Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9841–9845, October 1992
Medical Sciences

Glutamic acid decarboxylase autoantibodies in preclinical insulin-dependent diabetes

(immunoprecipitation/insulin-dependent diabetes mellitus/stiff man syndrome)

HENRY J. DE AIZPURUA, YVETTE M. WILSON, AND LEONARD C. HARRISON

Burnet Clinical Research Unit, The Walter and Eliza Hall Institute of Medical Research, Parkville 3050, Victoria, Australia

Communicated by G. J. V. Nossal, May 12, 1992

ABSTRACT **Insulin-dependent diabetes mellitus (IDDM) is associated with serum antibodies that precipitate a 64-kDa pancreatic islet cell protein reported to be glutamic acid decarboxylase (GAD; glutamate decarboxylase, EC 4.1.1.15). Previously, antibodies to GAD were found in the rare neurological disorder stiff man syndrome. To demonstrate directly antibodies to GAD, enzymatically active GAD was first purified from fresh human cerebellum. Brain GAD activity was precipitated by noninhibitory antibodies in the sera of 16/26 (62%) subjects defined as having preclinical IDDM (islet cell antibody-positive first-degree relatives of a person with IDDM), 3/13 (23%) with recent-onset IDDM, and 3/3 with the stiff man syndrome. In addition, sera of 5/26 (19%) preclinical and 2/13 (15%) recent-onset IDDM subjects contained antibodies that precipitated GAD but inhibited its activity. Thus, overall, 21/26 (81%) preclinical and 5/13 (38%) recent-onset IDDM subjects had antibodies that precipitated GAD activity. Antibodies to GAD were not detected in sera from subjects with other autoimmune diseases ($n = 29$) or healthy controls ($n = 14$). GAD affinity-purified to homogeneity (specific activity, 58 units/mg) was specifically immunoprecipitated as a single 60-kDa species by the IDDM sera. In an ELISA incorporating whole mouse brain GAD captured by the GAD-6 monoclonal antibody the frequencies of GAD antibodies for all subject groups were indistinguishable from those found by precipitation of human brain enzymatic activity. We conclude that (*i*) GAD is an (auto)antigen in a majority of subjects operationally defined as having preclinical IDDM, (*ii*) pancreatic islet and brain GAD are likely to be cross-reactive, and (*iii*) the majority of GAD antibodies are directed away from the catalytic site of the brain enzyme. The lower frequency of GAD antibodies in recent-onset IDDM subjects indicates either that immunoreactivity is lost with near-total β-cell destruction or that GAD antibodies denote a low risk of progression to clinical disease.**

The destruction of pancreatic islet β cells in insulin-dependent diabetes mellitus (IDDM) is believed to be mediated by autoimmune mechanisms in genetically susceptible individuals (1). At least three islet antigens have been identified as targets for antibodies in IDDM: a putative glycolipid postulated to account for the reactivity of islet cell antibody (ICA) with frozen sections of human pancreas (2), insulin (3), and a 64-kDa protein (4). Antibodies to the 64-kDa protein have been found in a majority of preclinical and recent-onset IDDM subjects (5). Baekkeskov *et al.* (6) reported evidence that the 64-kDa islet antigen is glutamic acid decarboxylase (GAD; glutamate decarboxylase, EC 4.1.1.15), the enzyme responsible for the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid. Antibodies to GAD were first described by Solimena *et al.* (7) in serum and cerebrospinal fluid (CSF) of patients with stiff man syndrome (SMS), a rare neurological disorder associated with organ-specific autoimmunity including IDDM. The connection between SMS and IDDM is tenable because GAD is present not only in the brain but also in the insulin-producing β cells of the islets (8). Baekkeskov *et al.* (6) found that the 64-kDa antigen and GAD were cross-immunoprecipitated by sera from a small number of IDDM subjects or by a sheep anti-GAD serum. To obtain direct evidence that GAD is a target (auto)antigen in IDDM, and to ascertain whether GAD antibodies are a marker of subjects defined as having preclinical IDDM, we tested the immunoreactivity of purified, enzymatically active brain GAD.

