detecting islet autoimmunity in a subset of patients with type 1 and 2 diabetes. IA-2ec autoantibodies identified islet autoimmunity in a subset of clinically diagnosed patients with type 2 diabetes that were otherwise negative for conventional islet autoantibody testing.

Table 1—Islet autoantibody status in patients with type 1 diabetes and clinically diagnosed type 2 diabetes with diabetes duration <6 months

	Type 1 diabetes	Type 2 diabetes
N	147	50
Male sex	72 (48.9)	33 (66)
Age (years)	13.3 ± 10	55.6 ± 9.8
IA-2ec Ab	12 (8.2)	3[a] (6)
IA-2ic Ab	105 (71.4)	0
GAD65 Ab	109 (74.1)	0
ZnT8 Ab	78 (53.1)	0
Single islet Ab	42 (28.6)	3 (6)
At least one islet Ab	143 (97.3)	3 (6)
Multiple islet Ab	101 (68.7)	0
Diet use	0	26 (52)
OHA use	0	18 (36)
Insulin use	147 (100)	5 (10)

Data are mean ± SD or n (%). Ab, antibody; OHA, oral hypoglycemic agent. [a]One patient was on insulin therapy.

Table 2—Islet autoantibody status in patients with type 1 diabetes and clinically diagnosed type 2 diabetes on insulin therapy

	Type 1 diabetes	Type 2 diabetes
N	150	73
Male sex	74 (49.3)	39 (53.4)
Age (years)	13.4 ± 10.4	54.7 ± 9
IA-2ec Ab	12 (8)	4 (5.5)
IA-2ic Ab	108 (72)	5 (6.8)
GAD65 Ab	111 (74)	11 (15)
ZnT8 Ab	79 (52.7)	4 (5.5)
Single islet Ab	43 (28.7)	9 (12.3)
At least one islet Ab	146 (97.3)	15 (20.6)
Multiple islet Ab	103 (68.7)	6 (8.2)
Insulin use	150 (100)	73 (100)

Data are mean ± SD or n (%). Ab, antibody.
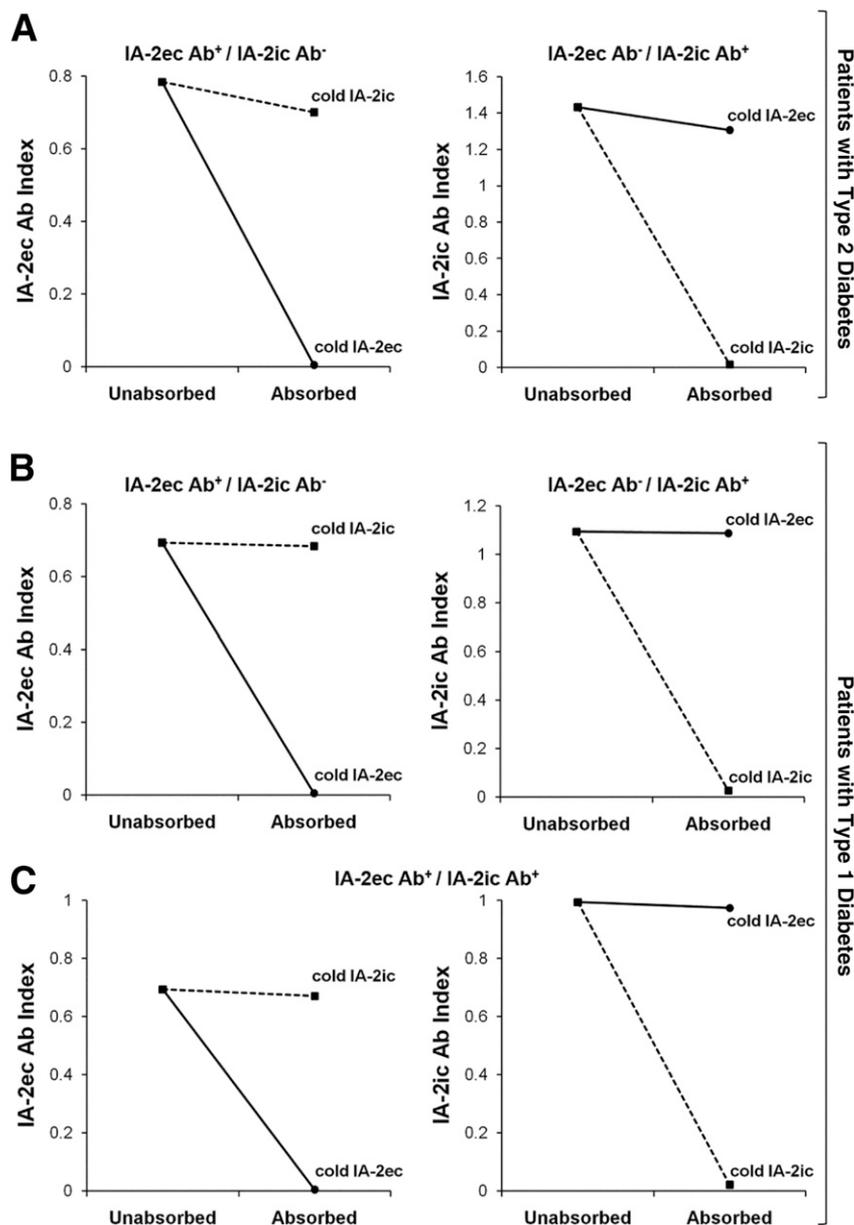
Figure 3—Specific inhibition of autoantibody binding to IA-2ec (circles) and to IA-2ic (squares) with sera from patients with type 1 and 2 diabetes. *A*: Binding to IA-2ec of serum of a patient with type 2 diabetes positive for IA-2ec antibodies was not inhibited by preincubation with unlabeled IA-2ic, whereas it was specifically inhibited by preincubation with unlabeled IA-2ec (left panel). Binding to labeled IA-2ic of serum of a patient with type 2 diabetes positive for IA-2ic antibodies was not inhibited by preincubation with unlabeled IA-2ec, but it was specifically inhibited by preincubation with unlabeled IA-2ic (right panel). *B* and *C*: Specific inhibition of autoantibody binding to IA-2ec and IA-2ic in patients with type 1 diabetes with different combinations of these autoantibodies. Ab, antibody.

