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M A Atkinson, … , A J Tobin, N K Maclaren

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Research Article

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Islet Cell Cytoplasmic Autoantibody Reactivity to Glutamate Decarboxylase in Insulin-dependent Diabetes

Mark A. Atkinson,* Daniel L. Kaufman,‡ Douglas Newman,‡ Allan J. Tobin,§ and Noel K. Maclaren*
**Department of Pathology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida 32610; and Departments of ‡Psychiatry and Biobehavioral Sciences, and §Biology, University of California, Los Angeles, Los Angeles, California 90024*

Abstract

Individuals with or at risk for insulin-dependent diabetes (IDD) frequently have autoantibodies against an islet cell cytoplasmic (ICA) antigen thought to be a sialoglycolipid. However, we now report that preabsorption of ICA-positive sera with recombinant glutamate decarboxylase (human GAD 65 and/or GAD 67) reduced or blocked the ICA reactivity of 5/18 (27%) new-onset IDD patients and 7/18 (39%) prediabetics. Interestingly, nondiabetic subjects with ICA of ≥ 5 yr in duration had GAD-reactive ICA significantly more often (16/24, 67%, $P < 0.04$) than the diabetic groups. ICA reactivity to GAD was not related to serum ICA titer nor the age of the individual, and in all cases tested was blocked by GAD 65 or GAD 67 with equivalent efficiency. The ICA observed in 21/25 (84%) IDD patients with ICA long after clinical onset of disease (9–42 yr) was reactive to GAD. A natural history analysis of three individuals showed conversions from ICA which was reactive to GAD to a non–GAD-reactive ICA nearer to their clinical onsets of IDD. This study further defines the autoantigens reactive to ICA, and suggests that, whereas ICA that are not reactive to GAD may identify an advanced and more prognostic lesion, GAD-reactive ICA may typify the early or inductive lesion that may or may not progress to clinically significant β cell injury. (*J. Clin. Invest.* 1993. 91:350–356.) Key words: autoantigens • autoimmunity • glutamate decarboxylase • islet cell autoantibodies • insulin-dependent diabetes

Introduction

Insulin-dependent diabetes (IDD,[1] type 1 diabetes) results from the autoimmune destruction of insulin-producing pancreatic islet β cells (1). Because of the significant long-term morbidity and mortality associated with IDD (2), the design of methods for early detection of the pathogenic process is actively being researched in the anticipation of developments for disease prevention (3). The disease has a long prodromal period before its clinical presentation, which is characterized by autoantibodies directed against self antigens of islet cells and their products. Such autoantibodies can serve as markers to identify anti–islet cell autoimmunity before the onset of clinical symptoms. The most widely studied of these "predictive" markers include anti-insulin (IAA) (4, 5), anti–64-kD or glutamate decarboxylase (GAD) autoantibodies (6–9), and anti–islet cell cytoplasmic antigen autoantibodies (ICA) (10–15). Whereas radioimmunoassay and immunoprecipitation techniques are employed to detect IAA and GAD autoantibodies, respectively, standard analysis of ICA involves indirect immunofluorescence of sera on frozen human pancreas sections. While a positive ICA test is considered to be diagnostic for IDD in persons with hyperglycemia, it is also a common admission criteria for individuals entering studies aimed at the prevention of IDD in humans (3). Therefore, the establishment of a standardized test for ICA identification (16, 17), as well as the biochemical characterization of the islet cell antigen(s) responsible for ICA reactivity is of significant clinical research interest.

Nayak and co-workers (18) have suggested that the islet cell antigen responsible for the ICA reaction is a sialic acid–containing glycolipid. Evidence supporting this claim included the similarity in biochemical properties of antigens recognized by anti-ganglioside monoclonal antibodies with that of ICA-positive sera, and the observation that ICA binding to islet cells is inhibited by a glycolipid extract which migrates as a monosialoganglioside (GM2-1) from human pancreas (18, 19). However, a report by Solimena and co-workers (20) suggested that patients with the neurological disorder Stiff-Man syndrome had an ICA-like autoantibody which represented an anti-GAD immune response. Another study (21) has suggested that ICA in IDD patients may represent a heterogenous population of autoantibodies, which could be categorized into two classes of autoantibodies that vary in their degree of predictive value for IDD and in the islet cell antigens which they recognize. Unfortunately, no antigenic characterizations or natural history studies were performed in that latter study (21).

