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Antigen presentation of detergent free glutamate decarboxylase (GAD65) is affected by human serum albumin as carrier protein

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1. Summary

The smaller isoform of glutamate decarboxylase (GAD65) is a major autoantigen in type 1 diabetes (T1D). Its hydrophobic character requires detergent to keep the protein in solution, which complicates studies of antigen processing and presentation. In this study an attempt was made to replace detergent with human serum albumin (HSA) for in vitro antigen presentation. Different preparations of recombinant human GAD65 complexed with HSA were incubated with Priess B cells (HLA DRB1*0401) and antigen presentation was tested with HLA DRB1*0401-restricted and epitope-specific T33.1 (GAD65 epitope 274-286) and T35 (GAD65 epitope 115-127) T cell hybridomas. Specific epitope recognition by T33.1 (274-286) and T35 (115-127) cells varied between the different GAD65/HSA preparations, and a reverse pattern of antigen presentation were detected by the two hybridoma. The HSA-specific T-cell hybridoma 17.9 response to the different GAD65/HSA preparations followed the same pattern as that observed for the T33.1 cells. The content of immunoreactive GAD65 measured with four GAD65 antibodies indicated that the lowest GAD65 concentration resulted in the highest 274-286, but the lowest 115-127 presentation. This suggests that HSA-GAD65 complexes qualitatively affect the epitope specificity of GAD65 presentation. HSA may enhance the 274-286 epitope presentation, while suppressing the 115-127 epitope.

Keywords

antigen presentation; GAD65; Human serum albumin; T cell assay; Type 1 diabetes; T cells; Diabetes; Autoimmunity; Antigen Presentation/Processing

2. Introduction

Type 1 diabetes mellitus (T1D) is an autoimmune disease that results in the specific destruction of the pancreatic islet beta cells. The smaller isoform of glutamate decarboxylase (GAD65) is implicated as a major contributing autoantigen in the pathogenesis of beta cell destruction [1, 2]. Autoantibodies directed to GAD65 (GAD65Ab) are present in the majority of new onset T1D patients [3,4] and GAD65-specific T cells have been identified in T1D patients and in spontaneously diabetic NOD mice and BB rats (for review see [5,6]). However, while GAD65Ab are readily detectable in radioligand binding assays with good correspondence between different laboratories [7], measurement of T cell responses to GAD65 vary markedly [8] and only some GAD65 preparations have shown reproducible results in international

standardization efforts [9]. The establishment of a standard T cell assay remains critical for the understanding of GAD65 antigen uptake, processing and presentation.

GAD65, which catalyzes the generation of the neurotransmitter GABA, is associated with intracellular membranes [10,11]. In vitro, the highly hydrophobic GAD65 requires detergents to remain in solution [12]. However, because detergents are extremely cytotoxic, it is critical that the detergent is removed prior to incubation with antigen-presenting cells (APC) and responder T cells.

In the present study, we tested the well known property of human serum albumin (HSA) to maintain proteins of hydrophobic character in solution (for review see [13,14]). The aim of our study was to test whether GAD65 complexed with HSA affected antigen presentation by human Priess B cells to two GAD65- and one HSA-specific specific HLA-matched T cell hybridoma as readouts of antigen presentation. The specific epitope response of the two GAD65-specific T cell hybridoma varied between the different GAD65/HSA preparations and appeared to be affected by the different GAD65/HSA ratios. These findings suggest that HSA-GAD65 complexes qualitatively affect the epitope specificity of GAD65 presentation. HSA may enhance the 274-286 epitope presentation, while suppressing the 115-127 epitope, possibly due to the hydrophobic character of the N-terminal region..

