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Expression of Recombinant Human Glutamic Acid Decarboxylase (GAD) in Myeloma Cells and Enzyme-Linked Immunosorbent Assay (ELISA) for Autoantibodies to GAD

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Detection of serum autoantibodies to glutamic acid decarboxylase (GAD) is a new method to differentiate insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). We established a transformed mouse myeloma cell line, SPG14, which expresses recombinant human GAD65, a major isomer of 65 kDa, inside the cells. GAD65 was partially purified by affinity chromatography using the mouse anti-GAD antibody (Ab). We also established a sandwich ELISA for anti-GAD Ab of the IgG class using GAD65 for coating and the anti-human IgG for detection and examined 54 sera of the IDDM patients and 45 sera of normal individuals. When the mean+2 SD of the color development of the sera of normal individuals was used as a cut-off level, 59.2% of patients with IDDM were positive. This indicates that the ELISA was effective to differentiate IDDM and NIDDM. The result also indicates that the autoantibody to GAD does not dissociate from the recombinant GAD rapidly, even when unbound anti-GAD Ab was fully removed. By using a perfusion culture system, we obtained as many as 4.2×10^{10} SPG14 cells, from which 5 mg of GAD65 could be obtained; this is sufficient for 5,000 assays. This system could be clinically useful for large-scale diagnosis of IDDM.

Key words: autoantibody, ELISA, GAD, IDDM, myeloma cell.

Glutamic acid decarboxylase (GAD) catalyzes the formation of γ-aminobutyric acid from glutamic acid and exists as two isoforms of 67 kDa (GAD67) (*1*) and 65 kDa (GAD65) (*2*), the latter of which is a major autoantigen in insulin-dependent diabetes mellitus (IDDM) (*3-6*). Serum autoantibody to GAD has been detected as a differentiation marker between IDDM and non-insulin-dependent diabetes millitus (NIDDM), and furthermore as a primary immunological marker in IDDM for predicting which patients with apparent NIDDM at diagnosis will subsequently progress to IDDM (*7-11*).

A number of different assays including enzymatic immuno-precipitation assay (*3*), radiobinding assay (RBA) (*7, 12-15*), enzyme-linked immunosorbent assay (ELISA) using regional peptides of GAD (*16*), immunofluorescence assay (*17*), and Western blotting (*18*) have been reported for measurement of autoantibodies to GAD and compared with each other (*19*). Of the various methods, the RBAs had relatively high scores in terms of disease sensitivity and specificity (*19*). However, sandwich ELISA for anti-GAD antibody (Ab) is desirable if its sensitivity and specificity are high enough, because it is applicable to a fully automated system for a large number of screenings. Such a system requires a large amount of recombinant GAD having the same structure as the native enzyme in terms of recognition patterns by a variety of serum autoantibodies to GAD. Although the expression of recombinant GAD has been reported in *Escherichia coli* (*20, 21*), insect cells (*22, 23*), CHO cells (*24, 25*), and COS cells (*8*), no system has been reported for producing batches of mg levels of recombinant GAD65 which can be recognized by a variety of serum autoantibodies to GAD.

In this study, we established myeloma cells highly expressing recombinant human GAD65 and established a two-step sandwich ELISA for the measurement of autoantibody to GAD in the sera of patients of IDDM.

MATERIALS AND METHODS

cDNA Cloning of Human GAD65 and Construction of Plasmids—A cDNA clone coding for human GAD65 was isolated from a human pancreas cDNA library (Clontech, USA) with 30 cycles of polymerase chain reaction (PCR) (94°C for 1 min, 54°C for 2 min, 72°C for 2 min) using Taq DNA polymerase and primers 5′-TTGTCGACATGGCATCTCCGGGCTCTGGC-3′ and 5′-GGGCGGCCGCTCTAGAAATCTTGTCCTAGGCGTTC-3′. The amplified product was cloned by use of a TA cloning kit (Invitrogen, USA), and cDNA for GAD65 was selected after sequencing analysis by the dideoxynucleotide chain-termination method with an automated sequencing apparatus (Applied Biosystems, USA). cDNA for GAD65 was digested with *Sal*I and *Not*I and inserted into the plasmid BCMGSneo (*26, 27*) to give the plasmid BCMGS-GAD (Fig. 1).