SUBJECTS AND METHODS

Subjects. Experimental procedures were approved by the Human Ethics Committee. Subjects with preclinical IDDM were asymptomatic, first-degree relatives of patients with IDDM who had circulating ICA ≥20 Juvenile Diabetes Foundation (JDF) units (U). Subjects with recent-onset IDDM, within 6 weeks of diagnosis, met the clinical criteria for IDDM and were insulin-dependent. Sera were from 26 preclinical subjects (12 male, 14 female; mean age, 28; range, 6–48) and 13 recent-onset (9 male, 4 female; mean age, 25; range, 10–41) (see Table 2). Other subjects included 3 with SMS, 10 with Graves disease and thyrotropin receptor antibodies, 10 with the CREST nondiffuse variant of scleroderma and centromere antibodies (where CREST indicates calcinosis, Raynaud phenomenon, esophageal dismotility, sclerodactyly, telangiectasia), 4 with pernicious anemia and gastric parietal cell antibodies, 5 males with infertility associated with anti-sperm antibodies, and 14 healthy controls.

Purification of GAD. GAD was semipurified from human cerebellum obtained with the consent of relatives within 2 hr of death by methods adapted from Wu *et al.* (9). GAD was immunoaffinity purified by the method of Chang and Gottlieb (10) with GAD-1 monoclonal antibody (ATCC no. HB184) purified from ascites fluid on Sepharose-protein A. Purified GAD was analyzed for homogeneity by SDS/PAGE and silver staining. It retained enzymatic activity for at least 2 months when stored at −70°C in a lightproof container.

GAD Enzymatic Assay. GAD activity was assayed by a modification of the method of Albers and Brady (11). Briefly, 50 μl of GAD solution was placed in duplicate sterile Eppendorf tubes containing 20 μl of 2 mM L-glutamate, 0.4 μCi of L-[U-^{14}C]glutamic acid (1 Ci = 37 GBq), 1 mM 2-aminoethylisothiouronium bromide, 0.2 mM pyridoxal 5-phosphate, and 50 mM potassium phosphate buffer (pH 7.2; buffer A), to a final volume of 100 μl. The tubes were sealed with lids into which a disc of Whatman 3MM filter paper was inserted presoaked with 30 μl of hyamine hydroxide to collect

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Abbreviations: GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; ICA, islet cell antibody(ies); SMS, stiff man syndrome; JDF, Juvenile Diabetes Foundation; IAA, insulin autoantibody(ies); U, unit(s); CSF, cerebrospinal fluid.

liberated $^{14}CO_2$. After incubation for 20 hr at 37°C, 100 μl of 2.5 M H_2SO_4 was injected into the tubes and 60 min later the filter discs were removed and placed into 2 ml of scintillation fluid for β counting. One unit of GAD activity is defined as 1 μmol of CO_2 evolved per min at 37°C.

Immunoprecipitation of GAD Enzymatic Activity. Thirty microliters of IDDM or control subject serum was bound to 50 μl (final bed volume) of Sepharose-protein A beads for 1 hr at 4°C. Beads were washed three times with buffer A and incubated overnight at 4°C with 50 μl of 1:100 dilution of semipurified brain GAD (i.e., 1.5 mU). The supernatant was recovered after centrifugation and stored at 4°C prior to assay for GAD enzymatic activity. The bead pellets were washed three times with buffer A, resuspended in 50 μl, and immediately used in the GAD enzymatic assay as described. Results were expressed as a percentage of total GAD activity in the supernatant after precipitation by Sepharose-protein A beads only.

Immunoprecipitation of Affinity-Purified ^{125}I-Labeled GAD (^{125}I-GAD). Affinity-purified human brain GAD was iodinated with ^{125}I by the chloramine-T method (12). The enzymatic activities of the unlabeled and radiolabeled GAD were assayed as described above and found to be identical. Samples of ^{125}I-GAD were precleared by incubating (2 hr at 4°C) aliquots (100 μl) with 25 μl of Sepharose-protein A prebound to immunoglobulins from 10 μl of normal human serum. Immunoglobulin in IDDM or control serum (2 μl) prebound to Sepharose-protein A, or sheep anti-GAD serum (S3) raised against rat brain (gift from Wolfgang Oertel, Munich) prebound to Sepharose-protein G, was incubated with precleared samples (0.5 μCi) overnight at 4°C with gentle rotation. Immune complexes were recovered by centrifugation in a Microfuge for 1 min at 4°C. The precipitates were washed once with phosphate-buffered saline (PBS), once with 0.5 M NaCl, and twice with PBS, then solubilized in SDS sample buffer, and analyzed by 10% SDS/PAGE under reducing conditions. Gels were stained with 0.2% Coomassie blue R-250 (Bio-Rad), dried, and autoradiographed at room temperature using Amersham Hyperfilm-MP and DuPont Lighting Plus intensifying screens.

