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Value of antibodies to GAD_{65} combined with islet cell cytoplasmic antibodies for predicting IDDM in a childhood population

H.-J. Aanstoot[1], E. Sigurdsson[2], M. Jaffe[1], Y. Shi[1], S. Christgau[1,2], D. Grobbee[3], G. J. Bruining[4], J. L. Molenaar[5], A. Hofman[3], S. Baekkeskov[1,2]

[1] Hormone Research Institute, Departments of Microbiology/Immunology and Medicine, University of California, San Francisco, California, USA
[2] Hagedorn Research Laboratory, Gentofte, Denmark
[3] Department of Epidemiology, Erasmus University Medical School, Rotterdam, The Netherlands
[4] Departments of Pediatrics, Division of Congenital/Hereditary Diseases, Erasmus University Medical School and Sophia Children's Hospital, Rotterdam, The Netherlands
[5] Department of Immunology, Stichting Samenwerkende Delftse Ziekenhuizen, Delft, The Netherlands

Summary The value of a test for islet cell cytoplasmic antibodies together with a test for GAD_{65} antibodies to predict the subsequent development of diabetes over a period of 11.5 years was assessed in an open childhood population comprising 2,805 individuals. A single serum sample was obtained from each individual between 1975 and 1977 and screened for islet cell cytoplasmic antibodies for which eight individuals were positive (0.29 %). During the average follow-up period of 11.5 years, four of eight islet cell antibody positive and three islet cell antibody negative individuals developed clinical diabetes. Sera from all individuals, who were islet cell antibody positive and/or developed diabetes (total of 11) and from 100 randomly selected control subjects were analysed for GAD_{65} antibodies. Six of eight islet cell antibody positive individuals were GAD_{65} antibody positive including all four who subsequently developed IDDM. Furthermore, one of the three islet cell antibody negative individuals who developed IDDM was GAD_{65} antibody positive both in 1976 and in 1989. Thus, a positive test for GAD_{65} antibodies alone correctly predicted diabetes in five of seven children, who developed the disease. Only one of the children, who developed diabetes was positive for insulin autoantibodies and this individual was also positive for islet cell cytoplasmic antibodies and GAD_{65} antibodies. One of the 100 control subjects was positive for GAD_{65} antibodies (1 %). The results suggest that a single GAD_{65} antibody test may have a higher sensitivity for predicting IDDM than a test for islet cell cytoplasmic antibodies, but that a combined positive test for both antibodies increases the specificity for predicting IDDM over a period of 11.5 years. [Diabetologia (1994) 37: 917–924]

Key words GAD_{65} antibodies, islet cell cytoplasmic antibodies, predictive value, IDDM in childhood.

The destruction of pancreatic beta cells, which precedes the clinical onset of IDDM, is mediated by autoimmune mechanisms [1]. The presence of islet specific autoantibodies in the pre-diabetic period [2–4] is likely to reflect the ongoing autoimmune process, one that eventually leads to critical beta-cell depletion and insulin dependency. A major goal of diabetes research is to develop immune interventions that block or otherwise interfere with the destruction of beta cells and the development of clinical diabetes. Concomitant with this goal is the necessity for methods for early accurate identification of susceptible individuals [5]. Antibody assays that detect early signs of humoral autoimmunity associated with beta-cell destruction are obvious candidates for this purpose. Furthermore, susceptibility to develop diabetes is associated with certain MHC-class II haplotypes [6] and analysis of both HLA haplotypes and autoantibodies provide a test of high predictive value in family members of IDDM patients [3, 7]. Since most new cases of IDDM involve individuals without a first degree relative with the disease [8, 9], predictive meth-

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Corresponding author: Dr. S. Baekkeskov, Hormone Research Institute, University of California San Francisco, 513 Parnassus Avenue, HSW 1090, San Francisco, CA 94143-0534, USA

Abbreviations: CL, Confidence limits; GAD, glutamic acid decarboxylase; IAA, insulin autoantibodies; ICA, islet cell autoantibodies; IDDM, insulin-dependent diabetes mellitus.

ods need to be easily applicable to analyses of the general population.

The classic method to analyse islet cell autoantibodies (ICA) is by immunohistology using fresh-frozen pancreatic sections. The ICA assay is however difficult to standardize [10]. A better method for a standardized analysis of early humoral responses in IDDM might involve a rapid and quantitative assay using a target antigen of humoral autoimmunity in IDDM such as insulin [11], or the GAD_{65} autoantigen [12].