Transfection of SP2/0 Cells and Selection of Transformants—The plasmid BCMGS-GAD (10 μg) was transfect-

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ed into 2×10^7 cells of a non-immunoglobulin-secreting mouse myeloma (CRL1581; ATCC) in PBS on ice by electroporation using a Gene Pulser (Biorad, USA) at 900 V and 25 μF. After incubation on ice for 10 min, the cells were cultured in E-RDF medium supplemented with 10% fetal calf serum for 24 h then further cultured in a selective medium (E-RDF medium containing 10% fetal calf serum and 600 μg/ml G418) for 3 weeks, and subjected to limiting dilution. Each clone was checked for expression of GAD65, as will be described later. One clone expressing GAD65 was selected and named SPG14.

Western Blot of GAD65—Each cellular extract was separated by 10% SDS-PAGE under non-reducing conditions, then transferred to a Hybond-C super membrane (Amersham, USA) using a mini-transblot cell (Bio-Rad) at 50 V for 2 h. For blocking, the membrane was soaked in the PBS buffer overnight containing 5% skim milk and 0.1% Tween 20 at 4°C. It was then incubated with mouse anti-GAD monoclonal Ab GAD6 (Developmental Studies Hybridoma Bank, USA) (1 μg/ml) in PBS containing 3% skim milk for 2 h at room temperature. After washing with PBS, the membrane was incubated with goat anti-mouse IgG coupled to horseradish peroxidase (1 μg/ml) in PBS containing 3% skim milk for 2 h at room temperature. These blots were developed using an ECL Western blotting detection system (Amersham, USA).

Purification of GAD65—SPG14 cells (8.4×10^8) were suspended in 10 ml of lysis buffer [25 mM potassium phosphate buffer, pH 7.0, 0.2 mM pyridoxal phosphate (PLP), 1 mM 2-aminothylisothiouronium bromide (AET), 1 mM benzamidine hydrochloride] and homogenized with a Teflon homogenizer at 0°C. The homogenate was subjected to high speed centrifugation, and the supernatant was collected as the cellular extract. Purified mouse anti-GAD monoclonal Ab GAD1 (HB184; ATCC, USA) (10 mg) was conjugated to the HiTrap NHS-activated resin (1 ml) (Pharmacia, Sweden) according to the manufacturer's instructions. The cellular extract was loaded into the column equilibrated with the binding buffer (50 mM potassium phosphate buffer, pH 7.2, 0.2 mM PLP, 1 mM AET) and washed with the same buffer (5 ml). The bound GAD65 was eluted from the column with the elution buffer (50 mM potassium phosphate buffer, pH 11.0, 0.2 mM PLP, 1 mM AET, 20 mM glutamic acid, 10 mM diethanolamine) and neutralized to pH 7.0 with 0.1 N HCl.

Radiobinding Assay for Anti-GAD Ab—An anti-GAD antibodies detection kit (Hoechst Japan, Tokyo) was used according to the manufacturer's instructions. Briefly, assay buffer (100 μl) and serum (20 μl) were mixed, ^{125}I-labeled porcine GAD was added, and the mixture was incubated at room temperature for 2 h. The reaction was stopped by adding 1 ml of precipitation reagent containing goat anti-human IgG. The tubes were agitated well, incubated for 30 min at room temperature, and centrifuged at $1,500\times g$ for 30 min at 4°C. The radioactivity of the pellet was measured with a γ-counter (Aloka, Tokyo).