3. Materials and Methods

Cells The HLA DRB1*0401 positive Priess B cell line was used as APC. The Priess cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.292 mg/ml L-Glutamine (Gibco, Grand Island, NY), and 1 mmol/L sodium pyruvate (Gibco, Grand Island, NY). GAD65-specific murine T-cell hybridomas T33.1 (274-286) and T35 (115-127) were kindly donated by Dr. Linda Wicker (University of Cambridge, Cambridge, U.K.). The T33.1 (274-286) and T35 (115-127) cells, generated in an HLA-DR4 (DRB1*0401) transgenic mouse, recognized the GAD65 274-286 and 115-127 epitopes, respectively [1]. The T cell hybridomas were maintained in RPMI 1640 and 10% FCS, 1.0 µM β-mercaptoethanol (BioRad, Hercules, CA), 1 mmol/L sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. T-cell hybridomas were passed to 0.35×10⁶ cells per ml every 2-3 days.

The DRB1*0401-restricted HSA-specific hybridoma 17.9 recognized the HSA 64-76 epitope [15], and was grown in RPMI 1640 with 10% FCS.

Recombinant Human GAD65

A total of four GAD65 preparations (batch #2, #3, #4, and #5) were obtained from Diamyd Medical AB, Stockholm, Sweden. One sample (control GAD) contained only buffer without GAD65. These GAD65-HSA preparation are available from Diamyd Medical AB (<http://www.diamyd.com/>) as T cell GAD65. The samples were thawed, aliquoted, and stored at -80°C. When samples were used, each sample was thawed only once to ensure consistency.

T cell assays

Priess cells were plated at 0.10×10⁶ cells/well in 96 well tissue culture plates (BD, Franklin Lakes, NJ) in 100 µl of serum free medium (X-VIVO 15, Cambrex Life Sciences, Walkersville, MD). Antigen was added to wells in a total volume of 20 µl at desired concentrations bringing the total volume for each well to 120 µl. Cells were incubated at 37°C overnight. T cell hybridomas (100 µl) were next added to the Priess cells and the co-cultures incubated overnight at 37°C. The cells were frozen to stop the experiment. In experiments using HSA as antigen, T cell culture medium (RPMI 1640, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml

streptomycin, 0.292 mg/ml L-Glutamine, and 1 mmol/L sodium pyruvate) was used to substitute for the serum free medium because it contained HSA. No antigen was added in the negative controls, positive controls contained GAD65 peptides (274-286 for T33.1 cells), (115-127 for T35 cells), and HSA (2.5×10^{-3} g) (17.9 cells). The two batches of HSA used from Baxter Healthcare Corporation, Glendale, CA (Lot # 2837733AA) and from ZLB Bioplasma AG, Berne, Switzerland (Lot# 02714-00043) were comparable in the ability to stimulate IL-2 production in 17.9 cells.

IL-2 ELISA

Frozen cells were thawed and centrifuged at $750 \times g$ for 10 minutes to pellet cellular debris. Cell supernatants were diluted 1:2 and analyzed using an IL-2-specific ELISA (eBioscience, San Diego, CA). A total of 100 μ l of supernatant was removed and used in the IL-2 ELISA according to the manufacturer's instructions.

GAD65 Radioligand Binding Competition Assay

GAD65 concentrations were assessed in a competitive radioligand binding assay using two different GAD65Ab-positive antisera and two GAD65-specific monoclonal antibodies. Serum #673 is the World Health Organization (WHO) standard 97/550 [16] and serum sample #622 was derived from a set of ten well characterized sera from T1D patients [17,18]. The monoclonal antibodies included GAD-6 [19] and N-GAD65mAb [20], and recognized epitopes located at the C- and the N-terminus, respectively. The sera and monoclonal antibodies were first titrated for binding capacity. The competition experiments were next performed at the respective half maximal binding concentrations. The GAD65Ab-positive sera or monoclonal antibodies were incubated with recombinant human GAD65 (Diamyd Medicals AB) in concentrations ranging from 0.78-125 ng/ml and 35 S-labeled human recombinant GAD65 generated by coupled in vitro transcription translation [21]. The radioligand binding assay was performed as previously described [22,23].

Western Blotting

Protein samples were separated on 8% SDS-PAGE under reducing conditions and subsequently electrotransferred onto Immobilon-P membranes (Millipore, Boston, MA). Membranes were blocked with 1% BSA and incubated with 0.5 μ g/ml N-GAD65mAb or GAD-6. Bound mAb was detected by horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Immuno Research, West Grove, PA) at a dilution of 1/20,000. The immunocomplexes were detected with Enhanced Chemiluminescence Detection system (ECL, Amersham, Buckinghamshire, UK).