We have recently reported the ability of IDD sera to specifically immunoprecipitate two forms of bacterially produced recombinant GAD polypeptides (9). The two isoforms of GAD have molecular weights of 65,000 and 67,000 (GAD 65 and GAD 67) which are encoded by different genes located on different chromosomes (human chromosome 2 for GAD 65 and 10 for GAD 67). The amino acid sequences of the two GADs are ~ 70% identical, and both proteins contain a putative bind-

Address reprint requests to Dr. Mark A. Atkinson, Department of Pathology, Box J-275, JHMHC, Gainesville, FL 32610.

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1. *Abbreviations used in this paper:* GAD, glutamate decarboxylase; IAA, insulin autoantibody(ies); ICA, islet cell cytoplasmic autoantibody; IDD, insulin-dependent diabetes; JDF, Juvenile Diabetes Foundation.

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ing site for pyridoxal phosphate, a cofactor necessary for the enzymatic activity of GAD (22–25). Both forms of GAD have been identified within neurons of the brain as well as in the pancreatic islet cells, however other reported locations include the testes, ovary, oviduct, and adrenal medulla (26). Sequence analysis (22–25) as well as biochemical characterization (27) have so far revealed no distinct differences between brain and the pancreatic islet GAD. Indeed, there is only one gene for each form of GAD (23).

To further define the antigen(s) responsible for the ICA reaction seen in association with IDD, we have tested for the ability of recombinant human GAD (65 or 67) and recombinant control *Escherichia coli* lysate to block the ICA reactivity associated with IDD sera. These studies suggest that a major antigen of the ICA reaction in IDD is GAD, and that autoimmunity to this antigen may represent an early state of anti-β cell immunity.

Methods

Patient sera, antibodies, and tissues. Sera from four groups of individuals were selected from our ongoing studies into the natural history of IDD (7, 13) for the analyses presented here. There were 18 new onset IDD patients (9 males/9 females, age 15.6±12.3 yr) as well as 18 IDD patients (6 males/12 females, age 17.9±13.7 yr) who had sera drawn from 2 to 90 mo before their documented onsets of IDD. 24 ICA-positive nondiabetic individuals (10 males/14 females, age 26.6±13.4 yr) who had ICA of ≥ 5 yr duration but without development of IDD were also analyzed. The mean duration of ICA positivity in the ICA-positive nondiabetic group was 7.6±1.9 yr. Finally, 25 ICA-positive IDD patients (15 males/10 females, age 33.2±14.7 yr) who had IDD for 9 to 44 years (mean 17.4±8.4 yr) duration were also studied. Diabetes was defined according to the World Health Organization criteria (28). Human leukocyte antigen typing was performed as previously described (29). Informed consent was obtained from the subjects and/or their parents according to the subject's age as approved by the University of Florida Institutional Review Board.

Mouse IgG_1 mAb GAD-6 (kindly donated by David Gottlieb [30]) and rabbit polyclonal antisera K-2 (31) were used for these studies. In conjunction with these primary antibodies, the FITC-labeled secondary antibodies (goat antiserum to mouse IgG [Kallestad, Austin, TX] and rabbit IgG [Sigma Chemical Co., St. Louis, MO]) were used at 1:160 dilution in PBS (pH 7.4). Fresh tissue (pancreas and cerebellum) from humans and rats were snap-frozen and cryopreserved at −80°C, and cryocut for immunohistochemical analysis. Human brain was provided by the Brain Institute at The University of Florida, and animal use was approved by The University of Florida committee on animal use.

