Brief Definitive Report

Glutamic Acid Decarboxylase 67-reactive T Cells: A Marker of Insulin-dependent Diabetes

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Summary

Glutamic acid decarboxylase (GAD) has been shown to be a target of autoantibodies in insulin-dependent diabetes (IDD). Two forms of GAD, with molecular weights of 67,000 and 65,000, have been cloned from separate genes. As pancreatic islet β cell destruction in IDD is an autoimmune process mediated by T cells, we sought to determine if recombinant GAD67 was recognized by T cells in IDD subjects and particularly their first-degree relatives with islet cell antibodies known to be at risk for IDD. The central regions of human islet and brain GAD67 (amino acids 208–404) were cloned as fusion proteins with glutathione-S-transferase (GST). Proliferation of peripheral blood T cells in the presence of recombinant GAD67 was significantly higher in both at-risk relatives and recent-onset IDD subjects than in other autoimmune disease subjects and human histocompatibility leukocyte antigen (HLA)-matched healthy controls. Thus, 12 of 29 (41%) at-risk relatives and 11 of 29 (38%) recent-onset IDD subjects responded to GAD67, compared with 1 of 7 (14%) other autoimmune disease subjects and 1 of 23 (4%) HLA-matched controls. T cell responses to GST alone or to tetanus toxoid were not different between the groups. These findings demonstrate that GAD67 is a target autoantigen of T cells in IDD and suggest the possibility that GAD-reactive T cells may delineate asymptomatic subjects at increased risk for IDD.

Insulin-dependent diabetes (IDD) is an autoimmune disease that results from T cell–mediated destruction of the pancreatic islet β cells (1). Islet-reactive T cells can be detected in the peripheral blood of recent-onset IDD subjects and asymptomatic islet cell antibody (ICA)-positive first-degree relatives of persons with IDD (2). In addition to ICA, which react with an uncharacterized antigen in frozen sections of human pancreas, the majority of recent-onset IDD subjects and at-risk first-degree relatives have antibodies to a 64,000–mol wt islet antigen identified as glutamic acid decarboxylase (GAD) (3). Such subjects have recently been shown to have antibodies that react directly with purified native (4) or recombinant (5) GAD.

GAD catalyzes the conversion of L-glutamate to γ-amino butyric acid and is found in high concentrations in neurones and β cells (6). It exists in two forms, derived from separate genes encoding proteins with predicted molecular weights of 65,300 (GAD65) and 66,660 (GAD67) that have 65% amino acid identity (7). It has been proposed that GAD65 is the major islet form of GAD that accounts for the 64,000–mol wt antigen (8). Atkinson et al. (9) reported that T cells from IDD subjects proliferate in response to recombinant GAD65. However, it has not been shown that T cell responses to GAD are IDD specific, i.e., absent in other autoimmune diseases. Furthermore, the key role of MHC molecules in presenting peptides to T cells and the association between specific MHC class II alleles and IDD (10) dictates that T cell responses of IDD subjects be compared with those of MHC class II–matched healthy controls to exclude the possibility that they are MHC rather than IDD specific.

Materials and Methods

Subjects. Samples of peripheral venous blood were obtained with informed consent and approval of the Human Ethics Committee (Royal Melbourne Hospital) from: (*a*) 29 first-degree relatives of persons with IDD defined as at-risk for IDD because of the presence of circulating ICA at ≥20 Juvenile Diabetes Foundation (JDF) units; (*b*) 29 subjects with recent-onset clinical IDD (<6 wk after diagnosis); (*c*) seven subjects with other autoimmune diseases (four Graves' disease, one scleroderma, two Sjögren's syndrome); and (*d*) 23 healthy control subjects HLA matched for HLA-DR and DQ with subjects in groups *a* and *b* (Table 1).

HLA Typing. HLA typing was performed serologically on all subjects by the standard microlymphocytotoxic technique for all recognized HLA class I and II alleles. The class II typings of at-risk and recent-onset IDD subjects were confirmed by sequence specific oligotyping following the 11th International Histocompatibility Workshop protocol.

