Autoimmunity to Two Forms of Glutamate Decarboxylase in Insulin-dependent Diabetes Mellitus

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Abstract

Insulin-dependent diabetes mellitus (IDDM) is thought to result from the autoimmune destruction of the insulin-producing β cells of the pancreas. Years before IDDM symptoms appear, we can detect autoantibodies to one or both forms of glutamate decarboxylase (GAD_{65} and GAD_{67}), synthesized from their respective cDNAs in a bacterial expression system. Individual IDDM sera show distinctive profiles of epitope recognition, suggesting different humoral immune responses. Although the level of GAD autoantibodies generally decline after IDDM onset, patients with IDDM-associated neuropathies have high levels of antibodies to GAD, years after the appearance of clinical IDDM. We note a striking sequence similarity between the two GADs and Coxsackievirus, a virus that has been associated with IDDM both in humans and in experimental animals. This similarity suggests that molecular mimicry may play a role in the pathogenesis of IDDM. (*J. Clin. Invest.* 1992. 89:283–292.) Key words: insulin-dependent diabetes mellitus • glutamate decarboxylase • diabetic neuropathy

Introduction

Insulin-dependent diabetes (IDDM;[1] type I diabetes) is one of the most serious and common of metabolic disorders, affecting approximately 1 person in 300 in the U.S., while epidemiological studies in Europe suggest that its incidence is increasing (reviewed in 1–3). The disease is thought to result from the autoimmune destruction of the insulin-producing β cells of the pancreas and the subsequent metabolic derangements. Al-

1. *Abbreviations used in this paper:* GAD, glutamate decarboxylase; ICA, islet cell antibodies; IDDM, insulin-dependent diabetes mellitus; JDF, Juvenile Diabetes Foundation; NIDDM, non-IDDM; NOD, nonobese diabetic; PAS, protein A-Sepharose; PCR, polymerase chain reaction.

though insulin therapy allows most patients to lead active lives, this replacement is imperfect since it does not restore normal metabolic homeostasis. Metabolic abnormalities are thought to be important in the subsequent development of common complications, which include retinopathy, cataract formation, nephropathy, neuropathy, and heart disease.

While the initiating agent of IDDM autoimmunity is not known, it ultimately provokes a loss of immunological tolerance to self-antigens present in insulin-secreting β cells within the pancreatic islets (4–6). IDDM begins with an asymptomatic stage, characterized by a chronic inflammatory infiltrate of the islets (insulitis), which selectively destroys the β cells. Only after the destruction of the majority of the β cells, often occurring over several years, do hyperglycemia and ketosis appear.

The pathogenesis of IDDM involves both genetic and environmental factors. One or more susceptibility factors are encoded by the major histocompatibility complex on chromosome 6, probably by the DQ A1 and B1 loci (7, 8). Studies of monozygotic twins, however, show a concordance for IDDM of < 40%, suggesting that environmental factors play an important role (9). Long suspected environmental causes of IDDM include a number of viruses, such as rubella, encephalomyocarditis virus, and especially Coxsackie virus B_4 (reviewed in 10–12).

Autoantibodies to a 64,000 M_r islet cell protein are associated with IDDM and have been detected years before the onset of symptoms (13–15). Other IDDM-associated autoantibodies, such as those against insulin and cytoplasmic gangliosides of islet cells (ICA), appear later, possibly as a consequence of the release of these antigens (or their precursors) from the damaged islet cells (16, 17). Antibodies to the 64,000 M_r proteins are, however, the earliest and most reliable predictive marker of IDDM in humans and are also present in the two animal models for IDDM, the nonobese diabetic (NOD) mouse and the Biobreeding rat (14, 15, 18, 19).

Baekkeskov et al. (20) reported that the 64,000 M_r islet cell autoantigen is a form of glutamate decarboxylase (GAD; E.C. 4.1.1.15), the enzyme responsible for the synthesis of γ-aminobutyric acid (GABA) in brain, peripheral neurons, pancreas, and other organs (21). We have recently shown that the brain contains two forms of GAD, which are encoded by two separate genes (22). The two GADs (GAD_{65} and GAD_{67}) differ in molecular size (with M_rs = 65,000 and 67,000) and amino acid sequence (with ~ 30% sequence divergence), as well as in their intracellular distributions and interactions with the GAD cofactor pyridoxal phosphate (22–25). In brain neurons, GAD_{65} is preferentially associated with axon terminals, while GAD_{67} is present in both terminals and cell bodies (25).

Previous studies of the 64,000 M_r IDDM autoantigen have used pancreatic extracts enriched for membrane-associated

proteins. In view of our demonstration that the brain contains two GADs, we set out to determine the molecular identity of islet cell GAD by immunohistochemistry with monospecific antibodies. We then used GAD_{65} and GAD_{67} produced in genetically engineered bacteria from our GAD cDNAs to examine the specificity of IDDM autoantibodies for the two GADs and for restricted sets of GAD epitopes.

Our results lead to two new suggestions concerning the pathogenesis of IDDM and its complications: (a) GAD autoimmunity may play a role in the pathogenesis of IDDM-associated neuropathies; and (b) IDDM autoimmunity may result from molecular mimicry of GAD and a Coxsackievirus peptide.

Methods

Patient sera. IDDM patients and individuals at high risk for later developing IDDM were selected from a previous study at the University of Florida Diabetes Clinics (15, 26). IDDM patients with peripheral neuropathies were selected from the University of Florida Diabetes Clinics and the UCLA Diabetes Clinic.