Islet cell cytoplasmic autoantibodies. Islet cell antibodies were determined by indirect immunofluorescence using unfixed, snap-frozen human pancreas as previously described (13). A test was considered to be positive for ICA when the intensity of the fluorescence and pattern of staining of an undiluted serum was the same or greater than a laboratory standard serum that had been calibrated to approximate 10 Juvenile Diabetes Foundation (JDF) units (16). All positive serum samples were expressed in JDF units by comparing the end point dilution of each positive serum to a standard calibration dilution curve using the international JDF reference serum accepted by the Immunology of Diabetes Workshops. Our laboratory at The University of Florida is a regular participant of the ICA proficiency program conducted under the auspices of the Workshops, and we currently administer serum collection and distribution for that program.

Insulin autoantibodies. Insulin autoantibodies were determined by a modification of a radiobinding assay (32). The assay used human insulin ligand monoiodinated at amino acid 14 of the A chain, which was generously provided by Eli Lilly & Co., Indianapolis, IN. Any serum found to have binding levels greater than 3 SD over the mean of 83 controls (107 nU/ml) submitted to the Third International Insulin Antibody Workshop was defined as positive. From an independent analysis of the results of the participants of this workshop, our assay was found to be highly specific and sensitive for IDD.

Glutamate decarboxylase. Human GAD 65 and GAD 67 (GAD; E.C.4.1.1.15) were amplified by polymerase chain reaction from their respective cDNAs (23), inserted into the open reading frame of maltose-binding protein fusion vector 997 (New England Biolabs, Beverly, MA), and transformed into XL1 Blue (Stratagene, La Jolla, CA) *E. coli.* Control (harboring nonrecombinant 997 vector) and GAD producing *E. coli* were grown, induced with iso-propyl-thio-β-D-galactoside, and collected by centrifugation. The bacterial pellet was taken up in 5–10 vol of 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.2 mM pyridoxal phosphate, and sonicated three times for 30 s. The resulting whole-cell lysate was used for subsequent immuno-adsorption. Quantification of GAD was performed by SDS-PAGE analysis against protein standards of known concentration.

ICA blocking studies. To analyze the effect of GAD in blocking ICA reactivity, serum samples were diluted to determine the JDF endpoint titer beyond which the ICA reaction was extinguished and the two dilutions prior to this end-point titer. A fixed quantity (10 μl) of either bacterially produced GAD 65 or GAD 67 (10 μg of GAD per sample) or an equivalent quantity (10 μl) of control bacterial extract was added to each serum sample which served as the primary diluent used as well as a specific internal control. Both the GAD-containing and control bacterial extracts were analyzed by SDS-PAGE analysis, and were adjusted to equivalent bacterial protein concentrations through volumetric dilutions. Serum samples (at a base volume of 10 μl of sera diluted in PBS when required) were incubated with the bacterial GAD or control extracts and rocked for 60 min at room temperature. After this incubation, samples were tested for ICA as previously described. Alternatively, sera were incubated at a 90% (vol/vol) sera/tissue ratio with snap frozen human cerebellum and or liver powder under the same incubation conditions prior to ICA analysis. A positive blocking effect was defined as a loss of fluorescence activity more than or equal to twofold dilutions from the JDF endpoint titer (onefold in the case of sera of 10 JDF units) in the presence of GAD containing extracts, coupled with lack of ICA blockage with control extracts. All visual analyses were performed in a blinded format, with samples assayed on side-by-side testing of consecutive cryocut sections of human pancreas. Statistical analyses were performed using the Fisher's exact test.

Results

Blocking of antibodies with recombinant GADs. Our initial studies established an anti-GAD antibody absorption protocol that tested the ability of recombinant GADs to compete for GAD antigen contained within the pancreatic tissue substrate. The reactivity of anti-GAD antibodies GAD-6 (mouse mAb) and K-2 (rabbit polyclonal) were specific for GAD 65 and GAD 67 respectively (31). In our studies, the GAD-6 mAb was very efficient at identifying human islet cells by indirect immunofluorescence using unfixed/cryocut human pancreatic sections at an antisera dilution range of 1:40 to 1:1,000. However, we were not able to identify islet cells within pancreatic sections using the K-2 antibody. This may have been due to a property of the K-2 antibody itself, or to a limited quantity of GAD 67 present within the islet cell sections (27). Therefore, we used sections of human and/or rat cerebellum for identification of cells containing GAD 67 with K-2 antibody. The Purkinje cell bodies, which contain GAD 67, were clearly visible by indirect immunofluorescence (dilution range 1:20 to 1:100).