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Table 1. *Clinical and Experimental Data for Individual Subjects*

Subjects	Age	Sex	HLA DR;DQ	T cell proliferation					
				Basal	GST	HIG	HBG	Tetanus	HIG*
	yr					*cpm*			*SI*
ICA⁺ first-degree relatives									
1	37	F	3,12; 2,7	4,044	16,811	22,168	16,406	10,651	1.3
2	11	M	2,4; 1,9	2,497	2,980	16,632	21,391	28,275	5.5
3	18	F	3,4; 2,8	5,923	6,225	15,325	42,746	22,516	1.5
4	6	M	3,4; 2,8	4,703	4,685	55,491	53,118	34,627	11
5	48	M	3,4; 2,8	2,417	2,638	9,660	6,447	17,688	2.9
6	17	F	3,3; 2,2	3,501	9,360	14,891	6,800	60,838	1.6
7	49	M	3,4; 2,8	5,510	4,314	30,766	25,934	24,554	4.8
8	49	F	3,4; 2,7	6,514	10,079	15,364	6,512	24,254	0.81
9	11	M	4,13; 1,7	5,044	5,376	26,875	75,874	21,871	4.3
10	33	M	4,13; 1,8	2,234	6,379	7,769	5,777	9,939	0.62
11	30	M	2,3; 1,2	3,258	18,271	35,889	29,719	45,356	5.4
12	13	M	1,3; 1,2	17,233	30,086	37,459	31,931	71,331	0.43
13	40	F	2,9; 1,9	2,539	85,376	118,842	77,310	86,180	13
14	44	F	3,3; 2,2	17,334	25,181	28,211	35,744	25,181	0.17
15	49	M	4,8; 4,7	5,250	5,059	7,044	6,848	17,366	0.38
16	10	F	4,4; 7,8	2,486	3,040	13,444	–	18,401	4.2
17	11	M	3,3; 2,2	5,645	4,804	35,112	38,853	20,438	5.4
18	16	M	3,4; 2,8	380	2,210	6,311	6,717	21,449	11
19	11	F	4,4; 8,8	1,527	7,474	19,602	–	33,719	7.9
20	19	M	–	877	756	2,363	725	30,924	1.8
21	5	M	–	13,533	13,222	17,595	50,553	72,337	0.32
22	6	M	1,4; 1,8	75	710	1,616	2,105	2,399	12
23	19	M	3,4; 2,8	6,517	9,307	10,787	6,064	38,730	0.22
24	43	F	3,4; 2,8	11,854	51,287	39,010	34,438	18,231	−1.0
25	21	F	4,11; 6,7	5,639	8,193	29,855	30,671	68,950	3.8
26	14	F	3,4; 2,8	20,677	21,763	73,153	20,273	45,305	2.5
27	14	M	3,3; 2,2	635	1,561	1,316	1,744	8,269	−0.38
29	39	M	3,7; 2,2	1,793	1,450	2,065	2,562	1,844	0.34
29	45	F	3,3; 2,2	9,213	18,346	31,828	14,774	6,600	1.5
Recent-onset IDD									
1	35	F	3,4; 2,8	4,260	6,327	43,265	62,995	54,057	8.7
2	12	F	3,4; 2,7	23,298	28,766	114,244	57,288	132,359	3.7
3	–	F	–	16,609	8,907	16,533	18,728	14,447	0.46
4	12	M	3,3; 2,2	1,139	1,419	7,424	8,009	27,497	5.3
5	35	M	4,13; 6,7	1,243	13,424	19,108	17,775	54,493	4.5
6	16	M	1,8; 1,8	76,450	62,764	106,254	74,274	32,583	0.57
7	11	M	–	2,314	3,489	4,750	3,951	21,235	0.54
8	10	F	4,6; 1,8	38,460	46,376	85,127	116,728	167,211	1.0
9	30	M	4,4; 8,8	4,786	543	6,222	–	72,892	1.2
10	28	M	4,4; 8,8	2,731	5,189	27,583	8,466	28,730	8.2
11	20	M	4,11; 7,7	23,032	28,279	14,116	28,591	18,725	−0.61