Nondiabetic controls and the individuals studied before the documented clinical onset were ascertained through ongoing prospective screening for islet cell antibodies of more than 5,000 first-degree relatives of IDDM probands, and 8,200 individuals from the general population, of whom 4,813 were school children. These studies were approved by the University of Florida's Institutional Review Board. All participating individuals first gave their written informed consent. Individuals at high risk for the development of IDDM were identified by the presence of high titers of ICAs, assayed by indirect immunofluorescence on cryostat sections of blood group O human pancreas. All results were interpreted on coded samples, with control negative and positive sera in each batch. The ICA levels were estimated as Juvenile Diabetes Foundation units, according to the standardization guidelines established by the Immunology Diabetes Workshop (IDW), as previously described. M. Atkinson and N. Maclaren subscribe to the IDW's ICA proficiency testing program, which they currently supervise.

GAD assays. Patient sera were assayed blind for their ability to bind GAD enzymatic activity from a cleared homogenate of human cerebellar cortex in "GAD buffer," which contained 60 mM potassium phosphate, pH 7.1, 0.5% Triton X-100, 1 mM PMSF, 1 mM 2-aminoethyl-isothiouronium bromide, and 0.1 mM pyridoxal phosphate. IgG from each serum was bound to protein A-Sepharose (PAS) by adding 40 μl of serum to 80 μl of a 1:1 slurry of preswollen PAS in GAD buffer, incubating for 30 min at 4°C with gentle rocking, isolated by centrifugation, and then washing four times in the same buffer. 100 μl of brain extract was then added to each sample and incubated for 1 h at 4°C with gentle rocking, washed four times, resuspended in buffer, and assayed for GAD activity as previously described (25). Values shown are means of three determinations.

Immunohistochemistry. Immunochemical detection of the two forms of GAD was performed as previously described for rat cerebellum (25).

Antigen preparation and immunoadsorption. Rat GAD_{65} and GAD_{67} cDNAs were subcloned in the NcoI site of pET 8C and the Nhe I site of pET-5C respectively and transformed into *Escherichia coli* BL21 (DE3) (20, 27). Control and GAD-producing *E. coli* were grown and induced with isopropyl-thio-β-D-galactoside, harvested by centrifugation, resuspended in GAD buffer, sonicated, and cleared by centrifugation at 55,000 *g* for 15 min. For immunocompetition, 30 μl of each patient serum was incubated with 100 μl of extract from control bacteria or from bacteria that produced either GAD_{65} or GAD_{67} for 1 h at 4°C. Human pancreatic islets were labeled with ^{35}S-methionine as pre-

viously described (15). A detergent extract (300 μl) was first precleared with human control serum. The material that bound to the control IgG was removed with protein A-Sepharose. The precleared islet cell detergent extract was then split into three fractions and then incubated (2 h on ice) with serum that had been absorbed with each of the *E. coli* lysates. IgG-bound material was isolated with protein A-Sepharose as described above, and the bound material was analyzed by polyacrylamide gel electrophoresis in SDS (SDS-PAGE), followed by fluorography.

Detection of GAD autoantibodies. E. coli expressing rat GAD_{65} and GAD_{67} cDNAs were grown in minimal medium and induced with isopropyl-thio-β-D-galactoside in the presence of a mixture of ^{35}S-labeled amino acids (Tran-^{35}S; ICN Pharmaceuticals, Inc., Irvine, CA). The bacteria were harvested, sonicated in GAD buffer, and centrifuged to remove debris. Sera were preadsorbed with extracts of unlabeled host bacteria and then added to a mixture of ^{35}S-labeled extracts of GAD_{65}- and GAD_{67}-producing bacteria. IgG-bound polypeptides were isolated with PAS and analyzed by SDS-PAGE. Initial experiments analyzed sera for their ability to precipitate GAD_{65} and GAD_{67} separately (data not shown). Using a mixture of the two extracts simplified the assay. A number of *E. coli* polypeptides were also immunoadsorbed by some patient and some control sera. One such band, with $M_r \sim 70,000$, is apparent in many samples.

Epitope mapping. Portions of GAD_{65} cDNA were amplified by the polymerase chain reaction (PCR; 28) to produce DNA segments encoding three polypeptide segments: amino acid residues 1–224 (segment A); 224–398 (segment B); and 398–585 (segment C). Each construct also contained a T_7 promoter, a consensus sequence for the initiation of translation and an initiating methionine codon (29). Each PCR product was then transcribed in vitro with T_7 RNA polymerase and translated in vitro in a rabbit reticulocyte cell-free system in the presence of ^{35}S-methionine, using conditions recommended by the supplier (Amersham Corp., Arlington Heights, IL). Each test serum (30 μl) was incubated with the resulting ^{35}S-polypeptides. The bound peptides were isolated with PAS and analyzed by SDS-PAGE in 15% polyacrylamide and fluorography.
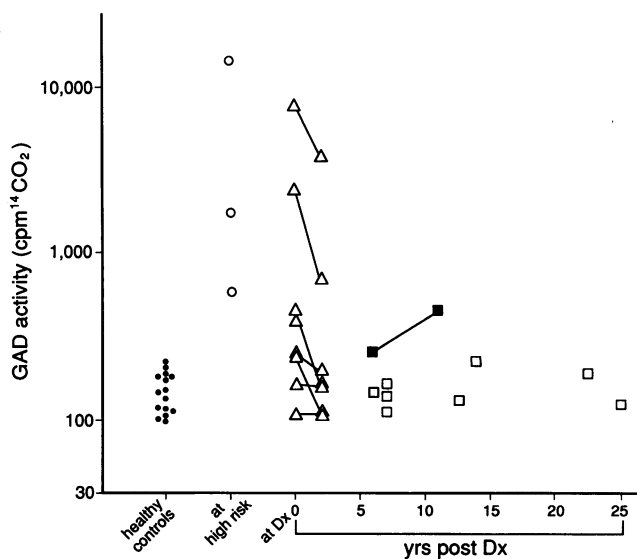

Figure 1. Immunoprecipitation of GAD activity by IDDM sera. GAD activity in brain extracts was immunoprecipitated with sera from healthy controls (●); individuals at high risk for IDDM (o); IDDM patients at diagnosis and two years later (△); and unrelated patients more than six years after diagnosis (□); one patient (■) developed a sensory neuropathy.