GAD Antibody Capture ELISA. GAD-6 monoclonal antibody (13) was coated at 10 μg per well in 60 mM carbonate buffer (pH 9.6) to plastic wells of a round-bottomed 96-well Costar serocluster microtitration plate overnight at 4°C. Wells were washed six times with wash buffer [PBS with 0.05% (vol/vol) Tween 20] followed by two washes with PBS at room temperature. All wells were exposed to blocking buffer (5% nonfat skim milk powder in buffer A) for 60 min and then washed six times with buffer A and twice with PBS. Semipurified mouse brain GAD (see Table 1) (20 μg per well in buffer A) was allowed to react with immobilized GAD-6 overnight at 4°C in the dark. Plates were again washed as described followed by the addition, in duplicate, of serum at 1:100 dilution in blocking buffer. After 90 min at room temperature the plates were washed as described. Anti-human immunoglobulins conjugated with alkaline phosphatase, at 1:1000 in blocking buffer, were incubated in each well for 60 min at room temperature followed by washing. Color was developed with *p*-nitrophenol phosphate chromogen at 1 mg/ml in diethanolamine buffer [9.7% (vol/vol) diethanolamine/0.01% (wt/vol) $MgCl_2$, pH 9.8] for 15 min at 37°C. The reaction was stopped by adding 50 μl of 3 M NaOH per well. The optical density at 405 nm was determined using a Titertek Multiscan MCC spectrophotometer.

Assays for Antibodies to Islet Antigens. ICA were measured in serially diluted serum by indirect immunofluorescence on frozen sections of human, organ donor pancreas and expressed in JDF units relative to reference control serum. Insulin autoantibodies (IAA) were measured by radioimmunoassay (14), the normal range being −15 to +40 nU of insulin bound per ml of serum. ICA and IAA assays have been included in all International Workshops on Standardization. Antibodies to the 64-kDa protein (64K antibodies) were detected by immunoprecipitation of solubilized [^{35}S]methionine-labeled fetal pig proislets (15), the intensity of the precipitated 64-kDa band being graded visually as +, ++, or +++.

RESULTS

GAD Purification. Isolation from fresh human cerebellum by three sequential chromatographic procedures resulted in a 940-fold purification of GAD with a specific activity of 3 U/mg (Table 1). When the GAD activity peak from Sephacryl S-200 was subjected to SDS/PAGE under reducing conditions a predominant band of 60 kDa was detected (Fig. 1, lane 4), but this preparation was clearly not homogeneous. Subsequent immunoaffinity chromatography of the semipurified GAD on a GAD-1 monoclonal antibody column resulted in a product that migrated as a single band at 60 kDa (Fig. 1, lane 5) and had a specific GAD activity of 58 U/mg. This represented a 20-fold purification over the semipurified GAD. Although GAD-1 monoclonal antibody has been reported to react with the 59- to 60-kDa and a 63-kDa form of rat brain GAD (10), our results show that it purified only a 60-kDa form from the human cerebellum.