Statistical Analysis

Data was analyzed using Microsoft Excel spreadsheets (Microsoft, Redmond, WA) and Graph Pad Prism software (Graph Pad, San Diego, CA).

4. Results

GAD65 T33.1 (274-286) T Cell Hybridoma responses

Antigen presentation by Priess cells of the GAD65-HSA batches #2, #3, #4, and #5 to T33.1 (274-286) T cells showed quantitatively different antigen presentations of the GAD65 274-286 epitope (Fig. 1, left panel). GAD65 preparations resulted in dose-dependent T cell responses. Presentation of batch #2 resulted in the highest T cell response, being 69% higher in IL-2 production over batch #4 (mean difference 51 pg/ml, $p=0.007$), 132% higher than batch #5 (mean difference 72 pg/ml, $p=0.0035$), and 348% higher than batch #3 (mean difference 98 pg/ml, $p=0.001$). The rank order for the 274-286 GAD65 epitope was batch #2 > #4 > #5 > #3.

GAD65 T35 (115-127) T cell Hybridoma responses

Compared to the T33.1 (274-286) cell responses, there was a distinctly different antigen presentation pattern of the GAD65 115-127 epitope recognized by the T35 (115-127) cells (Fig. 1, right panel). When T35 (115-127) cells were used as responders, batch #5 showed the maximum effect yielding a 37% increase in IL-2 production over batch #3 (mean difference of 14 pg/ml, $p=0.001$), a 118% increase over batch #4 (mean difference 28 pg/ml, $p=0.015$) and 314% increase over batch #2 (mean difference 39 pg/ml, $p=0.02$). The rank order for the 115-127 GAD65 epitope therefore was #5 > #3 > #4 > #2.

HSA T cell hybridoma responses

As the four GAD65 preparations showed essentially a reverse order in 274-286 and 115-127 GAD65 epitope presentation, we next tested whether this was reflected in different antigen presentation responses to HSA (Fig 2). Using a standard curve with known amounts of HSA to determine the contribution of HSA in the different GAD65 preparations (data not shown), we observed significant differences in IL-2 production by the 17.9 cells when GAD65 preparations were tested (Fig. 2).

Batch #3 showed the weakest ability to stimulate the 17.9 cells (mean IL-2 levels was 129 pg/ml), with intermediate T cell responses to batches #5 (mean 170 pg/ml IL-2) and #4 (252 pg/ml IL-2). Batch #2 showed a HSA T cell response similar to the HSA control (366 pg/ml IL-2). However, because of the limited quantity of this preparation the experiment could not be repeated and is therefore not included in the figure. Therefore, the 17.9 cells responded to HSA in the GAD65 batches with a tentative rank order of #2 > #4 > #5 > #3, a distribution similar to the GAD65 274-286 responses of the T33.1 (274-286) T cell hybridoma.

Radioligand binding Competition assay

We next investigated whether the differences in presentation between the GAD65 274-286 and 115-127 were due to different contents of immunoreactive GAD65. The contents of GAD65 in each preparation was determined by two T1D standard sera and two monoclonal GAD65-specific antibodies in competition assays between the different GAD65-HSA preparations and radiolabeled GAD65 (Fig. 3). All four GAD65 preparations showed similar affinities when competing serum 673 (Fig. 3c). Binding of GAD65 to the T1D GAD65Ab-positive 622 serum was competed only weakly by batch #2, while the three other preparations showed comparable levels of competition (Fig. 3d). The N-terminus-specific N-GAD65mAb showed the strongest competition with batch #4, followed by #5, #3, and #2 (Fig. 3a) and binding of GAD65 to the C-terminus specific GAD6 monoclonal antibody required higher concentrations of GAD65 for displacement, but the rank order was comparable to the pattern of displacement with monoclonal antibody N-GAD65mAb. EC₅₀ values calculated for each GAD65 preparation in each competition assay showed that the rank order for the two monoclonal antibodies were batch #4 > #5 > #3 > #2, while 673 ranked #5 > #3 > #4 > #2 and 622 #4 > #5 > #3 > #2 (Table I).