Establishment of ELISA for Anti-GAD Ab—Ninety-six-well microplates were coated with purified GAD65 (100 μl of 10 μg/ml) in coating buffer (0.1 M sodium carbonate, pH 9.6) and blocked with 1% BSA in PBS. A test sample of 100 μl (10 μl of serum plus 90 μl of 1% BSA in PBS) was added to each well and incubated at 37°C for 2 h. The bound autoantibodies to GAD65 were detected by sequential incubation with alkaline phosphatase-conjugated goat anti-human IgG and the enzyme substrate solution.

Patients—Sera from 54 patients with IDDM and 45 normal individuals were used. The diagnosis of IDDM was based on occurrence of ketoacidosis at onset or a history of predisposition to ketosis, daily insulin requirements of >0.7 U/kg/day, daily C-peptide excretion in urine, and serum C-peptide responses to glucagon (1 mg i.v.).

Suspension Fermentation of SPG14 Cells—A 3-liter fermentor (Axellon; Kirin Brewery, Tokyo) was used. Initially, 3×10^8 SPG14 cells were seeded in the medium based on E-RDF containing 10% FCS and 600 μg/ml G418 (Sigma, USA) and cultured for 3 days. When the cell concentration reached 1×10^6/ml, fresh medium was perfused at a rate of 3-liter/day for 3 days, resulting in a cell concentration of 1.4×10^7/ml. During the culture, the temperature was controlled at 37°C, and the pH was maintained at 7.0 using carbon dioxide gas and NaOH. The dissolved oxygen tension was 5 ppm. SPG14 cells were collected by centrifugation at 1,000 rpm at 4°C for 5 min, resuspended with ice-cold PBS and collected by centrifugation under the same conditions. These cells were frozen at −80°C before use.

RESULTS

Establishment of Human GAD65-Expressing Myeloma Cells—Our initial goal was to establish a cell line which highly expressed recombinant human GAD65 and could grow in a suspension culture. For this purpose, we selected mouse myeloma cells SP2/0, a non-immunoglobulin-secreting mouse myeloma, as the host and BCMGSneo as the expression vector. As shown in Fig. 1, BCMGS-GAD contains a DNA sequence encoding the human GAD65 under the transcriptional control of the cytomegalovirus promoter. It also contains 69% (5.4 kb) of the bovine papilloma virus gene to afford a high copy number (20–200). BCMGS-GAD was transfected into SP2/0 cells. After limiting dilution of G418-resistant cells, the expression levels of clones were determined by Western blotting using anti-GAD monoclonal Ab GAD6 and compared with each other. As shown in Fig. 2, one stable transformant which expressed GAD65 at a higher level than other transformants and the previously established CHO transformants

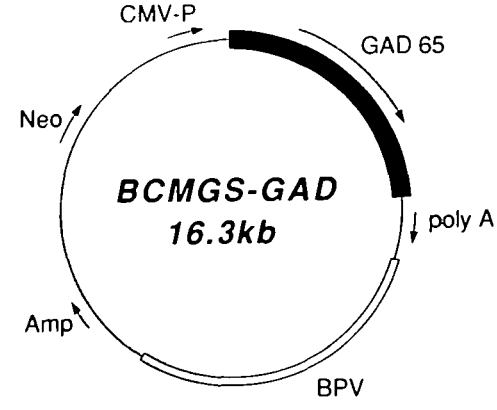

Fig. 1. **Construction of BCMGS-GAD.** CMV-P, cytomegalovirus promoter; BPV, bovine papilloma virus; Amp, β-lactamase; poly A, polyadenylate attachment signal.

expressing GAD65 (GAD-CHO) (*25*) was picked up, named SPG14, and used for further analysis. We speculate that the bands with an M_r of 90,000 are undenatured GAD65, because they did not appear when the cellular extract was boiled before loading onto gels (data not shown).