Immunoprecipitation of GAD Enzymatic Activity. Semipurified GAD was allowed to react with serum immunoglobulin from IDDM and control subjects prebound to Sepharose-protein A. Two types of GAD antibodies were detected. As depicted in Fig. 2, sera from 16/26 (62%) preclinical and 3/13 (23%) recent-onset IDDM subjects immunoprecipitated GAD activity at a level greater than the mean plus two standard deviations of the healthy control group. The ability of IDDM immunoglobulin to precipitate GAD activity was concentration-dependent (example shown in Fig. 3). Additionally, five other preclinical and two recent-onset sera immunodepleted ≥20% of GAD activity from the supernatant, without significant GAD activity being recovered in the immune precipitate (Fig. 2). Thus, in total, 21/26 (81%)

Table 1. Purification of GAD from human brain

Sample	Volume, ml	Total activity,* U	Total protein, mg	Specific activity, (U/mg) × 10^3	Yield, %	Fold purification
Homogenate	5875	300	94,000	3.2	100	—
Crude extract	940	75	3,230	23.2	30	7
Sephacryl S-300	212	31	293	105.8	10	33
DEAE-Sephadex	12	5.4	3.5	1,542	1.8	482
Sephacryl S-200	20.0	3.5	1.2	2,916	1.1	911
Semipurified GAD	1.0	3.3	1.1	3,000	1.1	940
Input GAD-1	1.0	3.3	1.1	3,000	—	940
Output GAD-1	0.2	2.9	0.05	58,000	—	18,125

*1 U = 1 μmol of product evolved per min at 37°C.

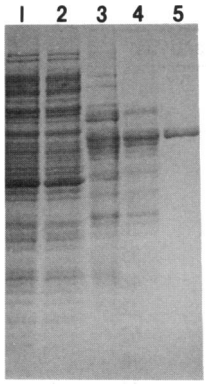

FIG. 1. SDS/PAGE during purification of human brain GAD. Lane 1, crude extract (20 μg of protein); lane 2, Sephacryl S-300 GAD activity peak (20 μg); lane 3, DEAE-Sephadex GAD activity peak (10 μg); lane 4, Sephacryl S-200 GAD activity peak (5 μg); lane 5, affinity-purified GAD activity peak (2 μg). Electrophoresis was performed in a 10% acrylamide gel under reducing conditions and the gel was then silver stained. Molecular masses are indicated in kDa.

preclinical and 5/13 (38%) recent-onset subjects had antibodies that precipitated GAD. All three subjects with SMS and the CSF from one of these subjects immunoprecipitated significant GAD activity. Sera from subjects in other disease groups, including Graves disease, pernicious anemia, scleroderma, and male infertility with sperm antibodies, failed to precipitate significant GAD activity.

Table 2 gives the clinical and immunogenetic data for each preclinical and recent-onset IDDM subject. Preclinical subjects, operationally defined by ICA positivity, had a low frequency of IAA (5/26 subjects); in recent-onset subjects the higher frequency of IAA (5/13 subjects) may reflect the response to treatment with insulin. The frequency of 64K antibodies (69%) and GAD antibodies (81%) in the preclinical subjects is significantly higher than in the recent-onset subjects (38% and 38%, respectively). Concordance between 64K and GAD antibodies was present in only 15/26 (58%) preclinical and 7/13 (54%) recent-onset subjects. Although there is a predominance of older females and younger males in the preclinical group, the presence of GAD antibodies does not appear to be age- or sex-associated. Finally, a majority (14/21) of the preclinical subjects with GAD antibodies have high titer ICA (≥40 JDF U).

Immunoprecipitation of Affinity-Purified ^{125}I-GAD. To directly confirm that GAD is a target of antibodies in IDDM, affinity-purified human brain GAD was iodinated with ^{125}I and immunoprecipitated with sera from IDDM subjects.

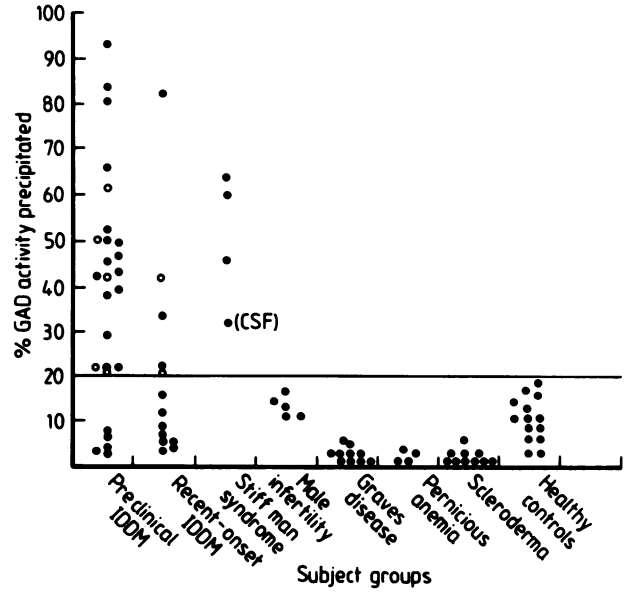

FIG. 2. Immunodepletion of human brain GAD enzymatic activity by serum noninhibitory (●) and inhibitory (○) IgG. The mean plus two standard deviations of the healthy controls (——) was used as the lower limit of positivity.