Western blot analyses—We further evaluated the GAD65 content in the different preparations using the N-terminus specific monoclonal antibody N-GAD65mAb in Western blot analysis. We found that batches #2 and #3 had significantly less GAD65 as compared with batches #4 and #5 (data not shown). This confirmed our results of the radioligand binding competition assay.

GAD65/HSA ratio—Based on our results from the radioligand binding competition assay and the Western blot analysis and the HSA concentrations as derived from the 17.9 cell antigen presentation assay, we ranked the GAD65-HSA ratio in the different GAD preparations as follows: batch #2 < #3 < #5 < #4 (Table II).

5. Discussion

We studied the T cell response of two well characterized GAD65-specific T cell hybridomas to different preparations of GAD65 complexed with HSA using Priess cells as APC. The major finding is the distinct epitope presentation pattern of different GAD65/HSA antigen preparations, as recognized by the two GAD65 epitope-specific T cell hybridomas. The 115-127 (MNILLQYVVKSFD) epitope (recognized by T35 (115-127)) was presented in the order 5→3→4→2, while the 274-286 (IAFTSEHSHFSLK) epitope (recognized by T33.1 (274-286)) was presented in the order 2→4→5→3. Likewise, the response to HSA was identical to that of epitope 274-286, i.e. 2→4→5→3. Based on these findings, we suggest that the presence of HSA in these GAD65 preparations affects the antigen presentation of both the 115-127 and 274-286 epitopes. To our knowledge this or similar findings have not been reported previously. Although the chemical nature of the GAD65-HSA complex including any residual detergent need to be determined, there were no indication that increasing concentrations of the GAD65-HSA preparations were cytotoxic to the APC or the responder T cells.

We detected different GAD65 concentrations between the GAD65-HSA preparations with monoclonal antibodies that recognize linear epitopes. Our data suggest that for in vitro experiments, a single antigen-antibody interaction is not necessarily a viable measure of how GAD65 will be perceived by the APC. Ultimately, this implies that the use of one specific GAD65 antibody, particularly a polyclonal antibody, may not be sufficient to characterize the binding affinity of a given GAD65 antigen, particularly that of T cell autoreactivity and GAD immunizations [24]. At present, there is no consensus on the quantitative effects of GAD65 on APC in vitro, despite exhaustive attempts by investigators worldwide to standardize GAD65-specific T cell responses [8,9]. Our study suggests that factors other than GAD65 concentrations have to be taken into account when analyzing the ability of GAD65 to stimulate T cells in vitro.

However, the observed differences in GAD65 concentrations between the GAD65-HSA preparations alone could not account for the observed differences in the T cell responses. When testing for possible variations in the ratio between GAD65 and the carrier protein HSA, we found significant differences. Preparation #2 showed the lowest GAD65/HSA ratio and resulted in the highest T cell response for the T33.1 (274-286) cells, while the T35 (115-127) cells and the HSA-specific 17.9 cells responded only poorly to this preparation. Our data may suggest that GAD65 complexed with HSA is co-processed for antigen presentation and that the HSA/GAD65 ratio affects antigen presentation.

Antigen processing and subsequent presentation may be affected by a number of factors. Antigen-specific antibodies [25,26], complement fragments, and alpha-2-macroglobulin have been shown to modulate antigen processing and presentation [27]. HSA is well known for its ability to bind to a wide variety of agents and transport them in the blood stream. The hydrophobic cavities in two of the three subdomains of HSA are thought to provide the principal regions for ligand binding [28]. Although the chemical nature of the GAD65/HSA complex requires analyses beyond the scope of the present study, we can only speculate that the ratio between the two proteins may affect antigen processing and subsequent presentation. The N-terminus of GAD65 is very hydrophobic [29,30] and we speculate that this portion may be bound to HSA, due to the binding characteristics described above. Preparation #2 has the lowest GAD65 to HSA ratio and therefore the N-terminus may be engulfed by the carrier protein. This may have subsequent consequences for the processing and presentation of epitopes residing in this region. Our observation that the N-terminal specific T35 (115-127) cells showed the lowest response to this antigen preparation, while the middle region T33.1 (274-286) cells showed the highest response supports our speculation that the processing and presentation of the N-

terminus of GAD65 may be impaired by HSA. It is noteworthy that in T1D GAD65Ab to the N-terminal region are particularly rare [31–33], which may be due to its binding with hydrophobic carrier proteins in circulation.