continued

Table 1. *(continued)*

Subjects	Age	Sex	HLA DR:DQ	T cell proliferation					
				Basal	GST	HIG	HBG	Tetanus	HIG*
12	26	F	3,4; 2,8	14,701	24,347	30,905	–	34,541	0.45
13	13	F	–	24,227	59,473	56,536	43,729	43,347	−0.12
14	11	M	–	10,020	10,990	27,201	32,581	13,260	1.6
15	17	M	1,4; 1,8	9,891	10,418	19,463	29,211	13,902	0.91
16	17	M	1,4; 1,8	3,145	12,562	38,110	20,092	93,221	8.1
17	19	M	3,4; 2,8	8,872	4,903	2,383	6,247	11,799	−0.28
18	45	F	–	4,828	6,740	24,684	9,073	8,228	3.7
19	20	F	4,4; 7,8	1,842	2,324	17,194	21,049	24,523	8.1
20	26	M	3,4; 2,8	8,795	8,442	53,244	–	53,039	5.1
21	22	M	3,4; 2,8	4,363	6,160	47,786	30,560	80,174	9.5
22	35	F	3,4; 2,8	4,306	20,991	34,864	–	96,886	3.2
23	24	F	4,8; 4,8	41,386	39,098	44,012	–	39,533	0.12
25	35	F	1,3; 1,2	61,432	84,724	116,462	–	88,052	0.52
26	12	M	3,4; 2,8	6,530	10,345	23,040	–	38,011	1.9
27	15	M	3,4; 2,8	38,414	76,673	82,711	111,631	114,511	0.16
28	17	M	3,4; 2,8	55,326	44,410	37,809	54,565	–	−0.12
29	39	M	3,3; 2,2	4,299	44,788	41,751	35,944	15,752	−0.70
Autoimmune disease controls (1–4, Graves' disease; 5, Scleroderma; 6 and 7, Sjögren's syndrome)									
1	38	F	9,11; 7,9	8,026	10,114	53,926	69,244	43,904	5.5
2	30	F	3,4; 2,7	4,910	4,080	4,941	–	7,365	0.17
3	64	F	3,11; 2,7	11,552	7,899	14,744	9,333	43,952	0.59
4	29	F	–	9,598	17,311	14,023	39,382	32,088	−0.34
5	40	F	–	680	948	1,015	972	1,119	0.10
6	48	F	2,10; 1–	1,930	1,902	2,452	2,378	1,436	0.28
7	23	F	2,3; 1,2	9,281	12,001	21,133	19,849	132,223	0.98
Healthy controls									
1	37	M	2,13; 1,6	16,306	15,587	20,458	20,651	122,493	0.29
2	24	M	3,BR; 1,2	14,840	28,631	39,208	33,215	18,871	0.71
3	34	M	1,4; 1,7	1,851	4,298	2,256	2,550	10,542	−1.1
4	30	F	3,4; 2,8	4,379	5,278	4,639	–	14,072	−0.15
5	30	F	3,3; 2,2	4,992	4,702	4,278	–	11,387	−0.085
6	27	F	3,4; 2,8	3,063	6,179	9,722	5,792	70,122	1.2
7	20	M	4,4; 7,7	30,402	35,130	66,277	–	59,000	1.0
8	30	F	4,7; 2,7	3,372	16,388	25,106	21,379	21,211	2.6
9	21	M	4,11; 7–	9,335	18,197	28,662	39,243	44,958	1.1
10	28	F	3,4; 2,8	2,543	8,967	18,054	15,047	76,630	3.6
11	38	M	2,4; 1,7	9,105	10,579	30,983	32,205	47,088	2.2
12	38	M	4,4; 8,8	13,433	2,974	2,932	–	4,868	−0.012
13	11	M	13,11; 1,7	16,246	36,983	29,438	47,240	81,429	−0.46
14	7	M	13,11; 1,7	8,750	26,835	35,926	32,426	92,180	1.0
15	30	F	4,4; 3,3	17,937	17,210	27,982	21,329	90,812	0.60
16	32	M	4,13; 1,8	42,955	65,488	44,390	81,628	87,938	−0.49
17	37	F	1,2; 1,1	3,348	2,779	4,233	1,863	11,454	0.43