Purification of Recombinant Human GAD65 from SPG14 Cells—For purification of recombinant GAD65 by affinity chromatography, we used mouse anti-GAD monoclonal Ab GAD1 instead of GAD6, because GAD65 bound so strongly to GAD6 that it could not be eluted (data not shown). The cellular extract from SPG14 was applied onto a GAD1-conjugated affinity column, and the eluate was subjected to SDS-PAGE under non-reducing conditions, followed by Coomassie Brilliant Blue (CBB) staining. As shown in Fig. 3, a protein band with an M_r of 65,000 was detected, indicating that the recombinant GAD65 was partially purified by a single affinity chromatography. We also estimated the concentration of this GAD65 by SDS-PAGE and CBB staining together with varying concentrations of bovine serum albumin (BSA) (data not shown). Based on this estimation, the total amount of GAD65 from 4.2×10^{10} SPG14 cells, the number obtained in one batch of 3-liter perfusion culture, was calculated to be approximately 5 mg.

Binding of Recombinant Human GAD65 to Serum Autoantibodies to GAD—To determine whether the recombinant GAD could be used as a component of the ELISA for serum autoantibody to GAD, we first examined the binding of recombinant GAD65 expressed in SPG14 cells to serum autoantibodies to GAD. For this purpose, a radiobinding assay kit for anti-GAD Ab, namely, an anti-GAD antibodies detection kit (Hoechst Japan), was used. As shown in Fig. 4, the cellular extract of SPG14 cells dose-dependently inhibited the binding of porcine-derived GAD with serum autoantibody to GAD, whereas the cellular extract of control SP2/0 cells did not. This result showed that the GAD65 in the cellular extract of SPG14 could be recognized by serum autoantibodies to GAD in competition with porcine-derived GAD. The partially purified GAD65 showed the same result (data not shown).

Establishment of ELISA for Autoantibody to GAD65 Using Purified GAD65—We established a sandwich

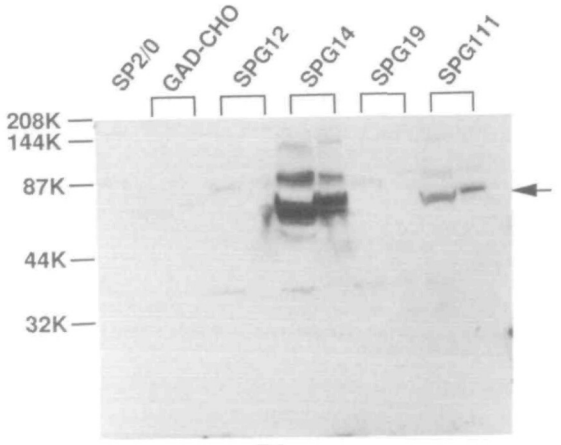

Fig. 2. **Expression of GAD65 in each clone.** The cellular extract (20 μl for the left and 5 μl for the right lane of two lanes of each clone) from the negative control cell (SP2/0), recombinant human GAD65-expressing CHO cells, and each transformant of myeloma cells was subjected to Western blotting analysis. The arrows indicate the position of the M_r corresponding to GAD65.

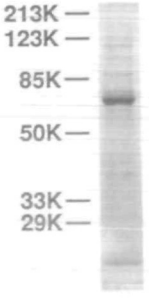

Fig. 3. **SDS-PAGE of purified GAD65.** Purified GAD65 was analyzed by electrophoresis on a 0.1% SDS/10% polyacrylamide gel under reducing conditions. The M_r of standards is shown on the left. The gel was stained with Coomassie Brilliant Blue.