Insulin autoantibodies (IAA's) are only detected in up to 50 % of newly-diagnosed patients and are most frequent in very young patients (< 5 years of age) [13]. IAA's alone have a limited predictive value and are not suitable as a marker to replace ICA's [5, 14, 15].

Incidence of 64 kDa or GAD_{65} autoantibodies in the pre-diabetic period and at clinical onset of IDDM measured by immunoprecipitation of ^{35}S-methionine labelled human islet cell protein is 75–90 % [4, 16]. Following identification of the 64 kDa protein as the smaller form of the enzyme glutamic acid decarboxylase [12, 17], now called GAD_{65}, several assays were developed and standardization is now beginning (1st GAD antibody workshop was held under the auspices of the 12th International Immunology of Diabetes Workshop, Orlando, April 1993). GAD_{65} antibodies precede IDDM by several years and are detected concomitantly with, and sometimes before, ICA and IAA [4, 16, 18].

GAD_{65} is a cytoplasmic protein and is amongst the target antigens of ICA's [19, 20]. Purified GAD_{65} however does not block all ICA reactivity in some sera [20] and the ICA reactivity is likely to involve other target molecules. Furthermore, sera that immunoprecipitate GAD_{65} do not always react with the protein by immunohistology and are therefore negative in the ICA assay [16, 21]. In most IDDM patients, ICAs only stain frozen and not fixed sections of human pancreas. Thus, ICA epitopes are dependent on conformation of the relevant antigen [22]. Similarly diabetes associated epitopes in the GAD_{65} molecule are predominantly conformational and only the sera of very few patients recognize a linear epitope in the protein [12, 19].

In addition to GAD_{65}, GAD is expressed as a second non-allelic form, GAD_{67} in neurons [23] and in rat and mouse beta cells [24]. Human islets only express the GAD_{65} protein [24, 25] and the incidence of GAD_{67} antibodies in IDDM is low [26, 27]. GAD_{67} does not seem to have an independent role as an autoantigen in IDDM. Rather this protein only seems to be recognized in cases where the immune response to GAD_{65} involves recognition of epitopes that cross-react in the two proteins [27].

Previous studies have suggested that islet ICA measured by immunohistology are of insufficient specificity to serve as a single marker of susceptibility [5, 28, 29]. One study of the predictive value of ICA in a Dutch childhood population [30] without emphasis on families of diabetes patients, found an incidence of 0.29 %, which is close to the incidence of IDDM in The Netherlands. This study used a human pancreas which was prepared with a very short ischaemia time resulting in frozen sections that enabled ICA analyses of very high specificity and sensitivity [30]. All the ICA-positive subjects had complement fixing ICA (CF-ICA) and the antibodies preceded the clinical onset of non-familial diabetes in some individuals by several years [30]. The predictive value of positive CF-ICA test on a single occasion to predict the development of IDDM in a subsequent 11.5-year follow-up period was 50 %. The sensitivity of CF-ICA to predict IDDM was 57 %.

The present study analysed GAD_{65} autoantibodies in all individuals in this population who were either CF-ICA positive or developed IDDM during the 11.5-year follow-up period, as well as in 100 randomly-selected subjects who remained healthy during this period. Although it was not possible to analyse GAD_{65} autoantibodies in the whole population with the methodology currently available, we have calculated the potential of a combined test for CF-ICA followed by GAD_{65} antibody analysis to predict diabetes in a childhood population at large, based on a single serum sample per child with a minimum follow-up of 10 years. The study shows that the combination of a positive test for CF-ICA and for GAD_{65} antibodies yields a strong predictive value for subsequent development of IDDM in individuals who are not relatives of IDDM patients and is more specific than a single positive test for either antibody.

Materials and methods

Description of the population: The population studied has been described in detail [30]. Briefly, 4860 schoolchildren aged 5–19 years of age from one township in The Netherlands participated in a longitudinal study on risk factors for cardiovascular and chronic diseases. The data collected included a history on IDDM. Serum was collected from these children between 1975 and 1977. Serum samples of 3383 individuals were available for ICA analysis. In 1988, a questionnaire was sent to all subjects for whom family data on diabetes were available. The three criteria of having a complete first-degree family history of IDDM, a stored serum sample and an adequate response to the questionnaire by the end of an average follow-up time of 11.5 years were fulfilled by 2805 children. CF-ICA's and IAA's were measured in this population and their predictive value analysed [30]. A second serum sample was obtained in 1989 from 10 of the 11 individuals, who were ICA positive in 1975–1977 and/or who developed IDDM in the interim period.