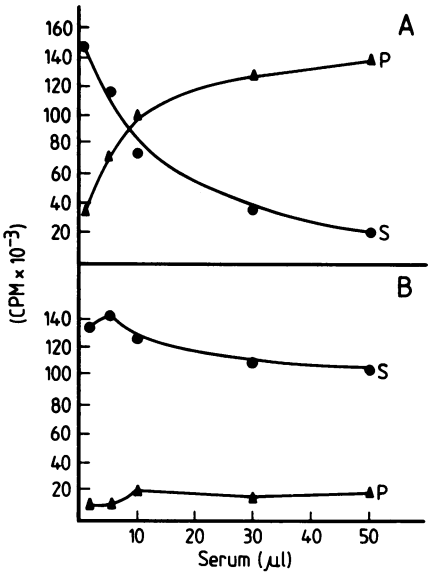

FIG. 3. Concentration dependence for precipitation of human brain GAD activity by serum immunoglobulin from a recent-onset IDDM subject (*A*) or a healthy control subject (*B*). GAD activity precipitated by immunoglobulin-Sepharose-protein A (P) is compared with enzymatic activity remaining in the supernatant (S). Similar results were obtained with sera from two other preclinical subjects.

IDDM and SMS sera that immunoprecipitated GAD enzymatic activity (Fig. 2) precipitated a single species of ^{125}I-GAD of 60 kDa (Fig. 4). Sheep anti-GAD serum also precipitated ^{125}I-GAD but other disease and control sera did not.

GAD Antibody Capture ELISA. To facilitate clinical screening for antibodies to GAD we developed a capture ELISA incorporating mouse brain GAD prebound to the GAD-6 monoclonal antibody. The ELISA detected antibodies to GAD in 8/11 (73%) preclinical and 3/8 (38%) recent-onset IDDM sera, 2/2 SMS, 0/5 Graves disease, 1/5 scleroderma, and 0/10 healthy control sera (Fig. 5). The eight preclinical and three recent-onset IDDM sera positive in the ELISA were also positive by immunoprecipitation of human brain GAD activity. The correlation coefficient (*r*) between the two sets of assay results was 0.89 for the preclinical subjects and 0.99 for recent-onset subjects.

DISCUSSION

Baekkeskov *et al.* (6) reported that the 64-kDa islet protein and GAD were coimmunoprecipitated from human islets by two IDDM sera and a sheep antiserum to rat brain GAD. We have found that human brain GAD activity is precipitated by antibodies in 81% (21/26) of sera from preclinical IDDM subjects and 38% (5/13) of sera from recent-onset IDDM subjects. In a minority of cases GAD activity was not recoverable in the precipitate, consistent with the presence of antibodies that bind at or near the catalytic site of GAD. Inhibitory and noninhibitory antibodies have been described in other organ-specific autoimmune diseases (16), but, to our knowledge, functional subtypes of antibodies in IDDM have not been reported previously. It will be of interest to determine if these subtypes of GAD antibodies have clinical correlates.