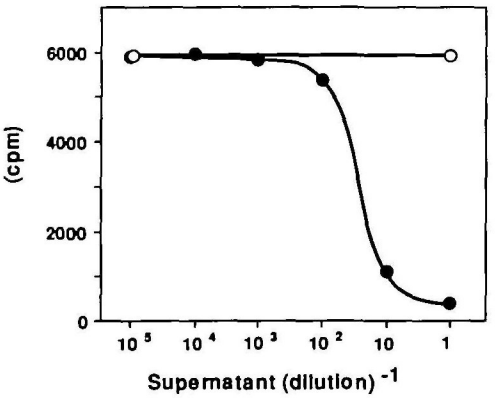

Fig. 4. **Inhibition by recombinant GAD65 of the binding of serum autoantibody to GAD to porcine-derived GAD.** The radiobinding assay was performed using sera (20 μl) containing 125 U/ml of autoantibodies to GAD using the anti-GAD antibodies detection kit (Hoechst Japan) and the cellular extracts (100 μl) of SPG14 (closed circles) or SP2/0 (open circles) which were serially diluted with PBS containing 1% BSA. The average values of duplicates are shown.

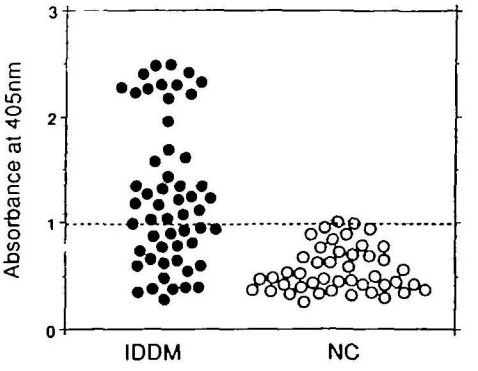

Fig. 5. **Detection of autoantibodies to GAD65.** Sera from patients with IDDM ($n=54$) and from normal controls (NC) ($n=45$) were diluted 10-fold and subjected to ELISA. All experiments were carried out in duplicate. Mean+2 SD of the sera from normal individuals is shown by the broken line.

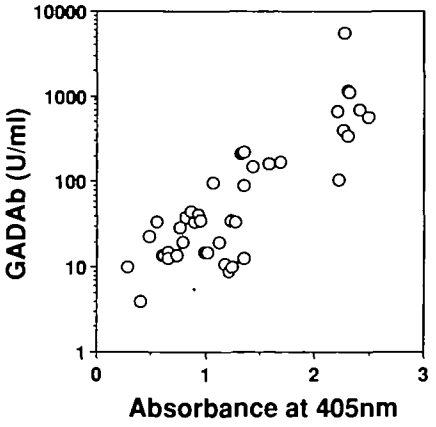

Fig. 6. **Comparison of the ELISA and the radiobinding assay for anti-GAD antibody.** The sera of patients were tested using both ELISA and the radiobinding assay with the anti-GAD antibodies detection kit (Hochest Japan). The results were then plotted. The average values of duplicates are shown.

ELISA for anti-GAD Ab of the IgG class using the purified GAD65 for coating and the anti-human IgG for detection, and examined whether the ELISA could differentiate sera from IDDM patients and normal individuals. Figure 5 shows a typical result: when the mean+2 SD of the color development in normal individuals was used as a cut-off level (broken line in Fig. 5), 59.2% (32/54) of the patients with IDDM were positive. The high cut-off level ($A=0.5$) might be due to non-specific binding of serum immunoglobulin to the blocking reagent, because a similar background level was observed for the sera containing autoantibody to GAD, which was supplied with the anti-GAD antibodies detection kit (Hoechst Japan).

In addition, as shown in Fig. 6, positive correlation was found for these 54 sera of IDDM patients between the color development by the ELISA we established and the calculated concentration of anti-GAD Ab assayed with the anti-GAD antibodies detection kit (Hoechst Japan). The results indicated that the ELISA we established could differentiate IDDM from normal individuals. The results also indicated that serum autoantibody to GAD does not dissociate from the recombinant GAD rapidly, because the bound anti-GAD Ab could be detected in the ELISA by means of sequential incubation of anti-human IgG and alkaline phosphatase after unbound anti-GAD Ab was fully removed.