continued

Table 1. *(continued)*

Subjects	Age	Sex	HLA DR:DQ	T cell proliferation					
				Basal	GST	HIG	HBG	Tetanus	HIG*
18	37	M	4,12; 7,8	9,842	14,180	30,373	19,014	44,798	1.6
19	39	M	4,11; 7,7	1,948	3,415	6,689	5,530	23,297	1.7
20	51	F	2,3; 1,2	1,770	2,433	2,655	2,952	2,154	0.12
21	30	M	3,3; 2,2	21,979	33,832	48,668	36,426	60,850	0.67
22	48	F	3,3; 2,2	1,859	3,646	6,505	6,095	21,249	1.5
23	40	M	3,4; 2,8	12,619	23,868	14,032	13,874	58,826	−0.78

* The stimulation index (SI) for HIG was corrected by subtracting the index for GST alone.

Recombinant Antigens. Cloning and sequencing of the central region (amino acids 208–404) of human brain GAD67 (HBG 584 amino acids) has been previously described (11). The equivalent region of human islet GAD67 (HIG) was obtained by reverse transcription of total islet RNA and amplified using the PCR (11). The predicted amino acid sequence of HIG was identical to HBG except for a leucine for phenylalanine substitution at position 248. Both partial GAD67 cDNA fragments were cloned into SmaI- and EcoRI-cleaved DNA of the pGEX-3 expression vector downstream from glutathionine-S-transferase (GST). HIG and HBG fusion proteins and the control GST protein were expressed in *Escherichia coli* and affinity purified on glutathione-agarose beads (11). Proteins were eluted from the beads with 10 mM reduced glutathione, 50 mM Tris pH 8.0, dialyzed against human tonicity PBS, filter sterilized, and stored at −70°C. Preparations were free of endotoxin as determined in the limulus lysate assay and were homogeneous by SDS-PAGE (Fig. 1).

Antibody Assays. ICA were assayed by indirect immunofluorescence on cryostat sections of human pancreas (blood group O), according to the protocol of the Second International Workshop on the Standardization of Islet Cell Antibodies.

T Cell Proliferation Assays. PBMC were isolated from heparinized blood by Ficoll-Hypaque density centrifugation, washed twice in RPMI 1640, and diluted to 2×10^6/ml in RPMI 1640 containing 5% autologous serum and 10^{-5} M 2-ME. Cells were distributed in 200-μl aliquots into wells of 96-well flat-bottomed Linbro trays, and 10 μl of antigen/well was added to quadruplicate wells. No antigen was added to cells in sets of quadruplicate wells at the beginning, middle, and end of the tray. Recombinant proteins (HIG, HBG, GST control) were added at 200, 2, and 0.2 μg to provide final concentrations of 10, 1, and 0.1 μg/ml. Tetanus toxoid (Commonwealth Serum Laboratories, Melbourne, Australia) without thiomersal was used as a positive control antigen at final concentrations of 1.8, 0.18, and 0.018 Lyons floculating units (LFU)/ml. After 7 d of incubation in 5% CO_2/air at 37°C, 1.0 μCi [^{3}H]thymidine was added to each well, the cultures harvested semi-automatically 7 h later, and thymidine incorporation measured by liquid scintillation counting. Proliferation was expressed as the stimulation index (SI), the median counts per minute (cpm) of the maximum response to antigen divided by the median cpm in the absence of antigen. SIs for HIG and HBG were corrected by subtraction of responses to GST alone. The threshold for a positive response was set at an SI of 3.0. This excluded all but one of the healthy controls. Differences between groups were analyzed using Wilcoxon's rank sum test. The correlation coefficient between paired responses was calculated by *t* test.

Sequence Screening. The GAD amino acid sequence was compared against all nucleic acid databases using the National Center for Biotechnology Information/National Library of Medicine T-BLAST program.