Results

IDDM patients have autoantibodies to GAD. We initially performed a blind trial to test for the presence of GAD autoantibodies in IDDM sera. We tested IDDM sera for the presence of GAD autoantibodies by assaying their ability to immunoprecipitate GAD activity from human brain homogenates (Fig. 1). We included sera from 35 individuals, which included 3 people judged to be at high risk for IDDM on the basis of their previously determined ICA titers, reduced responses to intravenous glucose, and their HLA DR/DQ haplotypes (15, 26), 8 IDDM patients studied at onset and 7 of these same patients two years later, 9 unrelated patients six or more years after IDDM onset, and 15 normal controls. Our results parallel those independently reported by Baekkeskov et al. (20).

The three high-risk individuals whose sera we examined had high anti-GAD titers, in one case comparable to those raised against purified brain GAD in experimental animals (data not shown). The levels of antibodies to GAD in five of eight newly diagnosed patients exceeded the mean + 1 SD of the control sera. Levels in these patients decreased by $\sim$ 50% during the subsequent two years, with only two of seven sera having levels more than the mean + 1 SD of the control sera. In most patients $\geq$ 6 years after diagnosis, the concentrations of antibodies to GAD were indistinguishable from controls. In one patient in this series, however, anti-GAD levels actually rose between 6 and 11 years after onset, during which time the patient developed a sensory neuropathy.

Levels of anti-GAD antibodies in these patients generally parallel the previously determined titers of autoantibodies to the 64,000 M_r antigen. Our assays of immunoprecipitated GAD enzymatic activity easily identified individuals with high titers of autoantibodies to the 64,000 M_r antigen, but did not often distinguish individuals with low titers from controls.

This study established that autoantibodies to GAD are present at and before the clinical diagnosis of IDDM and decline within a few years after diagnosis. We next addressed the question of the molecular identity of the GAD autoantigen.

Islet cells contain both GAD$_{65}$ and GAD$_{67}$. Immunohistochemical experiments with the GAD-6 monoclonal antibody, which recognizes only GAD$_{65}$, show the presence of GAD$_{65}$ in pancreatic islets (Fig. 2; references 20, 25, 30). Using our recently described K-2 antiserum, which recognizes only GAD$_{67}$, we show that islet cells also contain GAD$_{67}$ (Fig. 2; reference 25). Since both GAD$_{65}$ and GAD$_{67}$ are present in islets, either or both could be the autoantigen recognized by the IDDM sera surveyed in Fig. 1 and by Baekkeskov et al. (20).

The 64,000 M_r islet cell autoantigen is GAD$_{65}$. To define further the molecular identity of the IDDM autoantigen, we performed two sets of experiments. In the first experiment we used GAD-6 (the GAD$_{65}$-specific monoclonal antibody) to immunoadsorb GAD$_{65}$ both from detergent extracts of ^{35}S-labeled islet cells and from soluble extracts of ^{35}S-labeled GAD-producing bacteria. GAD-6 specifically recognized a 65,000 M_r immunoreactive polypeptide in both islet cells and GAD$_{65}$-producing bacteria with identical electrophoretic mobilities, which were distinct from bacterially produced GAD$_{67}$. Prior immunoadsorption with an IDDM serum removes immunoreactive GAD$_{65}$ (i.e., "64K") from both islet cell and bacterial extracts (data not shown).

In the second set of experiments, we examined the ability of

bacterially produced GAD$_{65}$ and GAD$_{67}$ to compete with the immunoadsorption of islet cell autoantigens by IDDM sera. Sera taken from two patients (patient 052 and 496 which recognize both GADs; see Table I) specifically precipitate a polypeptide of M_r 64–65,000 from detergent-phase extracts of ^{35}S-labeled islets in the presence of extracts of host bacteria (i.e., bacteria not engineered to produce GAD), containing 400 μg of protein (Fig. 3, lanes *1* and *2*). When we added extracts (also containing 400 μg of total protein) of genetically engineered bacteria that produce either GAD$_{65}$ or GAD$_{67}$, we found that an extract containing 100 μg of GAD$_{67}$ partially blocked the binding of the islet cell antigen, as would be expected if GAD$_{67}$ adsorbs some of the antibodies that recognize epitopes common to GAD$_{65}$ and GAD$_{67}$ (Fig. 3, lanes *5* and *6*). In contrast, an extract containing only 10 μg of GAD$_{65}$ completely blocked immunoadsorption of the 64K autoantigen (Fig. 3, lanes *9* and *10*). These data show that the previously identified 64,000 M_r autoantigen is immunologically indistinguishable from GAD$_{65}$. A serum (patient 476) that predominantly recognizes GAD$_{67}$ (which does not partition into the detergent phase of the islet cell extracts used in these studies) precipitated a very faint 64K band. The healthy control serum did not precipitate a 64K antigen.

IDDM sera differ in the recognition of GAD$_{65}$ and GAD$_{67}$. Antisera raised in experimental animals against purified brain GAD vary in their recognition of GAD$_{65}$ and GAD$_{67}$. With this in mind, we determined the specificity of individual IDDM sera for each species of GAD. We examined their ability to immunoprecipitate ^{35}S-labeled GAD$_{65}$ and GAD$_{67}$, produced from GAD cDNAs in a bacterial expression system. We examined sera from 59 individuals (to whose IDDM status we were blind), including 8 people at high risk for IDDM, 12 people who later (3–64 months) developed IDDM, 3 newly diagnosed IDDM patients, 12 patients 2–22 years after onset who had no neurological symptoms, and 9 patients 10–48 years after onset who developed sensory or autonomic neuropathies (Table I; Fig. 4). None of the control sera from 15 healthy individuals had detectable ICA, antibodies to the 64,000 M_r pancreatic antigen, or antibodies to either form of GAD.