In conclusion, our study of GAD65 complexed with HSA suggests that antigen processing and presentation by Priess cells may differ between preparations that vary in HSA concentration and the subsequent response by epitope-specific T cell clones.

Possible implications of these findings on antigen processing and presentation for in vitro studies and for the T1D-associated pathogenesis need to be determined.

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References

- [1]. Wicker LS, Chen SL, Nepom GT, Elliott JF, Freed DC, Bansal A, et al. Naturally processed T cell epitopes from human glutamic acid decarboxylase identified using mice transgenic for the type 1 diabetes-associated human MHC class II allele, DRB1*0401. *J Clin Invest* 1996;98:2597–603. [PubMed: 8958223]
- [2]. Nepom GT, Lippolis JD, White FM, Masewicz S, Marto JA, Herman A, et al. Identification and modulation of a naturally processed T cell epitope from the diabetes-associated autoantigen human glutamic acid decarboxylase 65 (hGAD65). *Proc Natl Acad Sci U S A* 2001;98:1763–8. [PubMed: 11172025]
- [3]. Liu E, Eisenbarth GS. Type 1A diabetes mellitus-associated autoimmunity. *Endocrinol Metab Clin North Am* 2002;31:391–410. [PubMed: 12092457]vii–viii
- [4]. Pihoker C, Gilliam LK, Hampe CS, Lernmark A. Autoantibodies in diabetes. *Diabetes* 2005;54(Suppl 2):S52–61. [PubMed: 16306341]
- [5]. Yoon JW, Jun HS, Santamaria P. Cellular and molecular mechanisms for the initiation and progression of beta cell destruction resulting from the collaboration between macrophages and T cells. *Autoimmunity* 1998;27:109–22. [PubMed: 9583742]
- [6]. Lernmark A. Controlling the controls: GAD65 autoreactive T cells in type 1 diabetes. *J Clin Invest* 2002;109:869–70. [PubMed: 11927613]
- [7]. Bingley PJ, Bonifacio E, Mueller PW. Diabetes Antibody Standardization Program: first assay proficiency evaluation. *Diabetes* 2003;52:1128–36. [PubMed: 12716742]
- [8]. Roep BO, Atkinson MA, van Endert PM, Gottlieb PA, Wilson SB, Sachs JA. Autoreactive T cell Responses in Insulin-dependent (Type 1) Diabetes Mellitus. Report of the First International Workshop for Standardization of T cell assays. *J Autoimmun* 1999;13:267–82. [PubMed: 10479395]
- [9]. Peakman M, Tree TI, Endl J, van Endert P, Atkinson MA, Roep BO. Characterization of preparations of GAD65, proinsulin, and the islet tyrosine phosphatase IA-2 for use in detection of autoreactive T-cells in type 1 diabetes: report of phase II of the Second International Immunology of Diabetes Society Workshop for Standardization of T-cell assays in type 1 diabetes. *Diabetes* 2001;50:1749–54. [PubMed: 11473034]
- [10]. Christgau S, Aanstoot HJ, Schierbeck H, Begley K, Tullin S, Hejnaes K, et al. Membrane anchoring of the autoantigen GAD65 to microvesicles in pancreatic b-cells by palmitoylation in the NH2-terminal domain. *J Cell Biol* 1992;118:309–20. [PubMed: 1321158]
- [11]. Dirks R Jr, Thomas A, Linsong L, Lernmark Å, Sherwin RS, De Camilli P, et al. Targeting of the 67-kDa isoform of glutamic acid decarboxylase to intracellular organelles is mediated by its interaction with the NH2-terminal region of the 65-kDa isoform of glutamic acid decarboxylase. *J. Biol. Chem* 1995;270:2241–6. [PubMed: 7836456]