We then tested the ability of an *E. coli* lysate containing recombinant GAD 65 or GAD 67 (0.1–5.0 μg) to inhibit their respective anti–GAD antibody binding to brain and pancreatic sections. Both the GAD-6 and K-2 antibodies were effectively removed by their preincubation (1 h, 24°C or 16 h, 4°C) with the recombinant GAD preparations before immunohistochemistry (data not shown). Blocking (i.e., total absorption) of the antibodies was maximally observed at the 5.0-μg dose. To prove the specificity of the antibody absorption, the anti–GAD antibodies were preabsorbed with an equivalent quantity (as measured by bacterial protein content) of control *E. coli* lysate. Under these same conditions in side-by-side analysis, the lysate alone did not reduce or block the immunofluorescent reactivity of the antibodies with the tissue substrate.

Blocking of islet cell autoantibodies with recombinant GAD. Having established a protocol for anti–GAD antibody absorption, we used this method to test the ability of recombinant GAD to block reactivity of ICA-positive sera. Fig. 1 (top) shows the serum reactivity of patient HS (Table I) to the islet cell cytoplasm of a pancreatic section. This sample was diluted to an end-point dilution of 160 JDF units with bacterial control lysate. In contrast, the lower panel shows the total loss of ICA reactivity from the same serum sample which was preincubated with GAD 65 before ICA analysis. The fluorescence observed with sera from other ICA-positive individuals was not affected by addition of GAD. The fluorescence staining pattern of islet cells observed with a patient's sera could not be reliably used to predict which form of ICA (i.e., GAD-reactive or non–GAD-reactive) was present.

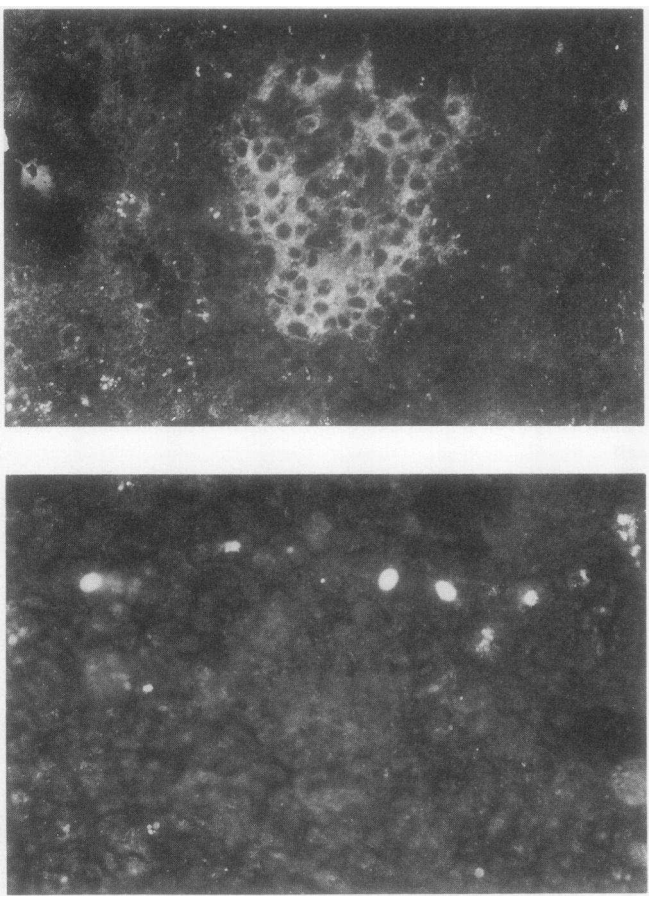

Figure 1. Effect of recombinant GAD on blocking ICA binding to human pancreatic islets. Panels depict activities of sera from patient HS. Top panel shows immunofluorescence activities of sera in the presence of bacterial control lysates, whereas lower panel shows immunofluorescence with sera preincubated with GAD 65 containing bacterial lysates.