Levels of GAD autoantibodies were generally highest in the sera of individuals who were likely to have been in the process of developing the disease; those who were known to develop IDDM some time after their sera were drawn and those thought to be at high risk for IDDM on the basis of their previously determined ICA levels and autoantibodies to 64K. Levels were much lower in the sera of patients examined a few years after IDDM onset. None of the nine patients without neuropathies tested long after onset ($\geq$ 5 years) had detectable antibodies to GAD. The intensity of the ^{35}S-labeled GAD$_{65}$ immunoprecipitated by the IDDM sera generally paralleled the previously determined titers of autoantibodies to the 64,000 M_r islet cell polypeptide (15, 26), again supporting the latter's identification as GAD$_{65}$. There was no obvious correlation between ICA titers and the levels of autoantibodies to either GAD form.

Among IDDM sera, the ability to precipitate each of the two GADs varied among individuals. Of the 23 individuals tested whom we thought to be at early stages of IDDM (8 at high risk, 12 tested before subsequent onset, and 3 newly diagnosed), 15 recognized both GADs, 3 recognized only GAD$_{65}$, and 4 recognized only GAD$_{67}$. We found no obvious correlation between the time before or after diagnosis and the specific-
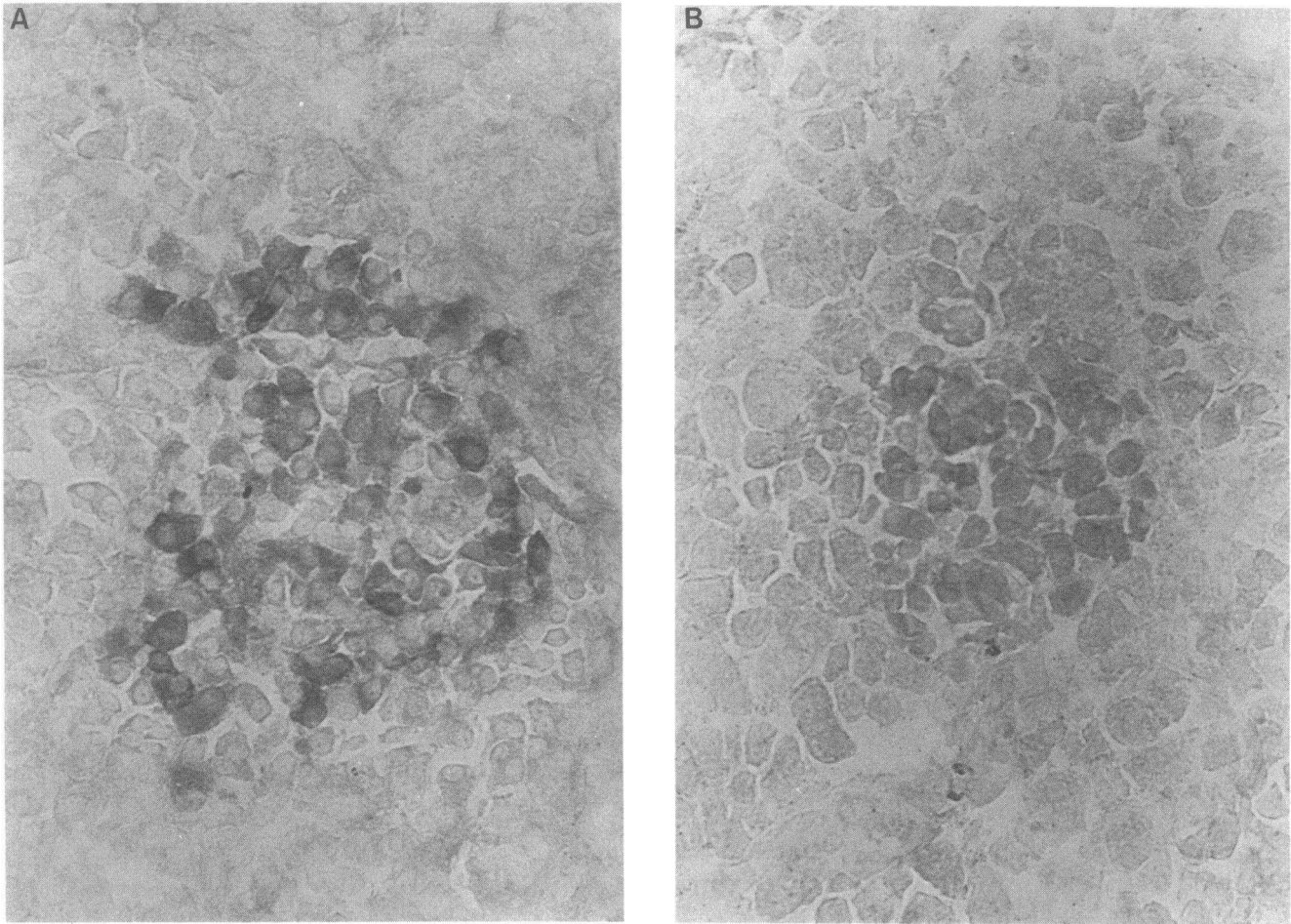
Figure 2. Pancreatic islets contain both GAD_{65} and GAD_{67}. (*A*) Immunohistochemistry with the GAD-6 monoclonal antibody specific for GAD_{65}, and (*B*) with the K-2 antiserum specific for GAD_{67}.

ity of autoantibodies for either form of GAD. A more sensitive assay (for example, one using human rather than rat GADs) might in fact reveal antibodies to both forms of GAD.

Altogether 78% (18/23) of sera from early stage IDDM individuals recognized GAD_{65}, a frequency similar to that reported in previous studies of 64,000 M_r autoantibodies (reviewed in 15, 20). When we tested for both GADs, however, we could detect autoantibodies to either or both in 96% (22/23) of the early stage individuals tested.

The sera of NOD mice also show immunoreactivity both to GAD_{65} and to GAD_{67} (Fig. 4, lane *22*). This finding further underscores the similarity of the disease processes in human IDDM and in NOD mice.