- [12]. Christgau S, Schierbeck H, Aanstoot H-J, Aagaard L, Begley K, Kofod H, et al. Pancreatic β cells express two autoantigenic forms of glutamic acid decarboxylase, a 65-kDa hydrophilic form and a 64-kDa amphiphilic form which can be both membrane-bound and soluble. *J. Biol. Chem* 1991;266:21257–64. [PubMed: 1939164]
- [13]. Fasano M, Curry S, Terreno E, Galliano M, Fanali G, Narciso P, et al. The extraordinary ligand binding properties of human serum albumin. *IUBMB Life* 2005;57:787–96. [PubMed: 16393781]
- [14]. Kragh-Hansen U. Structure and ligand binding properties of human serum albumin. *Dan Med Bull* 1990;37:57–84. [PubMed: 2155760]
- [15]. Reijonen H, Elliott JF, van Endert P, Nepom G. Differential presentation of glutamic acid decarboxylase 65 (GAD65) T cell epitopes among HLA-DRB1*0401-positive individuals. *J Immunol* 1999;163:1674–81. [PubMed: 10415074]
- [16]. Mire-Sluis AR, Das RG, Lernmark Å. The World Health Organization International Collaborative Study for Islet Cell Antibodies. *Diabetologia* 2000;43:1282–92. [PubMed: 11079747]
- [17]. Ludvigsson J, Heding L, Liedén G, Marner B, Lernmark Å. Plasmapheresis in the initial treatment of insulin-dependent diabetes mellitus in children. *Br Med J* 1983;286:176–8. [PubMed: 6401518]
- [18]. Marner B, Lernmark Å, Ludvigsson J, MacKay P, Matsuba I, Nerup J, et al. Islet cell antibodies in insulin-dependent (Type 1) diabetic children treated with plasmapheresis. *Diabetes Res* 1985;2:231–6. [PubMed: 3905187]
- [19]. Gottlieb DI, Chang Y-C, Schwob JE. Monoclonal antibodies to glutamic acid decarboxylase. *Proc Natl Acad Sci* 1986;83:8808–12. [PubMed: 2430303]
- [20]. Hampe CS, Lundgren P, T.L. D, Hammerle LP, Marcovina SM, Lernmark Å. A Novel Monoclonal Antibody Specific for the N-Terminal End of GAD65. *J Neuroimmunology* 2001;113:63–71. [PubMed: 11137577]
- [21]. Grubin C, Daniels T, Karlsen AE, Boel E, Hagopian WA, Lernmark Å. The cDNA-directed, in vitro-synthesized nascent peptide of glutamic acid decarboxylase (GAD2) is the autoantigen in insulin-dependent diabetes. *Clin Research* 1992;40:299A.
- [22]. Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, et al. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* 1994;37:344–50. [PubMed: 8063033]
- [23]. Falorni A, Örtqvist E, Persson B, Lernmark Å. Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using ^{35}S or ^3H recombinant human ligands. *J Immunol Methods* 1995;186:89–99. [PubMed: 7561152]
- [24]. Agardh CD, Cilio CM, Lethagen A, Lynch K, Leslie RD, Palmer M, et al. Clinical evidence for the safety of GAD65 immunomodulation in adult-onset autoimmune diabetes. *J Diabetes Complications* 2005;19:238–46. [PubMed: 15993359]
- [25]. Watts C. Suppressive effect of antibody on processing of T cell epitopes. *J Exp Med* 1993;178:1459–63. [PubMed: 7690836]
- [26]. Simitsek PD, Campbell DG, Lanzavecchia A, Fairweather N, Watts C. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J. Exp. Med* 1995;181:1957–63. [PubMed: 7539034]
- [27]. Jacquier-Sarlin MR, Gabert FM, Villiers MB, Colomb MG. Modulation of antigen processing and presentation by covalently linked complement C3b fragment. *Immunology* 1995;84:164–70. [PubMed: 7890301]
- [28]. Zhong D, Douhal A, Zewail AH. Femtosecond studies of protein-ligand hydrophobic binding and dynamics: human serum albumin. *Proc Natl Acad Sci U S A* 2000;97:14056–61. [PubMed: 11106379]
- [29]. Bu DF, Tobin AJ. The exon-intron organization of the genes (GAD1 and GAD2) encoding two human glutamate decarboxylases (GAD67 and GAD65) suggests that they derive from a common ancestral GAD. *Genomics* 1994;21:222–8. [PubMed: 8088791]
- [30]. Bu D-F, Erlander MG, Hitz BC, Tillakaratne NJK, Kaufman DL, Wanger-McPherson CB, et al. Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. *Proc Natl Acad Sci* 1992;89:2115–9. [PubMed: 1549570]