The preabsorption analyses were then applied to the three study groups. Because the analysis required dilution of samples with recombinant GADs, only sera from persons with ICA of ≥ 10 JDF units could be included within this study. Of the 18 newly diagnosed IDD patients test (onset samples), 5 (27%) had their ICA reactivity blocked by prior incubation with GAD 65 (Table I). Similarly, 7 of the 18 (39%) ICA-positive persons who subsequently developed IDD ("prediabetic") had their ICA reactivity blocked with GAD 65 (Table II). For analysis of these prediabetic individuals, the earliest available samples were tested for ICA absorption. Finally, 16 of the 24 (67%) nondiabetic individuals with ICA of ≥ 5 yr in duration without development of IDD had their ICA reactivity blocked with GAD 65 (Table III, $P < 0.04$ vs. new onset patients). For individuals listed in Table III, the only most recent available sample was tested. The ability of GAD 65 to block ICA reactivity did not correlate with ICA titer, presence of IAA, nor age of the subject (Tables I, II, and III). With respect to genetic susceptibility, no statistically significant associations between HLA-DR or HLA-DQ type and the presence or absence of ICA reactive to GAD (Tables I, II, and III) were identified. However, ICA to non-GAD was present in the two rare HLA-DR2 IDD patients, whereas three of four long-term ICA-positive HLA-DR2 individuals without IDD had GAD-reactive ICA.

We next examined whether GAD 65 and GAD 67 differed in their ability to block ICA reactivity, and whether absorption with GAD 67 might identify individuals whose ICA could not be blocked by GAD 65. Only a subgroup of 37 individuals (randomly selected from each group) were selected for these studies. Of 14 individual samples identified with ICA reactive to GAD, all were blocked with equal efficiency by either GAD 65 or GAD 67 (Tables I, II, and III). In addition, preincubation of these sera with cerebellum was also effective at specifically reducing the ICA activity, whereas no change in ICA activity was observed with liver absorption.

We next analyzed these two forms of ICA with respect to their natural history before disease onset. To date we have identified three prediabetic individuals (Table II) whose ICA at initial contact (42–48 mo before disease onset) was reactive to GAD, whereas a second analysis performed on serum samples closer to the time of clinical diagnosis of IDD showed that the predominant form of the antibody had changed to a non–GAD-reactive ICA. The time period between the conversion of one ICA type to the other was as little as 6 mo. Limited natural history analyses of four other prediabetic persons have not shown such conversions in ICA specificity in either direction (i.e., GAD-reactive ICA to non–GAD-reactive ICA or non–GAD-reactive ICA to GAD-reactive ICA) over the period which could be studied.

Finally, we examined our population of long-term IDD patients (≥ 9 yr) whom continued to test positive for ICA well beyond the time of diagnosis. Of 949 such individuals (458 males/491 females), 117 (12.3%) were positive for ICA of ≥ 10 JDF units. 25 of these individuals were then randomly

Table I. Physical Characteristics and Autoantibody Analysis of Sera from New Onset IDD Patients

Subject	Sex	Onset age	HLA DR	HLA DQ	Method of accrual	ICA JDF	ICA blocked by GAD 65	ICA blocked by GAD 67
MA	F	15	10, 4	NT	Rel	20	+	NT
BC	M	10	1, 3	NT	Rel	40	−	NT
JA	M	13	4, X	3	Rel	40	−	NT
SH	F	5	4, X	3	Rel	80	−	−
EB	M	10	3, X	2	Rel	80	−	NT
JG	F	11	3, X	2	Rel	80	+	+
JW	F	43	3, 4	2, 3	Rel	80	−	NT
JB	M	11	4, 13	6, 3	Rel	160	−	NT
KV	F	20	3, 6	1, 2	Rel	160	−	NT
KE	M	16	1, 5	1, 3	Rel	160	−	NT
MT	M	3	4, 3	2, 3	Rel	160	−	−
SH	M	17	4, 7	2, 3	Rel	160	+	+
DB	M	8	6, 4	1, 3	Rel	320	−	−
AC	F	10	NT	NT	Rel	320	−	−
WB	F	23	6, 4	1, 3	Rel	320	−	NT
BW	M	11	3, 4	NT	Rel	320	+	+
HS	F	50	3, 4	NT	Rel	640	+	+
CB	F	6	4, 1	1, 3	Rel	2560	−	NT