Individual sera vary in epitope recognition. To examine the individual variability in epitope recognition of IDDM autoantibodies, we determined the ability of sera from four individuals to recognize three polypeptide segments of GAD_{65} (Fig. 5). Each of these individuals was at a different stage in the progression of the disease: 052 (high risk), 723 (a patient who subsequently developed IDDM), 705 (at diagnosis), and UC2 (advanced neuropathy). We used PCR amplification followed by in vitro transcription and translation of the PCR products to produce ^{35}S-labeled polypeptides that represented the amino-terminal (A), middle (B), and carboxy-terminal (C) thirds of

GAD_{65}. None of the four sera reacted with the segment A, two (052 and UC2) reacted with segments B and C, one (705) with the carboxy-terminal segment C only, and one (723) with none of the GAD_{65} segments. Our inability to immunoprecipitate polypeptides with serum 723 (which, as shown in Fig. 4, does precipitate both GAD_{65} and GAD_{67} as intact molecules) may have resulted from a lack of sensitivity of the assay or from the inability of any of the utilized peptides to fold into the recognized epitope. While the three peptides that we investigated are unlikely to have formed all their native epitopes, our epitope mapping data, like our studies of the differential recognition of GAD_{65} and GAD_{67}, suggest that each of the tested sera has a distinctive profile of anti-GAD antibodies. Although IDDM autoantibodies recognize different GAD epitopes, we do not know which epitopes are recognized by the self-reactive T lymphocytes, which contribute to both humoral and cellular auto-immunity.

Persistent autoimmunity to GAD is often associated with peripheral and autonomic neuropathy. The occurrence of antibodies to GAD (and to the previously determined 64,000 M_r antigen), is unusual in patients many years after onset (Fig. 1, Table I, and M. Atkinson, unpublished data). Autoantibodies to GAD were, however, present in 8 of 9 IDDM patients with sensory or autonomic neuropathies, long (10–41 years) after
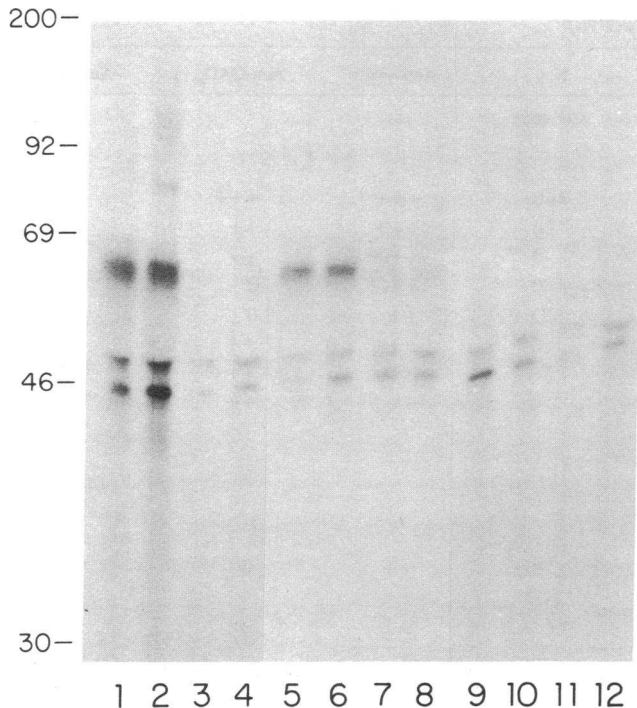
Figure 3. The 64,000 M_r autoantigen is GAD$_{65}$. The ability of sera which recognize both GADs (patients 052 and 496) to bind the previously described 64,000 M_r islet cell autoantigen was not blocked by preadsorption with an extract of wild-type BL21 (DE3) E. coli. Preadsorption with an extract of GAD$_{67}$-producing bacteria produced only partial blocking of these sera's ability to bind the pancreatic antigen. In contrast, preadsorption with extracts of GAD$_{65}$-producing bacteria, abolished the serum's ability to bind 64K antigen. Lanes 1–4 preadsorbed with 400 μg of a wild-type E. coli extract. Lanes 5–8 preadsorbed with a 400-μg extract containing 100 μg of GAD$_{67}$. Lanes 9–12 preadsorbed with a 400-μg extract containing 10 μg of GAD$_{65}$. Lanes 1, 5, 9; patient 052. Lanes 2, 6, 10; patient 496. Lanes 3, 7, 11; healthy control. Lanes 4, 8, 12; patient 476, whose serum predominantly recognizes GAD$_{67}$ (Table I), does bind the 64K antigen very weakly which is not apparent in the photograph.

the onset of diabetic symptoms (Table I; Fig. 4, lanes 18–21). Six of the sera examined had detectable levels of autoantibodies to both GAD$_{65}$ and GAD$_{67}$, while two had detectable autoantibodies only to GAD$_{67}$. Two patients with rapidly progressing autonomic neuropathies (UC1 and UC2) had especially high levels of autoantibodies to GAD. In contrast, none of the nine patients who were free of IDDM-associated complications examined at or more than five years after onset had detectable antibodies to GAD. The GAD autoantibodies in neuropathy patients may result from the restimulation of the immune system by GAD released from damaged neurons, or they may be involved in the actual pathogenesis of this complication. In either case, GAD autoantibodies may serve as a useful marker of an ongoing degenerative process.

Sequence similarities between GAD and Coxsackievirus. Although we observe high levels of autoantibodies to GAD before IDDM onset, their presence may merely reflect an immune reaction to the exposure of previously sequestered antigens following β cell damage. Indeed, the initiating agent of the autoimmune response in IDDM is completely unknown, though the increasing incidence of IDDM and its frequent discordance in monozygotic twins has led to the suggestion that an environmental agent triggers autoimmunity (31, reviewed in 3, 12). In other autoimmune diseases, pathogenesis is thought to involve "molecular mimicry," in which a bacterial or viral antigen triggers an immune response that then reacts with a similar self antigen (reviewed 4, 32, 33).