Measurement of GAD_{65} antibodies using rat islets: Autoantibodies to GAD_{65} expressed in rat islets were measured by immunoprecipitation of Triton X-114 (Calbiochem, San Diego, Calif., USA) detergent phase purified cytosolic protein fraction [12]. Immune complexes were analysed by SDS-PAGE using 10 % slab gels. Gels were processed for fluorography [31]. Fluorograms were obtained by 2, and 4 week exposure of each

gel and analysed visually by three independent observers, who did not know the sample identity and scored positive if the GAD_{65} α/β doublet was visible. A quantitative estimate of GAD_{65} in positive immunoprecipitates was obtained by densitometric scanning of autoradiograms using a BioRad model 620 Video Densitometer with 1D Analyst II and version 3.10 software (BioRad, Richmond, Calif., USA) and a control serum which is used as a positive standard for quantitative analyses of GAD_{65} autoantibodies and has an arbitrary value set at 10 [21]. GAD_{65} antibody values in other sera were calculated from integrated peak areas by the formula: 10 × (value for unknown serum – value for negative control serum)/(value for positive control serum – value for negative control serum). The prevalence of GAD_{65} antibodies in newly-diagnosed IDDM patients using this method (immunoprecipitation of Triton X-114 detergent phase purified rat islet cytosolic proteins) (n=73) is 82.2 %. The prevalence in healthy control individuals (n=69) is 1.4 %.

Measurement of GAD_{65} antibodies using recombinant GAD_{65} expressed in COS-cells: The eucaryotic expression vector pMT2 (kindly donated by Dr. R. Kaufman, Genetics Institute, Boston, Mass., USA) was used for transient expression of rat GAD_{65} in COS-7 cells. Oligonucleotide directed mutagenesis was used to change the sequence surrounding the translation initiation site of GAD_{65} into the Kozak consensus sequence [32] for optimum expression in mammalian cells before subcloning into the pMT2 vector. A 2.4 kilobase cDNA fragment containing the coding region of rat GAD_{65} was released from a cDNA clone (kindly donated by Dr. A. Tobin, University of California Los Angeles, Calif., USA) by EcoR I restriction enzyme digestion and inserted into the EcoR I site of pMT2. The resulting plasmid was named pMT2-rGAD_{65}-10. This clone was transfected into COS-7 cells (American Tissue Culture Collection, ATCC) using lipofectamine (GIBCO/BRL Grand Island, New Y., USA) according to the manufacturer's instructions. Forty eight hours after transfection cells were labelled with 400 µCi of ^{35}S methionine (Amersham SJ-1515, Arlington Heights, Ill., USA) per 10-cm plate. After labelling, cells were washed three times in an ice-cold harvest buffer (10 mmol/l Hepes/NaOH pH 7.4, 150 mmol/l NaCl, 10 mmol/l benzamidine/HCl, 0.1 mmol/l p-chloromercuriphenyl sulphonic acid (PCMBS), and 0.1 mmol/l phenylmethylsulphonyl fluoride (PMSF). All following procedures were performed at 4 °C. The cells were collected by scraping into 1 ml of HEAP buffer (10 mmol/l Hepes NaOH, pH 7.4, 1 mmol/l $MgCl_2$, 0.2 mmol/l pyridoxal-5-phosphate, 1 mmol/l aminoethylisothiouronium bromide hydrobromide) containing 0.1 mmol/l PMSF and 1 % Triton X-114, and extracted for 1 hour by repeated pipetting through a bent pipette tip. The extract was cleared by ultra centrifugation at 265,000 × g for 1 h in a Beckman TLA-100 ultracentrifuge with a TLA 100.3 rotor (Beckman, Fullerton, Calif., USA). The supernatant was subjected to a temperature induced TX-114 phase separation [33]. The detergent phase, containing the soluble amphiphilic GAD_{65}, was collected and diluted in HEMAP buffer (HEAP buffer with 1 mmol/l $MgCl_2$). Aliquots containing 200,000 cpm per immunoprecipitate were precleared using normal human serum. Precleared aliquots were incubated with test sera for 16 h in a total volume of 220 µl. Immunecomplexes were isolated by incubation for 1 h with protein-A Sepharose (Pharmacia, Piscataway, New J., USA) which had been coated with cold COS-7-cell lysate to minimize non-specific absorption. Immunocomplexes bound to Protein-A Sepharose were washed 5 times in IMP-washing buffer (10 mmol/l Hepes NaOH, pH 7.4, 150 mmol/l NaCl, 10 mmol/l benzamidine/HCl, 0.5 mmol/l methionine, 5 mmol/l EDTA, 0.1 % bovine serum albumin) and once in autoclaved H_2O. Immunocomplexes were eluted from Protein-A Sepharose, analysed by SDS-PAGE and processed for fluorography. Fluorograms were obtained from a 2- and 4-week exposure of each gel, analysed for a positive $GAD_{65}\alpha/\beta$ signal and quantitated as described above. In addition quantitative analyses of GAD_{65} on gels were performed using a model 425 Phospor-Imager (Molecular Dynamics, Sunnyvale, Calif., USA) following both 1- and 3-day exposure. The results of the Phosphor-Imager analyses and the densitometric analyses were similar except that the former measurement is linear over a longer range. Using the COS-cell material the prevalence of GAD_{65} antibodies is 80.8 % in newly-diagnosed IDDM patients (n=182) and 2.2 % in healthy control individuals (n=271). This assay yielded 100% sensitivity and 100% specificity in the first GAD antibody workshop.