We analyzed the prevalence of islet autoantibodies in recent-onset type 1 and 2 diabetes with the assumption that the degree of glycemic control is similar in those two groups according to previously published observations (27,28). The vast majority of newly diagnosed patients with type 2 diabetes evaluated in this study was either on dietary or oral hypoglycemic therapy. It is noteworthy to acknowledge that 33.3% of newly diagnosed patients with type 2 diabetes using insulin had detectable levels of IA-2ec autoantibodies with no evidence of other islet autoantibodies (Table 1).

Although canonical immune responses to IA-2ic are considered to be immunodominant (29–31), we found autoantibody and T-cell responses specifically directed to IA-2ec in individuals with or without antibodies directed to IA-2ic. Our results

reinforce the notion that long-lived autoantibody responses in the natural history of autoimmune disorders, such as type 1 diabetes, are generally regarded to be polyclonal and yet not restricted to one portion of a given self-antigen (32–35).

The presence of islet autoantibodies in clinically diagnosed patients with type 2 diabetes by conventional criteria (American Diabetes Association or World Health Organization) is not uncommon among older patients with diabetes, being 5–10% or higher, especially those on insulin therapy (36–38). In the current study, we found islet autoantibodies in ~21% of clinically diagnosed patients with type 2 diabetes on insulin therapy (Table 2). Thus, theoretically, there are at least as many islet autoantibody–positive older patients with diabetes as there are children affected by type 1 diabetes. This is a public health issue. Additional immunological markers of autoimmune diabetes, such as IA-2ec autoantibodies, may identify even larger numbers of clinically diagnosed patients with type 2 diabetes. Given its relative simplicity, larger studies are needed to measure IA-2ec antibodies to potentially identify clinically diagnosed patients with type 2 diabetes who may rapidly progress to insulin-requiring diabetes.

A limitation of this study is the different durations of diabetes between subjects with type 1 and 2 diabetes; the latter had long-standing diabetes for the most part.

We previously reported that islet autoantibody–positive long-term patients with type 2 diabetes exhibited profound impairment of insulin secretion as well as reduced β-cell mass seemingly determined by an immune-mediated injury of pancreatic β-cells (37,39). Further studies aimed at investigating the significance of autoimmunity and the pancreas immunopathology in this subset of patients with type 2 diabetes are required.