Analysis of the deduced amino acid sequences of GAD$_{65}$ and GAD$_{67}$ shows an extensive and surprising sequence similarity to the P2-C protein of Coxsackievirus B$_4$. Coxsackievirus B$_4$ is a picornavirus with a worldwide distribution. It causes a mild upper respiratory infection and can also infect β cells (reviewed in 10–12). It has a small genome (7,395 bases), and its P2-C protein appears to contribute to the membrane-bound replication complex (34). A core polypeptide segment of six amino acid residues is identical in sequence between GAD$_{65}$ and P2-C (Fig. 6; 22, 34). The immediately adjacent polypeptide segments also share a high level of similarity both in sequence and
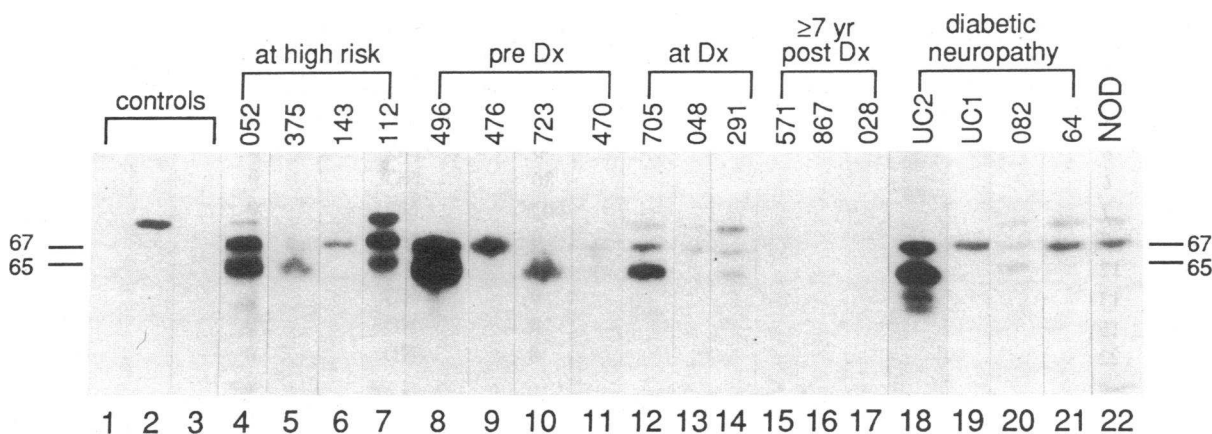

Figure 4. Detection of autoantibodies against GAD$_{65}$ and GAD$_{67}$ in IDDM sera. Sera were incubated with a mixture of ^{35}S-labeled lysates of GAD$_{65}$- and GAD$_{67}$-producing E. coli, and IgG-bound polypeptides were analyzed by SDS-PAGE. The composite photo shows representative data from controls and from individuals at different stages of IDDM: three controls (lanes 1–3), four people at high risk for IDDM (lanes 4–7), four who later developed IDDM (lanes 8–11), three patients at diagnosis (lanes 12–14), three IDDM patients more than seven years after diagnosis (lanes 15–17), four IDDM patients with neuropathies (lanes 18–21), and NOD mice (lane 22).

Table I. Analysis of Characterized Sera for GAD₆₅ and GAD₆₇ Immunoreactivity

Patient ID					ICA	Anti-64K	Anti-GAD$_{65}$	Anti-GAD$_{67}$
					JDF units			
Individuals at high risk for IDDM								
052					160	+++	+++	+++
825					20	+++	++	0
375					0	+++	++	+
143					40	++	0	++
692					ND	++	0	+++
356					160	+++	++	+++
112					80	ND	+++	+++
410					0	++	++	++
Individuals who later developed IDDM								
	Months before IDDM diagnosis							
624	12				20	++	0	++
UF1	4				ND	+	+	++
584	24				0	++	+	0
035	64				40	+	+	0
496	6				160	+++	+++	+++
171	3				40	+	0	+
470	13				0	+++	+	+
055	8				40	+	0	0
438	42				320	++	+	+
840	9				0	+	+	+
723	14				ND	+++	+++	+
476	11				ND	+	+	+++
At onset of clinical symptoms								
048					320	+++	+	++
705					160	+++	+++	++
291					0	+++	++	++
IDDM patients without neuropathies								
	Years after diagnosis							
147	2				ND	+	0	0
476	3				0	ND	0	+
604	3				0	ND	+	0
113	5				160	ND	0	0
238	6				0	ND	0	0
997	6				80	ND	0	0
867	7				ND	ND	0	0
382	7				0	ND	0	0
052	12				0	ND	0	0
571	13				0	ND	0	0
M31	15				0	ND	0	0
025	22				0	ND	0	0
IDDM patients with neuropathies								
	Years after diagnosis	Autonomic neuropathy	Peripheral neuropathy					
UC1	35	+	+		0	ND	0	++
UC2	10	+	+		0	ND	+++	+++
UC3	13	+	+		0	ND	+	+
082	11	+	−		0	+	+	+

Table I. (Continued)

Patient ID				ICA	Anti-64K	Anti-GAD$_{65}$	Anti-GAD$_{67}$
038	21	+	+	ND	ND	+++	+++
344	33	−	+	0	ND	0	+
194	41	−	+	0	ND	+	+
310	48	−	+	0	ND	0	0
64	29	−	+	0	ND	+	++