NT, not tested.

selected and the ability of GAD 65 to block ICA reactivity was tested as previously described. 84% (21/25) of these long term ICA positive persons had their ICA specifically blocked by GAD, indicating the presence of ICA reactive to GAD.

Discussion

The ICA, first described in 1974, provided strong evidence for an autoimmune etiology and pathogenesis of IDD (33). The

Table II. Physical Characteristics and Autoantibody Analyses of Sera from Prediabetic Patients

Subject	Sex	Onset age	HLA DR	HLA DQ	Method of accrual	IAA	ICA JDF	ICA absorbed by GAD 65	ICA absorbed by GAD 67	Number of mo before onset
LB	M	21	3, 4	3	Sch	30 (30)	10 (5–10)	+	NT	90
BS	F	13	NT	NT	Rel	30 (5–30)	40 (40–80)	−	−	3
PT	F	36	2, 4	1, 3	Rel	(46 (40–46)	40 (40)	−	−	2
DN*	F	13	1, 3	1, 2	Sch	106 (−66–106)	40 (0–40)	+/−	NT/−	48/27
BR	F	10	4, −	1, 3	Sch	145 (127–145)	40 (5–40)	+	NT	4
RS*	M	42	3, 4	2, 3	Rel	84 (−17–84)	40 (40–80)	+/−	NT/−	44/20
JR	F	57	3, 4	2, 7	Rel	38 (38–76)	40 (5–80)	+	+	10
JT	F	7	4, 6	1, 3	Rel	332 (204–322)	80 (80)	−	−	10
JG	M	12	3, 3	2	Rel	33 (33–106)	80 (40–80)	−	−	2
JB	F	10	3, 4	2, 3	Rel	50 (−14–50)	80 (80)	−	−	15
SL	M	4	3, 4	2, 3	Rel	620 (420–620)	80 (20–80)	−	−	5
SA	F	11	4, 7	NT	Rel	256 (5–783)	80 (10–160)	−	−	18
JF	M	9	NT	NT	Rel	26 (26)	160 (160–640)	−	−	15
DC	F	16	2, 8	1, 3	Sch	782 (−21–782)	160 (0–160)	−	−	52
BY	M	8	NT	NT	Rel	353 (−19–353)	160 (160)	+	NT	51
RB	F	20	4, 6	1, 3	Sch	131 (13–81)	160 (40–160)	−	NT	80
DR*	F	14	3, 4	2, 3	Sch	329 (−5–502)	320 (160–640)	+/−	NT/−	42/36
BI	F	20	1, 3	NT	Rel	68 (−12–69)	320 (80–320)	−	NT	54

The onset values for IAA and ICA are listed. However, the earliest antibody samples were tested for GAD absorption, with time before onset listed as months. The range of values during observation period are designated within parentheses where appropriate. Abbreviations: Rel, relative of IDD patient; Sch, school population; NT, not tested. * Persons who have samples which converted from GAD-ICA to non-GAD-ICA activity.