The difference in the frequency of GAD immunoreactivity between preclinical and recent-onset IDDM subjects suggests either that GAD autoreactivity is lost as β-cell destruction nears completion, consistent with loss of antigenic drive, or that GAD autoreactivity is not a good marker of preclinical subjects who progress to clinical disease. The proposal (17) that GAD antibodies are preferentially found in older females

Table 2. Clinical and laboratory data for preclinical and recent-onset patients

Subject	Sex	Age, years	HLA-DR	ICA, JDF U	IAA, nU/ml	64K antibody	% GAD activity	
							Immuno-precipitated	Immuno-depleted
Preclinical IDDM								
1	♀	43	3, 4	640	29	+	80	83
2	♀	48	3, 4	640	−5	++	93	90
3	♂	46	3, 4	40	22	−	24	20
4	♀	17	3, 4	40	8	−	38	35
5	♀	41	3, 4	20	7	++	45	48
6	♀	41	3, 7	40	21	++	7	22
7	♀	35	3, 11	20	32	++	8	62
8	♀	39	3, 12	320	5	−	32	36
9	♀	44	3, −	20	14	−	44	44
10	♂	10	3, −	40	0	+	42	43
11	♀	44	3, −	20	78	+	39	38
12	♂	12	4, 13	320	5	−	3	51
13	♂	33	4, 13	20	85	++	49	47
14	♀	22	4, 11	20	31	+	21	48
15	♂	15	4, 9	160	−9	+	50	53
16	♂	48	4, 8	20	10	+	52	50
17	♀	40	4, −	80	30	−	4	5
18	♂	6	1, 4	40	68	+	3	5
19	♂	30	2, 3	40	17	+	7	5
20	♂	12	1, 3	40	51	−	66	69
21	♂	10	ND	80	27	+	3	43
22	♂	7	ND	40	19	−	3	20
23	♂	10	ND	20	31	+	5	8
24	♀	44	4, 11	80	25	+	87	90
25	♀	17	3, 4	20	39	+	3	5
26	♀	8	7, 12	160	100	++	45	47
Recent-onset IDDM								
1	♂	26	1, 4	80	230	−	5	6
2	♂	33	4, 13	80	30	+	12	20
3	♀	10	ND	60	4	−	23	27
4	♀	41	2, 9	20	15	−	5	8
5	♂	30	3, 4	40	2	−	6	43
6	♂	22	ND	80	54	−	33	30
7	♀	20	3, −	640	15	−	5	8
8	♂	34	ND	0	11	−	5	5
9	♀	25	ND	160	470	−	16	19
10	♂	41	3, −	640	1400	++	82	80
11	♂	19	ND	20	4	+	7	9
12	♂	30	3, 4	0	190	+	8	10
13	♂	15	4, 7	40	60	+	6	4

ND, not done.

with polyendocrine autoimmunity who progress more slowly to clinical IDDM cannot be corroborated as none of our subjects was in this category. Although 10/11 (91%) of our older (>20 years) preclinical females were GAD antibody positive, so also were 6/8 (75%) of younger (<20 years) preclinical males; the numbers in the other sex and age categories are too small to analyze. It is interesting, however, that GAD antibodies in the preclinical subjects were present in twice as many with high-titer ICA (≥40 JDF U) than with lower-titer ICA. High-titer ICA are predictive of clinical IDDM (reviewed in ref. 1) and are associated with the presence of islet-reactive T cells in the peripheral blood of preclinical subjects (15). Together, these findings provide evidence for the specificity of GAD antibodies as a marker of preclinical IDDM. The significance and predictive value of GAD antibodies should be resolved by longitudinal measurements in preclinical subjects.

There is evidence that 64K antibodies are an early marker of preclinical IDDM and may precede the appearance of ICA (18). Although this seemingly supports a key role for GAD, some caution should be exercised in equating GAD and the 64-kDa antigen. In our subjects, the concordance of antibodies to GAD and the 64K antigen was <60%. This may reflect the species difference of the tissues used for these assays. However, there is also a lack of concordance between the biochemical properties of GAD (19–22) and those reported for the 64-kDa antigen (23, 24). The deduced molecular masses of the recombinant GAD isoforms range from 64 to 67 kDa (25–28) and native GAD is not a single species that migrates uniquely at 64 kDa (19, 20). Furthermore, analysis of tryptic fragments of the 64-kDa antigen (29) reveals a GAD-associated 50-kDa product that is immunologically distinct from a 37- or 40-kDa fragment. These data suggest that GAD may not account for all 64-kDa antigen.