Patient sera were obtained and assayed for ICA and autoantibodies to the 64,000 M_r protein as part of a previous study (15, 26) or from the UCLA Diabetes Clinic. Patients that are part of the University of Florida's database are identified by three digit numbers. Other patients are identified by sequential numbers, with UF numbers representing patients seen in Gainesville and UC patients seen in Los Angeles. ICA titers are expressed in JDF units. +++, high titers; ++, intermediate; +, detectable; ND, not determined. Patients UC1, UC2, and UC3 had rapidly progressing sympathetic neuropathies. None of the sera from 15 healthy controls had detectable ICA, antibodies to the 64,000 M_r protein, or antibodies to either form of GAD.

in the positions of charged residues. In the 24 residue segments of GAD$_{65}$ and P2-C that are illustrated in Fig. 6, 19 residues are either identical or conservative differences. The three peptides shown in Fig. 6 have nearly identical hydrophobicity profiles (data not shown). The high charge density and the presence of a proline residue in the shared core suggest that the segments are highly antigenic. No other significant similarities were found between GAD and other viruses implicated in IDDM, such as rubella, mumps, encephalomyocarditis virus, and cytomegalovirus. A generally similar sequence similarity is also present in the P2-C region of other members of the Coxsackievirus family. If specific members of the Coxsackievirus family (such as B$_4$ and B$_5$) are indeed involved in the etiology of IDDM, their pathogenicity may involve factors such as their particular amino acid sequences, virulence, and cell trophism, as well as the host immune repertoire.

Discussion

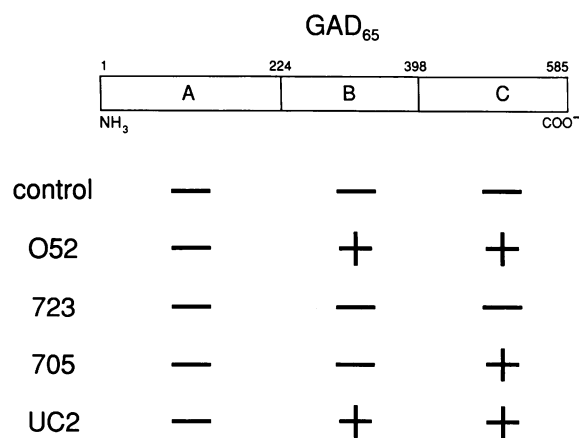In a blind clinical study, we tested IDDM sera for the presence of GAD autoantibodies by their ability to immunoprecipitate

GAD$_{65}$

Figure 5. Epitope mapping of GAD$_{65}$. Three labeled segments containing the amino-terminal (A), middle (B), and carboxy-terminal (C) portions of GAD$_{65}$ were immunoprecipitated with four IDDM sera that were initially characterized in the experiment shown in Fig. 4.

GAD enzymatic activity from brain homogenates. We found the highest levels of GAD autoantibodies in individuals at high risk for IDDM and in newly diagnosed IDDM patients. Levels of GAD autoantibodies decreased by ∼ 50% within two years after diagnosis. Six years after IDDM onset, the patients whose sera we examined had GAD autoantibody levels indistinguishable from controls. One patient, however, displayed increased GAD antibodies years after onset, during which time the patient developed a sensory neuropathy.

Our studies of GADs in the brain have shown that neurons express two forms of GAD, which derive from separate genes (22). Pancreatic β cells also express GAD and use GABA to regulate glucagon secretion by α cells (35). Our immunohistochemical data, using antibodies monospecific for GAD$_{65}$ and GAD$_{67}$, show that β cells, like most GABA neurons, contain both GAD$_{65}$ and GAD$_{67}$. Although our enzymatic studies, and those of Baekkeskov et al. (20), demonstrated GAD autoimmunity in IDDM, they did not distinguish the two forms of GAD.

We used GAD$_{65}$ and GAD$_{67}$ cDNAs to express large amounts of each GAD in a bacterial expression system and tested the ability of each form to compete with the immunoadsorption of the 64,000 M_r autoantigen from ^{35}S-labeled islet cells. Only GAD$_{65}$-containing lysates effectively competed, suggesting that the 64,000 M_r autoantigen corresponds to GAD$_{65}$.

The islet cell homogenates previously used to characterize IDDM autoantigens were enriched for membrane-associated molecules and may preferentially have included GAD$_{65}$. In contrast, both our studies of the soluble fraction and those of Christie et al. (36) show a complex pattern of antigens recognized by IDDM autoantibodies (data not shown). Since islet cells contain both GAD$_{65}$ and GAD$_{67}$, (Fig. 2) we sought to characterize the GAD autoantibodies by testing the ability of IDDM sera to recognize bacterially produced GAD$_{65}$ and GAD$_{67}$.

We could detect autoantibodies to either GAD$_{65}$ or GAD$_{67}$ or both in almost all people who later developed IDDM, in some cases years before the onset of clinical symptoms. Of 23 early stage IDDM individuals tested, we found antibodies to both GADs in 15, to GAD$_{65}$ alone in 3, and to GAD$_{67}$ alone in 4. By testing for antibodies to both forms of GAD we were able to detect GAD antibodies in 96% of the individuals tested.

HUMAN GAD 67   258 S I M A A R Y K Y F P E V K T K G M A A V P K L 281

HUMAN GAD 65   250 A M M I A R F K M F P E V K E K G M A A L P R L 273

COXSACKIEVIRUS   28 F I E W L K V K I L P E V K E K H E F – L S R L 50
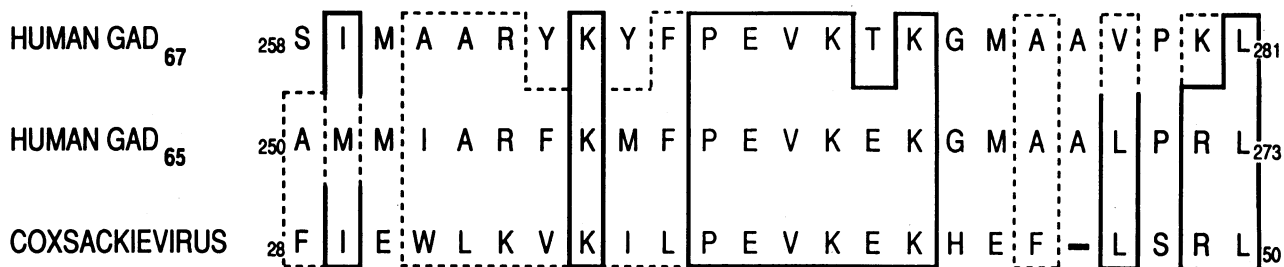
Figure 6. GAD and Coxsackievirus P2-C share common sequences. Solid line encloses identical amino acid residues. Dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity. Numbers refer to the amino acid residues in GAD_{65}, GAD_{67}, and Coxsackievirus protein P2-C. The human GAD amino acid sequences, which are almost identical to the rat GAD sequences, were determined by Bu Dingfang et al. (manuscript submitted for publication).