Table III. Physical Characteristics and Autoantibody Analyses of Sera from Long-Term ICA-Positive Nondiabetic Individuals

Sub	Sex	Age	Yr ICA+	HLA DR	HLA DQ	Method of accrual	IAA	ICA JDF	ICA blocked by GAD 65	ICA blocked by GAD 67
CD	M	14	8	4, 8	3	Rel	43 (25–43)	10 (5–10)	+	NT
AK	F	16	8	4, 8	3	Rel	19 (−30–98)	20 (20–360)	−	−
CF	F	41	7	4, 7	3	Rel	5 (5–46)	40 (40–80)	−	−
KM	F	44	8	4, X	NT	Rel	25 (−25–58)	20 (20–40)	+	+
BD	M	16	9	3, 4	NT	Rel	102 (−4–224)	20 (0–80)	+	+
JS	F	19	7	1, 3	1, 2	Sch	416 (212–416)	20 (20)	+	NT
KO	M	45	9	3, 4	NT	Rel	0 (−29–26)	20 (10–20)	+	+
CB	M	20	7	4, 5	3	Sch	18 (−15–75)	20 (0–20)	+	NT
SG	F	15	7	3, 4	2, 3	Sch	58 (−61–105)	20 (10–20)	+	NT
KM	F	7	6	6, 7	NT	Rel	1241 (32–1776)	2 (0–20)	+	+
TH	M	15	6	3, 4	2, 3	Rel	82 (6–176)	40 (40–160)	−	−
KA	M	25	7	4, 2	1, 3	Rel	88 (77–265)	40 (40–160)	−	−
RS	F	36	11	3, X	NT	Rel	39 (−6–39)	40 (40–160)	−	−
RM	F	12	7	5, 6	1, 3	Sch	14 (−24–61)	40 (5–40)	−	−
MG	M	17	8	6, 4	1, 3	Sch	64 (61–545)	40 (10–40)	+	NT
AK	F	15	5	2, 4	1, 3	Rel	−55 (−55–0)	40 (0–40)	+	NT
RT	M	19	8	2, 4	1, 3	Sch	50 (28–69)	40 (20–40)	+	NT
HO	F	40	5	3, 8	NT	Rel	−27 (−27–1)	80 (20–80)	+	+
VW	F	47	13	2, 4	NT	Rel	98 (1–98)	80 (80–160)	+	+
DO	M	33	9	3, 4	NT	Add	7 (7–52)	80 (20–160)	+	+
DG	F	38	5	4, 9	3	Sch	46 (−5–58)	160 (20–160)	−	NT
VM	F	45	8	4, X	NT	Rel	851 (292–1042)	160 (160–1280)	−	−
DA	M	15	5	3, 4	2, 3	Sch	168 (12–169)	160 (10–320)	+	+
BB	F	45	10	3, 4	2, 3	Add	135 (135–541)	2560 (320–2560)	+	+

Current values are listed, with range of values during observation period designated within parentheses where appropriate. Abbreviations: Rel, relative of IDD patient; Sch, school population; Add, Addison's disease; NT, not tested.

assay procedure most used involves an indirect immunofluorescence test of cryofrozen human pancreas: sera containing ICA react with autoantigens usually presented within the cytoplasmic compartment of all endocrine cells of the pancreatic islet.

A review of the literature would suggest that the autoantigen to which ICA react has been identified, but only partially characterized. Specifically, Nayak and co-workers (18) suggested that the antigen of ICA was a sialic acid containing glycolipid. Whereas the glycolipid extract from whole pancreas is primarily composed of GM3 and GD3 gangliosides, islet cells are enriched in monosialogangliosides (including GM2). Interestingly, the suppression of pancreatic islet activity in experimental animals by transplantable islet tumors correlated with the loss of the pancreatic islet cell cytoplasmic antigen (34). This loss was demonstrated by a loss of a specific ganglioside which on thin layer chromatography migrated between GM2 and GM1 (34). Conversely, hyperexpression of GM2-1 gangliosides in nonobese diabetes islets has been reported (35), and the amount of this ganglioside has been shown to decrease significantly with progression to beta cell destruction.