Results and Discussion

PBMC from 12 of 29 (41%) ICA-positive first-degree relatives responded to HIG compared with 11 of 29 (38%) recent-onset IDD subjects, 1 of 7 (14%) subjects with other autoimmune diseases, and 1 of 23 (4%) HLA-matched, healthy controls (Fig. 2). Proliferative responses to HIG were significantly greater than those of healthy controls in the first-degree relatives ($p < 0.01$) but just failed to reach significance in recent-onset IDD subjects; responses to HBG in first-degree relatives and recent-onset IDD subjects were both significantly greater than in healthy controls ($p < 0.003$ and $p < 0.05$, respectively). Autoimmune controls were not significantly different from healthy controls in response to either antigen. As expected, given their near identity, proliferative responses to HBG were overall no different from those to HIG. The tight

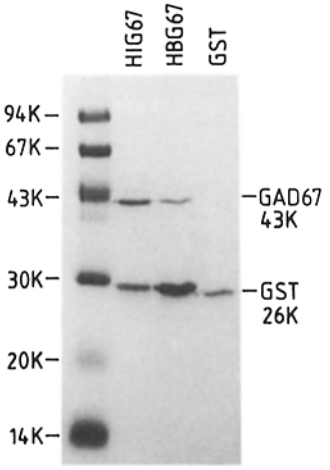

Figure 1. SDS-PAGE analysis of glutathione affinity purified recombinant GST fusion proteins. Proteins were subjected to electrophoresis in a 12.5% acrylamide gel and stained with coomassie blue. Lane *1*, molecular size markers; lane *2*, GST-human islet GAD67; lane *3*, GST-human brain GAD67; lane *4*, GST.

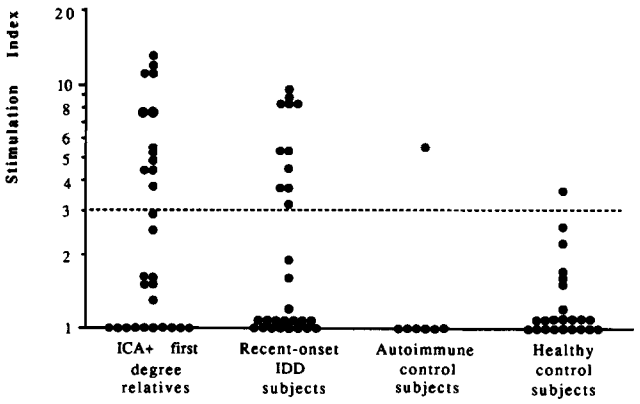

Figure 2. Proliferation of peripheral blood T cells in response to recombinant human islet GAD67 (amino acids 208–404).

correlation (r = 0.90) between the SI values for HIG and HBG for all subjects provided an internal measure of the specificity and precision of the results. In contrast to HIG or HBG, proliferative responses to tetanus toxoid were not significantly different between groups.

These results demonstrate that peripheral blood T cells that proliferate in response to the central region of human GAD67 can be detected not only in some recent-onset IDD subjects but also in an equivalent proportion of asymptomatic, ICA-positive first-degree relatives of IDD subjects. GAD-reactive T cells were not detected in most subjects with other autoimmune diseases, indicating that GAD67 responses are specific for IDD. The association of IDD with specific MHC class II alleles (e.g., HLA-DR3,4; DQ 2,8) predicts that APC bearing these alleles will preferentially process and present diabetogenic peptides to T cells. If all individuals possessed potentially autoreactive T cells and if GAD peptides in susceptible MHC molecules trigger GAD-specific T cell proliferation, then healthy control subjects with the same MHC alleles might also be expected to respond. However, only one of nine (11%) healthy controls with HLA-DR3 had significant T cell responses, compared with 5 of 18 (28%) of ICA-positive first-degree relatives and 6 of 13 (46%) recent-onset IDD subjects. 1 of 14 (7%) healthy controls with DR4 had significant T cell responses compared with 9 of 17 (53%) ICA-positive relatives and 9 of 19 (47%) IDD subjects. One of seven (14%) healthy controls with DQw8 had significant responses, compared with 6 of 12 (50%) ICA-positive relatives and 7 of 17 (41%) IDD subjects. For the full-susceptibility phenotype DR3,4;DQ2,8, one of four (25%) healthy controls had significant responses, in comparison with three of eight (38%) ICA-positive relatives and four of nine (44%) IDD subjects. Thus, the higher responses in ICA-positive relatives and IDD subjects are disease rather than MHC specific. These findings may be explained by a higher frequency of GAD-specific T cells in a subset of subjects with β cell autoimmunity. T cell responses were specific not only for IDD but also for GAD because the responses to GST and tetanus toxoid were not significantly different between the subject groups.