Levels of GAD autoantibodies were usually highest before IDDM onset and, in our patient sample, appeared as early as five years before onset of symptoms. GAD antibody levels declined after onset, presumably in parallel with the loss of GAD-containing β cells and the extinction of antigen-driven autoimmunity.

Patients showed varying immunoreactivity to GAD_{65} and GAD_{67}, which share ~ 70% amino acid similarity and are most divergent at their amino termini (22). They also varied in their ability to recognize individual polypeptide segments of GAD_{65}. These data suggest a diverse B lymphocyte response to different epitopes of GAD. While not all IDDM sera recognize the GAD_{65} polypeptide that contains the sequence shared with Coxsackievirus (segment B in Fig. 5), the antibodies may recognize GAD epitopes different from those that originally activated T lymphocytes.

Because our initial survey of IDDM patients detected increased levels of GAD autoantibodies in a patient who developed a sensory neuropathy long after the onset of diabetes itself, we further studied GAD autoimmunity in patients with IDDM-associated neuropathies. We found that 8/9 patients who had developed clinical IDDM symptoms 10–41 years earlier, showed significant levels of autoantibodies to GAD_{65}, GAD_{67}, or both. Of the eight patients in this group for whom we had ICA data, none had detectable ICA, and their low basal C-peptide did not respond to intravenous glucagon, suggesting that the continued high levels of anti-GAD autoantibodies did not result from the persistence of GAD-containing β cells.

The production of anti-GAD autoantibodies in patients with diabetic neuropathy may reflect continued stimulation of the immune system by GAD in the peripheral nervous system. Consistent with this hypothesis, Rabinowe et al. (37) have shown that, in some IDDM patients, autoantibodies to sympathetic ganglia are present at the diagnosis of IDDM, before the onset of clinical neuropathy. In addition, postmortem examination has revealed lymphocytic infiltration of sympathetic ganglia in IDDM patients with autonomic neuropathy (38). Our data suggest that autoimmunity to GAD, together with the metabolic effects of hyperglycemia, may play an important pathogenic role in diabetic neuropathy in IDDM.

The surprising similarity of the amino acid sequences of GAD_{65} and GAD_{67} to the P2-C protein of Coxsackievirus suggests that IDDM autoimmunity may arise by molecular mimicry, as a consequence of infection by Coxsackievirus. Epidemiological studies have shown that 39% of newly diagnosed

IDDM patients have IgM responses to Coxsackievirus, compared to 6% of controls (39, 40). The molecular mimicry hypothesis suggests a mechanism to explain both the epidemiological association of Coxsackievirus B_4 with human IDDM and its ability (in contrast to other viruses epidemiologically associated with IDDM) to produce diabetes in mice and primates (31, 41–44). Direct association of Coxsackie B_4 infection and subsequent onset of human IDDM has been documented in a few cases (45, 46).

Coxsackievirus infection (perhaps of β cells themselves) may, in genetically susceptible individuals, initiate the characteristic autoimmune attack on pancreatic β cells. Viral peptides would then be presented to T lymphocytes, probably on the surface of antigen-presenting cells in the context of class II molecules. Although the sequences of both GADs suggest that they are cytosolic molecules, GAD polypeptides may be presented on the cell surface in the context of MHC molecules (as discussed in 47 and 48). Christie et al. (36), moreover, have demonstrated the association of the molecule we now know to be GAD_{65} with β cell membranes. GAD epitopes on the surface of β cells, in the context of either class I or class II molecules, could thus become the targets of immune responses initially directed against a Coxsackievirus epitope. The resultant destruction of β cells would then release more GAD_{65} and GAD_{67}, including GADs from the cytoplasm. The released GAD could then continue to stimulate lymphocytes already primed to the Coxsackievirus peptide, thus perpetuating the immune response long after the termination of the viral infection. This molecular mimicry would then lead to the continued autoimmune destruction of β cells and eventually to the development of clinical diabetes.

Assays for antibodies to recombinant GADs should allow a straightforward means of distinguishing IDDM from other forms of diabetes mellitus. This should be especially useful for evaluating adult patients presenting with the more common type II, non–insulin-dependent diabetes mellitus (NIDDM). Patients with true NIDDM do not have ICAs or autoantibodies to the 64,000 M_r protein or to insulin. Of adult onset patients initially diagnosed as having NIDDM, however, 10–15% are true type I (IDDM) diabetics and will eventually require insulin therapy.

Clinical trials are now under way to test the effectiveness of general immunosuppressive agents (such as cyclosporin and azathioprine) in delaying the onset of IDDM in individuals at high risk, that is, who already have islet cell autoantibodies

(49). Autoantibodies to GAD are the earliest indication of autoimmunity in IDDM and the two GADs are therefore excellent candidates for the initial targets for autoimmunity. Future experiments will determine whether the epitope shared by GAD and Coxsackievirus contributes to IDDM pathogenesis. If GAD is indeed involved in the etiology of IDDM, it may be possible to devise specific, rather than nonspecific, immunosuppressive strategies to block the function of specific MHC and T cell receptor molecules.

Acknowledgments

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