Fermentation of SPG14—We used a 3-liter fermentor (Axellon; Kirin Brewery, Tokyo) for culture of the SPG14 cells. We inoculated 3×10^8 cells and obtained 4.2×10^{10} cells in one batch of fermentation, from which 5 mg of GAD65 was obtained. This amount is sufficient for 5,000 assays if 1 μg of GAD65 (100 μl of 10 μg/ml GAD65) is used for coating one well.

DISCUSSION

In this report, we have described the transformed myeloma cell line SPG14 highly expressing human GAD65 and the usefulness of recombinant GAD65 for sandwich ELISA for detecting autoantibodies to GAD in the sera of patients with IDDM.

The recombinant antigen, as a component of the ELISA for antigen-specific antibody in sera, should be recognized by a variety of serum autoantibodies to the same antigen. Therefore, we chose the mammalian expression system because, in general, the recombinant protein expressed in the mammalian cells retains the native structure, while that expressed in *E. coli* or yeast does not. For example, the recombinant thyrotropin receptor (thyroid stimulating hormone receptor; TSHR) expressed in the mammalian system bound a variety of serum autoantibodies to TSHR, whereas that expressed in *E. coli* did not (*28*, and unpublished data). Indeed, the recombinant GAD65 we prepared, as well as other recombinant GAD65s expressed in mammalian systems, could bind serum autoantibody to GAD whereas that expressed in *E. coli* or yeast could not (unpublished data).

In general, the quantity and purity of the components required for ELISA are much higher than those for RIA, although ELISA is better in terms of manipulation. This might explain why such diagnostic items as thyrotropin binding-inhibiting immunoglobulin (TBII) can still be performed only by RIA. In the case of anti-GAD Ab, EIA is inferior to RIA in terms of specificity (*19*): its sensitivity was fairly low (15-36%), which we speculate might be due to the productivity of the recombinant GAD65. Of various mammalian expression systems, we chose myeloma expression systems because the suspension culture could be easily performed. Indeed, we obtained 5 mg of recombinant GAD65 from one batch of culture, which was sufficient for 5,000 ELISAs. In our study, when the mean+2 SD of the color development in normal individuals was used as a cut-off level, the specificity was 95.6%, and the sensitivity was 59.2% (32/54). To our knowledge, the productivity of our system is much higher than that of other systems so far reported.

In order to quantitate serum autoantibody to GAD by ELISA, a purified standard substance is preferable. We presume that a mixture of genetically engineered chimeric anti-GAD monoclonal Abs of mouse Ig-derived antigen binding regions and human Ig-derived constant regions might work as a standard. Preparation of various monoclonal Abs to the recombinant GAD65 is currently under way.

The binding constants such as the affinity rate constant (k_{ass}), the dissociation rate constant (k_{diss}) and the affinity constant ($K_A=k_{ass}/k_{diss}$) of serum autoantibodies to GAD have not been well characterized. We showed that serum autoantibodies to GAD did not dissociate from the recombinant GAD65 rapidly even after the removal of unbound Ab and could be detected by sequential incubation with anti-human IgG and alkaline phosphatase. The binding constants might also be obtained by surface plasmon resonance measurement by immobilizing the recombinant GAD65 and injecting the serum of patients with IDDM.

The availability of GAD65 at mg levels might allow establishment of GAD-reactive T cells and anti-GAD Ab-producing B cells from the patients with IDDM. Also, the recombinant GAD might be used for administration to experimental animals.

