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 \( \beta \) 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.

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

| Subjects | Age | Sex | HLA DR/DQ | Basal | GST | HIG | HBG | Tetanus | HIG* | 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 | 5,393 | 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 | 45,356 | 30,924 | 1.8 |
| 27 | 14 | M | 3,3; 2,2 | 290 | 2,210 | 6,311 | 6,717 | 21,449 | 11 |
| 28 | 39 | M | 4,4; 8,8 | 4,786 | 543 | 6,222 | – | 27,583 | 8.2 |
| 29 | 45 | F | 3,4; 2,2 | 6,064 | 38,730 | 0.22 |
| 30 | 26 | M | 1,4; 1,8 | 75 | 710 | 1,616 | 2,105 | 2,399 | 12 |
| 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 | 16,333 | 18,728 | 14,447 | 0.46 |
| 4 | 12 | M | 3,3; 2,2 | 1,139 | 6,149 | 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 | 116,278 | 167,211 | 1.0 |
| 9 | 30 | M | 4,4; 8,8 | 4,786 | 543 | 6,222 | – | 27,583 | 8.2 |
| 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**
| 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 | 38,110 | 13,902 | 93,221 | 8.1  |
| 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)

| Subjects | Age | Sex | HLA DR:DQ | T cell proliferation |
|----------|-----|-----|-----------|---------------------|
| 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

| Subjects | Age | Sex | HLA DR:DQ | T cell proliferation |
|----------|-----|-----|-----------|---------------------|
| 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 | 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 Smal- and EcoRI-cleaved DNA of the pGEX-3 expression vector downstream from glutathione-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). 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 Smal- and EcoRI-cleaved DNA of the pGEX-3 expression vector downstream from glutathione-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 0, 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 days of incubation in 5% CO2/air at 37°C, 1.0 μCi [3H]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. SI's 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.](image-url)
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; DQ2,8) predicts that APC can be detected not only in some recent-onset IDD subjects but also in a subset of subjects with/3 cell autoimmunity. 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 GAD67 central region contains a sequence (amino acids 208–404) with homology to the P2-C protein of Coxsackie virus B4 (14). Eight residues are identical to the Coxsackie

Figure 2. Proliferation of peripheral blood T cells in response to recombinant human islet GAD65 (amino acids 208–404).

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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