It is noteworthy to acknowledge that the presence of autoantibodies reacting with portions of both IA-2ec and IA-2ic has been associated with LADA (40,41). Brooks-Worrell et al. (42) reported islet-reactive T cells in up to 50% of seronegative patients with type 2 diabetes. As there are interactions between CD4 T cells and B cells leading to antibody production, the possibility exists that some of those autoantibody-negative patients with type 2 diabetes may carry IA-2ec autoantibodies.
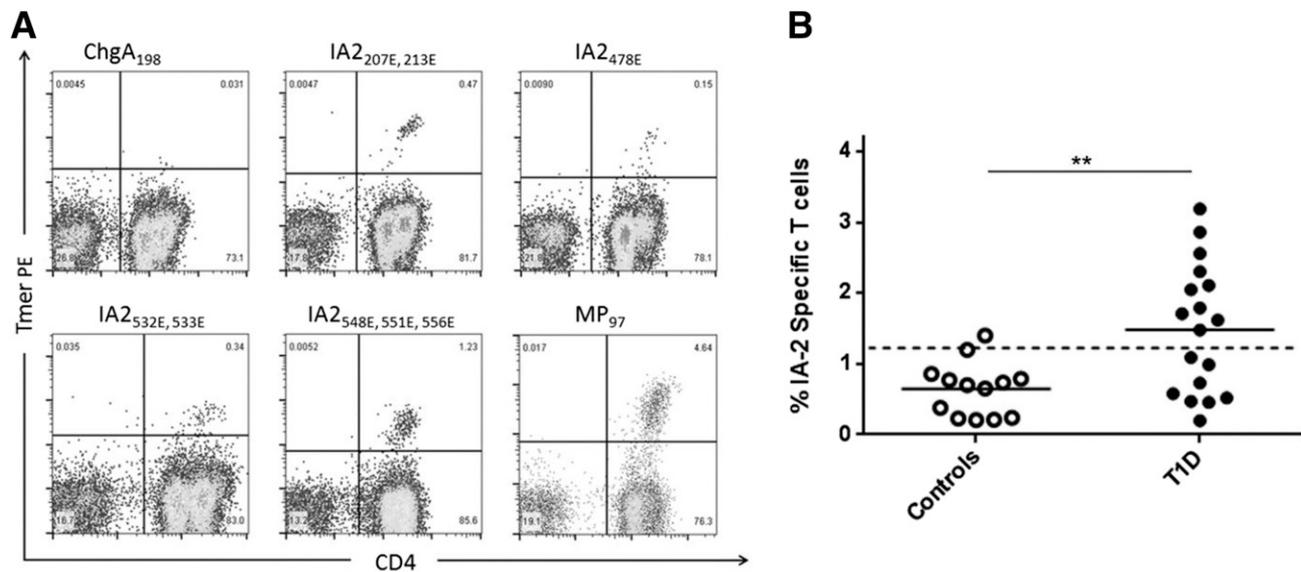
Figure 4—CD4^{+} T-cell responses to IA-2ec peptides are elevated in subjects with type 1 diabetes. *A*: Representative tetramer staining of T cells using tetramers loaded with a negative control peptide (chromogranin A 198 [ChgA$_{198}$]), DQ8-restricted IA-2 peptides (as indicated on each panel), or an influenza peptide (MP-97) after 2 weeks of in vitro expansion. *B*: Comparison of IA-2–specific T-cell responses measured in 18 subjects with type 1 diabetes (T1D) and 13 HLA-matched control subjects (all with DQ8^{+} haplotypes) by tetramer staining (combined percentages for all four epitopes). Responses >1.1% (1 SD above the mean response for controls, indicated by the dashed line) were above the positive threshold. The magnitude of responses to IA-2ec peptides were significantly higher in subjects with type 1 diabetes ($P = 0.0017$, two-tailed t test with Welch's correction).

We assessed IA-2ec CD4 T-cell responses and observed disease-associated responses to epitopes within the extracellular domain with tetramer staining of PBMCs. Similar to type 1 diabetic autoantibodies, T-cell responses to islet proteins may be biomarkers and important contributors of progressive decline in β-cell function observed both in patients with type 1 and 2 diabetes. We also show that peptides derived from IA-2ec are immunogenic in vitro and provide evidence that the precursor frequency of HLA-DQ8–restricted T cells in peripheral blood is higher in patients with type 1 diabetes than in healthy control subjects. Although initial studies have focused on HLA-DR4 epitopes in IA-2ic (30), recent observations demonstrated that naturally processed and post-translationally modified IA-2 peptides within the extracellular domain proved to be targets of autoreactive CD4^{+} T cells in patients with type 1 diabetes (21,43). These studies demonstrated that during inflammation both human islets and dendritic cells, which process and present antigen to CD4 T cells, can activate tissue transglutaminase to deamidate islet autoantigens (44,45). Of note, the amino acid sequence of HLA-DQ8–restricted epitopes of IA-2ec described in our study are different than those identified in the latter reports.

In summary, our findings suggest that IA-2ec autoantibodies and T cells can be detected in patients with type 1 diabetes. IA-2ec autoantibodies identify a subgroup of adult autoimmune phenotypic patients with type 2 diabetes who test negative for conventional islet autoantibodies. Autoimmunity to new IA-2 epitopes within the extracellular domain will provide impetus for efforts in developing mechanistic biomarkers, which are embedded in disease pathogenesis, as well as new IA-2 peptide-based immunotherapies for autoimmune diabetes and possibly assessing responsiveness to therapy.

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