Sera analysed for GAD_{65} antibodies by both assays included all seven individuals who developed IDDM, the four individuals who were ICA-positive, but did not develop diabetes, and three control subjects. Ninety seven age-matched control subjects were analysed using the COS-cell assay only.

HLA haplotyping, CF-ICA analyses, and IAA analyses: HLA haplotyping was performed as described by Giphart and coworkers [34]. The ICA assay was described earlier [30]. The same pancreas, which gave an unusually low background and high specificity in the analyses of the 1975–1977 samples [30], were used for analyses of the 1989 samples. The Juvenile Diabetes Foundation world standard serum for ICA analyses and containing 80 JDF units, was positive on this pancreas up to a dilution of 1 : 256. Dilution of this standard serum showed a strict linear titration curve, 0.625 JDF units corresponding to a titre of 1 : 2. ICA levels in samples were expressed in reciprocal titres rather than JDF titres because the calculated JDF titres are lower than the positive range for analyses using pancreases of lower quality. This assay participates in the International Diabetes Workshops on ICA proficiency. IAA were measured in all seven individuals who developed IDDM, and in the four individuals who were ICA positive, but did not develop diabetes, using both an ELISA assay [35], and a radioimmunoprecipitation assay [11]. Both assays participate in the International Diabetes Workshops on IAA proficiency. A positive value is defined as larger than 3 × a SD value obtained for 100 control individuals amongst whom none are positive in the radioimmunoprecipitation assay and 3.7 % are positive in the ELISA assay. The corresponding values for newly-diagnosed IDDM patients are 15 % (n=115) and 13 % (n=84) in the two assays, respectively.

Results

The GAD_{65} antibody assay: The analyses of GAD_{65} autoantibodies in this study included: 1) immunoprecipitation of the soluble hydrophobic form of GAD_{65} partially purified from rat islets [12, 17] (Fig. 1 A and C), and 2) immunoprecipitation of partially purified membrane and soluble forms of recombinant GAD_{65} expressed in COS-7 cells (Fig. 1 B and D). The first assay is a derivative of the standard immunoprecipitation assay using GAD_{65} expressed in islet cells [4, 21] but uses only the hydrophobic soluble fraction of islets. Since very few cytosolic proteins in rat islets, except GAD_{65}, are hydrophobic, the use of this fraction results in a very low background in im-