T cell responses to GAD67 in ICA-positive relatives mirror those to whole fetal pig proislets (2), in accord with the evidence that T cell–mediated destruction of β cells precedes the onset of symptomatic disease. Nevertheless, less than half of our ICA-positive first-degree relatives exhibited T cell responses to GAD. Although ICA are predictive for IDD, not all ICA-positive first-degree relatives and even fewer ICA-positive individuals without a relative with IDD will develop IDD (12). In addition, GAD is clearly not the only antigen responsible for T cell reactivity to islets in IDD (13). The combination of two or more tests of islet immunity should improve the prediction of disease. The first test, if highly sensitive like ICA, would exclude individuals not at risk and effectively increase the number with preclinical IDD in the remainder, who could then be screened with a second, preferably more specific test. As β cell destruction is a T cell–mediated process, it would be logical if the second test measured T cell reactivity to islet antigens. Accordingly, it is tempting to speculate that ICA-positive individuals who also have T cell reactivity to GAD represent a higher risk subgroup. Longitudinal studies will establish whether this hypothesis is correct. The group of ICA-positive relatives who were T cell responders contained relatively more males (M/F, 8:4) than the nonresponders (M/F, 9:8) and were younger (mean age, 18.5 vs 29.8 yr, respectively). The only ICA-positive relatives to develop IDD during the 18-mo course of this study were two young males (17 and 18 in Table 1) within the T cell responder group.

An increased risk of IDD in relatives who are T cell responders would predict a higher frequency of T cell responders in recent-onset IDD subjects, but this was not the case in our cross-sectional study. It is possible that the near-total loss of β cells at the time of symptomatic disease removes the antigenic drive to maintain T cell activation. T cell responses to whole fetal pig proislets were less frequent and lower in recent-onset IDD subjects than in ICA-positive relatives (2). Alternatively, recent-onset IDD subjects may respond to a greater number of epitopes in GAD than is present in the 208–404 amino acid region tested here. Atkinson et al. (9) found T cell proliferative responses to full-length recombinant GAD65 in five of eight (63%) ICA-positive relatives and 12 of 18 (67%) recent-onset IDD subjects. Comparison of GAD65 with the central 208–404 amino acid region of GAD67 used by us reveals 22 (11%) nonconservative substitutions and 24 (12%) conservative substitutions. Therefore, this central region of GAD could contain T cell epitopes unique to each of the two forms of GAD. The frequency of responses to GAD65 was apparently higher than to GAD67 but the number of subjects, especially ICA-positive relatives, was considerably less than in the present study. Although the differences are consistent with the existence of T cell epitopes in the NH_2- and COOH-terminal thirds of the protein, or reactivity with unique epitopes in the central region of GAD65, our epitope mapping studies using overlapping 13-mer GAD peptides indicates that the central region of GAD contains the dominant epitope (M. C. Honeyman and L. C. Harrison, unpublished results).

The GAD67 central region contains a sequence (amino acids 258–281) with homology to the P2-C protein of Coxsackie virus B4 (14). Eight residues are identical to the Coxsackie

sequence; nine residues are identical in the equivalent sequence of GAD65. When we screened this sequence of GAD67 against all nucleic acid data bases, several other homologous viral sequences were revealed in Kunjin virus, Japanese encephalitis virus, Western Nile virus, and Murray Valley encephalitis virus. This spectrum of viruses with sequence homology to GAD provides a plausible basis for molecular mimicry at the level of the T cell epitope.

The identification of GAD as a T cell autoantigen in IDD raises a number of critical questions. How early in the course of preclinical IDD are GAD-reactive T cells detected and do they predict clinical IDD better than antibodies to islet antigens? Definition of the primary T cell epitope of GAD might provide insight into a possible viral etiology of IDD and should refine the diagnosis of preclinical IDD and facilitate therapeutic strategies such as tolerance induction.

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