Autoantibodies that recognize at least two epitopes in human brain GAD were present in a majority of subjects operationally defined as having preclinical IDDM. It is reasonable to assume that such antibodies reflect autoreactivity to β-cell GAD, as the two major isoforms of GAD, GAD 67 and GAD 65, except for allelic differences, are the same in brain and islets (25–28, 30). A recent report (31) has demonstrated antibodies to both isoforms of recombinant brain

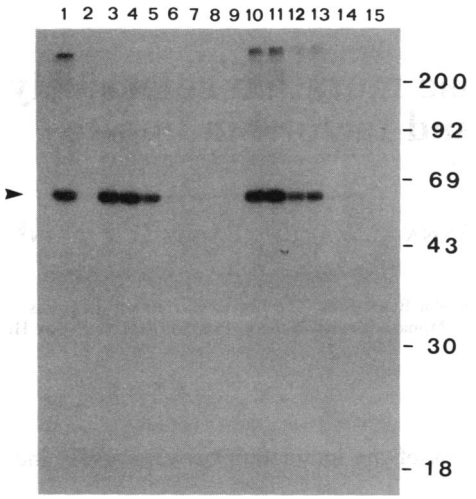

FIG. 4. Autoradiograph of affinity-purified ^{125}I-GAD immunoprecipitated by serum and analyzed by 10% SDS/PAGE. Lane 1, sheep anti-rat brain GAD serum; lane 2, normal sheep serum; lanes 3 and 4, SMS sera; lane 5, CSF; lanes 6–9, Graves disease (lanes 6 and 7) and preclinical IDDM sera (lanes 8 and 9) that failed to immunodeplete GAD enzymatic activity; lanes 10–13, preclinical IDDM sera that precipitated GAD enzymatic activity; lanes 14 and 15, control sera. Molecular masses are indicated in kDa.

GAD in a small number of clinical IDDM subjects. The availability of the recombinant proteins will allow GAD epitopes to be mapped, which may help to shed light on why IDDM and SMS are distinct clinical entities.

Our findings demonstrate that native human and mouse brain GAD is recognized by antibodies present in the majority of subjects operationally defined as having preclinical IDDM, consistent with immunological cross-reactivity between islet and brain GAD. Most GAD antibodies were not directed at epitopes in the catalytic site of the enzyme. The detection of GAD antibodies in a majority of preclinical IDDM subjects implies that autoimmunity to GAD may have a pathogenetic role in β-cell destruction.

We thank Mrs. Jan Caudwell and Dr. Peter Colman for ICA and IAA assays, Dr. Marjorie Dunlop for advice on the GAD assay, Dr. Grant Walker for the sera from SMS subjects, Prof. Henry Burger for sera from male infertility subjects, Drs. Stella Clark, David Cram, and Margo Honeyman for critically reading the manuscript, and Margaret Thompson for secretarial assistance. H.J.D.A. is supported by Diabetes Australia and L.C.H. is a Senior Principal Research Fellow of the National Health and Medical Research Council of Australia. The study was financially supported by a grant from the Victorian Health Promotion Foundation.

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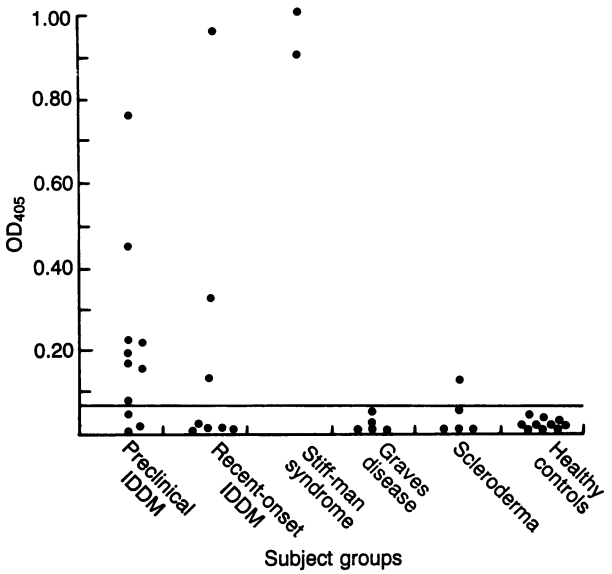

FIG. 5. GAD antibody capture ELISA. The mean plus three standard deviations of healthy controls (——) was used as the lower limit of positivity.

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