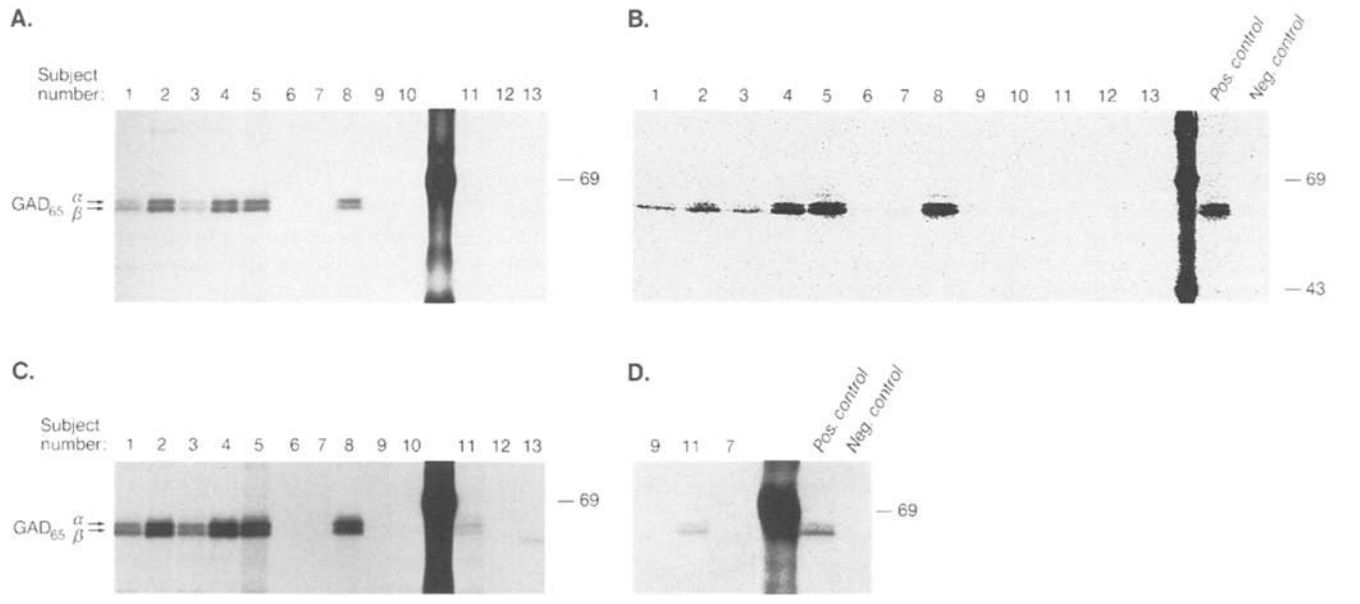

Fig. 1 (A–D). Analysis of GAD_{65} autoantibodies by immunoprecipitation. GAD_{65} autoantibodies were measured in a single serum sample drawn from subjects at baseline (1975–1977) (panels A–C) or in 1989 (panel D). Subjects 1–8 were ICA positive, subjects 9–14 were ICA negative. Subjects 1–4 and 9–11 developed IDDM 1.8–7.1 years following the serum sampling. Subjects 12 and 13 were healthy ICA-negative children.
A, Short exposure of a fluorogram of immunoprecipitates of rat islet cell GAD_{65} on an X-ray film
B, Short exposure of a fluorogram of immunoprecipitates of COS-7 cell GAD_{65} on a Phosphor-Imager
C, Long exposure of the fluorogram in panel A
D, Short exposure of a fluorogram of immunoprecipitates of COS-7 cell GAD_{65} on an X-ray film

munoprecipitates. Both methods are equally sensitive in detecting GAD_{65} autoantibodies in low-high titre sera. However, the first method is more sensitive for analyses of very low titre sera, because it has a better signal to noise ratio and allows specific detection of very low levels of the characteristic α and β doublet of GAD_{65} in immunoprecipitates [31] by long exposure of autoradiograms. This enhanced sensitivity was necessary for detection of GAD_{65} antibodies in the baseline serum from subject number 11 (Fig. 1 C) and the 1989 serum from subject number 4 (Fig. 2) in long exposure of fluorograms. By comparison the COS-7 cell assay did not detect a GAD_{65} signal above background in immunoprecipitates with those two samples after long exposures. Both assays however detected GAD_{65} antibodies in the same individuals in either earlier (subject number 4) or later (subject number 11) samples (Figs. 1 and 2, Table 1).

Prevalence of IDDM in the follow-up period: Seven children developed IDDM, which gave a cumulative incidence of IDDM during an average follow-up period of 11.5 years of 0.25 % (7 of 2805; CL: 0.10–0.51 %). None of those seven children had a first degree family member with IDDM or NIDDM.