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REFERENCES

1. Kobayashi, Y., Kaufman, D.L., and Tobin, A.J. (1987) Glutamic acid decarboxylase cDNA: Nucleotide sequence encoding an enzymatically active fusion protein. *J. Neurosci.* **7**, 2768-2772
2. Erlander, M.G., Tillakaratne, N.J.K., Feldblum, S., Patel, N., and Tobin, A.J. (1991) Two genes encode distinct glutamate decarboxylase. *Neuron* **7**, 91-100
3. Bakkeskov, S., Aanstoot, H.J., Christau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H., and De Camilli, P. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* **347**, 151-156
4. Karlsen, A.E., Hagopian, W.A., Petersen, J.S., Boel, E., Dyrberg, T., Grubin, C.E., Michelsen, B.K., Madsen, O.D., and Lernmark, A. (1992) Recombinant glutamic acid decarboxylase (representing the single isoform expressed in human islets) detects IDDM-associated 64,000-M_r autoantibodies. *Diabetes* **41**, 1355-1359
5. Velloso, L.A., Kampe, O., Hallberg, A., Christmanson, L., Betsholtz, C., and Karlsson, F.A. (1993) Demonstration of GAD-65 as the main immunogenic isoform of glutamate decarboxylase in Type 1 diabetes and determination of autoantibodies using a radioligand produced by eukaryotic expression. *J. Clin. Invest.* **91**, 2084-2090
6. Karlsen, A.E., Hapgopian, W.A., Grubin, C.E., Dube, S., Disteche, C.M., Adler, D.A., Barmeier, H., Mathewes, S., Grant, F.J., Foster, D., and Lernmark, A. (1991) Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. *Proc. Natl. Acad. Sci. USA* **88**, 8337-8341
7. Rowley, M.J., Mackay, I.R., Chen, Q.-Y., Knowles, W.J., and Zimmet, P.Z. (1992) Autoantibodies to glutamic acid decarboxylase discriminate major type of diabetes mellitus. *Diabetes* **41**, 548-551
8. Tuomi, T., Groop, L.C., Zimmet, P.Z., Rowley, M.J., Knowles, W., and Mackay, I.R. (1993) Autoantibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with non-insulin dependent onset of diabetes. *Diabetes* **42**, 359-362
9. Hagopian, W.A., Karlsen, A.E., Gottsater, A., Landin Olsson, M., Grubin, C.E., Sundkvist, G., Petersen, J.S., Boel, E., Dyrberg, T., and Lernmark, A. (1993) Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 65K autoantibody positivity at onset predicts diabetes type. *J. Clin. Invest.* **91**, 368-374
10. Zimmet, P.Z., Tuomi, T., Mackay, I.R., Rowly, M.J., Knowles, W., and Cohen, M. (1994) Latent autoimmune diabetes mellitus in adults (LADA): The role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabetic Med.* **11**, 299-303
11. Kawasaki, E., Takino, H., Yano, M., Uotani, S., Matsumoto, K., Takano, Y., Yamaguchi, Y., Akazawa, S., and Nagataki, S. (1994) Autoantibodies to glutamic acid decarboxylase in patients with IDDM and autoimmune thyroid disease. *Diabetes* **43**, 80-86
12. Mauch, L., Seissler, J., Haubruck, H., Cook, N.J., Abney, C.C., Berthold, H., Wirbelauer, C., Liedvogel, B., Scherbaum, W.A., and Northeman, W. (1993) Baculovirus-mediated expression of human 65 kDa and 67 kDa glutamic acid decarboxylase in SF9 insect sells and their relevance in diagnosis of insulin-dependent diabetes mellitus. *J. Biochem.* **113**, 699-704
13. Grubin, C.E., Daniels, T., Toivola, B., Landin-Olsson, M., Hagopian, W.A., Li, L., Karsen, A.E., Boel, E., Michelsen, B., and Lernmark, A. (1994) A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* **37**, 344-350
14. Petersen, J.S., Hejnaes, K.R., Moody, A., Karsen, A.E., Marshell, M.O., Hoier-Madsesn, M., Boel, E., Michelsen, B.K., and Dyrberg, T. (1994) Detection of GAD65 antibodies in diabetes and other autoimmune disease using a simple radioligand assay. *Diabetes* **43**, 459-467