- [31]. Hampe CS, Hammerle LP, Bekris L, Orqvist E, Kockum I, Rolandsson O, et al. Recognition of Glutamic Acid Decarboxylase (GAD) by Autoantibodies from Different GAD Antibody-Positive Phenotypes. *J Clin Endocrinol Metab* 2000;85:4671–9. [PubMed: 11134126]
- [32]. Kim J, Namchuk M, Bugawan T, Fu Q, Jaffe M, Shi Y, et al. Higher autoantibody levels and recognition of a linear NH2-terminal epitope in the autoantigen GAD65, distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. *J Exp Med* 1994;180:595–606. [PubMed: 7519242]
- [33]. Falorni A, Ackefors M, Carlberg C, Daniels T, Persson B, Robertson J, et al. Diagnostic sensitivity of immunodominant epitopes of glutamic acid decarboxylase (GAD65) autoantibodies in childhood IDDM. *Diabetologia* 1996;39:1091–8. [PubMed: 8877294]

Figure 1.

T cell responses to different GAD65 preparations

GAD preparations #2 (black squares), #4 (black inverted triangle), #5 (black diamond), and #3 (black triangle) were tested at the indicated concentrations with T cell hybridoma T33.1 specific to GAD65 peptide fragment 274-286 (left panel) and T cell hybridoma T35 (115-127) specific to GAD65 peptide fragment 115-127 (right panel), IL-2 responses of the T cell hybridoma is reported. IL-2 production in the presence of no antigen (black circle) is shown. The mean of four independent experiments is represented in the graph, standard error is indicated.

Figure 2.

Different GAD65 preparations induced different T cell responses by HSA specific T cell hybridomas, indicating different HSA-concentrations. Control GAD contains only the preparation buffer without GAD65,

Figure 3.

Different GAD preparations #4 (black diamond), #5 (black circle), #3 (black inverted triangle), and #2 (black triangle) were used in radioligand binding competition assays with monoclonal antibodies N-GAD65mAb (3a), and GAD6 (3b), human polyclonal serum 673 (3c), and human polyclonal serum 622 (3d). Competition observed in the presence of GAD buffer (black square) and BSA (white square) is shown.

Table IEffective concentration of GAD65 at 50 percent displacement (EC_{50}) in the GAD65Ab radioligand binding assay

Antibody	GAD65-HSA batch EC_{50}			
	#2	#3	#4	#5
N-GAD65mAb	78.4	19.7	4.9	10.2
GAD6	N/A	215.6	54.2	208.7
Serum 673	0.76	0.35	0.43	0.19
Serum 622	66.2	20.8	8.5	12.52

EC_{50} values (ng/ml) are shown for each GAD preparation in all four competition assays.

NA is not analyzed.

Table II
Estimated GAD65/HSA ratio and T cell responses to GAD65 274-286 and 115-127 epitopes

Batch #	GAD65 (arbitrary units)	HSA (mg/ml)	GAD65/HSA ratio	T35 (115-127)	T33.1 (274-286)
2	0.1	0.078	1.3	+	++++
3	0.3	0.025	12	+++	+
5	0.5	0.033	15	++++	++
4	1	0.05	20	++	+++

GAD65 concentrations in arbitrary units, HSA concentrations, GAD65/HSA ratio, and relative T cell responses are indicated for each GAD65 preparation.