The question as to the uniform sialoglycolipid nature of ICA has, however, needed to be readdressed owing to the recent findings that ICA associated with Stiff-Man syndrome, which is itself associated with IDD, are reactive to the protein enzyme GAD (20). Our studies clearly document that more than one antigen is involved in the ICA reactivity associated with IDD. One of these antigens appears to be the protein enzyme GAD; however, either isoform of this protein enzyme appears to be immunoreactive with ICA in that recombinant GAD 65 or GAD 67 can equally block the ICA reactivity associated with IDD. This observation suggests but is not limited to the proposal that at least one antigenic epitope of common autoantibody reactivity exists between the two GAD isoforms. As previously indicated, we could not reliably use the fluorescence staining pattern of islet cells to distinguish ICA reactivity to GAD vs. non-GAD antigen(s). Were the expression of GAD restricted to β cells (36), one might anticipate a β cell restricted pattern of ICA reactive to GAD. Future studies using dual staining techniques with anti-insulin, anti-glucagon, and anti-somatostatin antibodies may assist in distinguishing cellular staining patterns of the various types of ICA.

It is important to emphasize that, by this study design, this assay has the ability to measure which form of ICA is predominant. It cannot in all cases distinguish whether or not both forms of ICA coexist within a given serum sample. Indeed, although GAD completely removed ICA in many individuals, only a partial yet specific reduction was observed in others. This suggests that ICA reactive to a non-GAD antigen(s) can coexist in a single serum. We base this statement on the observation that many high-titered samples required dilution to near end-point titer in order to observe the effect of GAD blocking. In those patients, the addition of GADs to serum samples at concentrations that saturate the binding ability of GAD mAb and polyclonal antibodies had no measurable effect of reducing

islet cell fluorescence. We believe that the immunofluorescence remaining at these dilutions represents a non–GAD-reactive ICA response. Once the non–GAD-reactive ICA antigen(s) is readily available, we will attempt to further address this issue. A second limitation of our study involves the use of recombinant GADs expressed in *E. coli,* where completely native protein folding would not be anticipated. Since many antigenic epitopes are thought to be conformational dependent, some potential autoantibody reactivity may have not been observed with the GADs used in this study. However, similar fluorescence observations were observed with absorption with human cerebellum containing GAD. We are attempting to produce GADs in mammalian systems, which should provide native protein folding, in order to alleviate this concern.

In that autoimmunity formation is thought to be an antigen-driven process, the persistence of ICA reactive to GAD long after diagnosis of disease is consistent with the loss of the non–GAD-reactive ICA antigen with complete β cell ablation, and the continued availability of GAD to the immune system because of the presence of GAD antigen in tissues other than β cells (36, 37). The recent study by Rowley et al. (38) also observed anti–GAD immunity in persons with long duration of IDD, however no relationship to ICA reactivity to GAD was analyzed in that study.

A recent study (21) has analyzed the ICA primarily associated with IDD and reported that ICA represent a diverse group of autoantibodies defined by the pattern of islet cells stained and the species of pancreatic substrate used for autoantibody detection. The authors concluded that one form of ICA exists which is β cell specific, and they termed these antibodies "restricted ICA." They also reported that restricted ICA actually identify a subpopulation of individuals with a lower rate of progression to IDD than that expected for ICA positive persons. We believe that so-termed restricted ICA most likely represent our GAD reactive ICA. Whereas our long-term, ICA-positive but nondiabetic patients did have a significantly higher frequency of ICA reactive to GAD than the other groups, we believe the sum of our data support a broader hypothesis. Our natural history studies of individuals who developed IDD showed the early appearance of a GAD-specific ICA, which was later replaced by non–GAD-specific ICA closer to clinical onset.

These data suggest that GAD autoimmunity may represent a more primary autoimmune response which in many but not all cases leads to significant pancreatic β cell injury possibly through cellular immune mechanisms. In fact, we have previously shown (39) evidence of lymphocyte directed immunity toward GAD. Once β cell damage has become established, secondary autoimmune responses may occur involving constituents other than GAD released by damaged β cells. Thus non–GAD-reactive ICA may provide a marker for the advanced lesion which would have a more prognostic significance for IDD, whereas ICA reactive to GAD may typify the early or inductive lesion that may or may not progress to clinically significant β cell injury. Further studies are planned to address this important possibility.

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