15. Yano, M., Moriuchi, R., Kawasaki, E., Watanabe, M., Saito, K., Takino, H., Akazawa, S., Miyamoto, T., and Nagataki, S. (1995) Autoantibodies against glutamic acid decarboxylase 65 in Japanese patients with insulin-dependent diabetes millitus (IDDM). *J. Autoimmun.* **8**, 83-96
16. DeAizpurua, H.J., Harrison, L.C., and Cram, D.S. (1992) An ELISA for antibodies to recombinant glutamic acid decarboxylase in IDDM. *Diabetes* **41**, 1182-1187
17. Gonovese, S., Bonifacio, E., McNally, J.M., Dean, B.M., Wagner, R., Bosi, E., Gale, E.A.M., and Bottazzo, G.F. (1992) Distinct cytoplasmic islet cell antibodies with different risks for type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* **35**, 385-388
18. Solimena, M., Folli, F., Denis-Donini, S., Comi, G.C., Pozza, G., De Camilli, P., and Vicari, A.M. (1988) Autoantibodies to glutamic acid decarboxylase in a patient with stiff-man syndrome, epilepsy, and type I diabetes mellitus. *N. Engl. J. Med.* **318**, 1012-1020
19. Schmidli, R.S., Colman, P.G., Bonifacio, E., and Participating Laboratories (1995) Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. *Diabetes* **44**, 636-640
20. Atkinson, M.A., Kaufman, D.L., Cambell, L., Gibbs, K.A., Shah, S.C., Bu, D.F., Erlander, M.G., Tobin, A.J., and Maclaren, N.K. (1992) Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet* **339**, 458-459
21. Pleau, J.-M., Fernandes-Saravia, F., Esling, A., Homo-Delarche, F., and Dardenne, M. (1995) Prevention of autoimmune diabetes in nonobese diabetes female mice by treatment with recombinant glutamic acid decarboxylase (GAD65). *Clin. Immunol. Immunopathol.* **76**, 90-95
22. Moody, A.J., Hejnaes, K.R., Marshall, M.O., Larsen, F.S., Boel, E., Svendsen, I., Mortensen, E., and Dyrberg, T. (1995) Isolation by anion-exchange of immunologically and enzymatically active human islet glutamic acid decarboxylase 65 over expression in Sf9 cells. *Diabetologia* **38**, 14-23
23. Mauch, L., Seißler, J., Haubruck, H., Cook, N.J., Abney, C.C., Berthold, H., Wirbelauer, C., Liedvogel, B., Scherbaum, W.A., and Northmann, W. (1993) Baculovirus-mediated expression of human 65 kDa and 67 kDa glutamic acid decarboxylase in SF9 insect cells and their relevance in diagnosis of insulin-dependent diabetes mellitus. *J. Biochem.* **113**, 699-704
24. Solimena, M., Aggujard, D., Muntzel, C., Dirkx, R., Butler, M., Camilli, O. De, and Hayday, A. (1993) Association of GAD-65, but not of GAD67, with the golgi complex of transfected Chinese hamster ovary cells mediated by the N-terminal region. *Proc. Natl. Acad. Sci. USA* **90**, 3073-3077
25. Kawasaki, E., Moriuchi, R., Watanabe, M., Saitoh, K., Brunicardi, F.C., Watt, P.C., Yamaguchi, T., Mullen, Y., Akazawa, S., Miyamoto, T., and Nagataki, S. (1993) Cloning and expression of large isoform of glutamic acid decarboxylase from human pancreatic islet. *Biochem. Biophys. Res. Commun.* **192**, 1353-1359
26. Karasuyama, H. and Melchers, F. (1988) Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* **18**, 97-104
27. Karasuyama, H., Tohyama, N., and Tada, T. (1989) Autocrine growth and tumorigenicity of interleukin 2-dependent helper T cells transfected with IL-2 gene. *J. Exp. Med.* **169**, 13-25
28. Matsuba, T., Yamada, M., Suzuki, H., Kanai, A., Isozaki, O., Yoshida, T., Tsushima, T., and Yasukawa, K. (1995) Expression of recombinant human thyrotropin receptor in myeloma cells. *J. Biochem.* **118**, 265-270