Prevalence of CF-ICA, GAD_{65} antibodies, and IAA in 1975–1977: Eight of 2,805 (0.29 %; CL: 0.12–0.56 %) children (numbers 1–8, Table 1) were positive for CF-ICA at the baseline timepoint between 1975–1977. Four of these (numbers 1–4, Table 1) were among the seven who developed IDDM during the follow-up period. Sera from the seven individuals who developed diabetes as well as from the other four ICA-positive individuals were assayed for GAD_{65} autoantibodies (Fig. 1 A–C; Table 1) and IAA. Six of the eight ICA-positive individuals (number 1–4, 5 and 8, Fig. 1 A–C; Table 1) were positive, including all four who subsequently developed IDDM. One of those individuals (number 3), was the only IAA-positive individual by the ELISA assay whereas none were positive by the radio immunoprecipitation assay.

Among the three ICA-negative children who developed IDDM during the follow-up period, two (number 9 and 10, Fig. 1 A–C; Table 1) were also negative for GAD_{65} antibodies in the serum sample collected between 1975–1977. One subject (number 11, Fig. 1, Table 1) was scored negative for GAD_{65} antibodies based on a short exposure of fluorograms of rat islet immunoprecipitates and COS-7 cell immunoprecipitates (Fig. 1 A). However, prolonged exposure of rat islet immunoprecipitates clearly revealed that this serum immunoprecipitated the characteristic α and β doublet of the GAD_{65} autoantigen (Fig. 1 C). Amongst 100 healthy ICA-negative subjects included in the GAD_{65} antibody analyses, one individual, a 16-year-old female, was GAD_{65}-antibody-positive (GAD_{65} antibody index 3.5).

GAD_{65} antibody status 12–13 years after the initial sampling: In 1989 sera were collected from all the

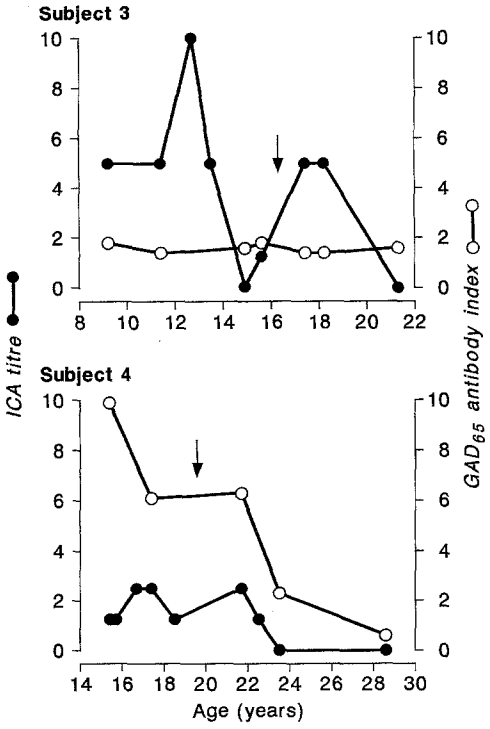

Fig. 2. Longitudinal analyses of GAD_{65} autoantibodies (open circles) and ICA (closed circles) in subjects 3 (top panel) and 4 (lower panel). ICA titres are converted to JDF units. Arrows indicate onset of IDDM

eight subjects who were ICA-positive at baseline (number 1–8) and from two of the ICA-negative subjects (number 10 and 11) who had developed IDDM. Subject number 9 was not available for testing in 1989. Sera were assayed for GAD_{65} autoantibodies and ICA (Fig. 1 D, Table 1). The ICA and GAD_{65} antibody-positive individuals who had developed IDDM had either become antibody negative or decreased significantly in titre. The ICA-negative, GAD_{65} antibody-positive individual (number 11) had a stronger GAD_{65} immunoreactivity in 1989 than in 1977 (Fig. 1 D, Table 1). The two individuals who were ICA/GAD_{65} antibody-positive at the baseline timepoint (number 5 and 8) but did not develop IDDM during the observation period, were still ICA and GAD_{65} antibody positive in 1989, which was 12 and 13 years later, respectively. Neither of those individuals has a first degree family member with IDDM or NIDDM. However, a niece to subject number 8 had a clinical onset of IDDM in 1989. Subject number 6 was still GAD_{65} antibody-negative and had also become ICA-negative in 1989. Subject number 7 was still GAD_{65} antibody-negative and ICA-positive in 1989. Subject number 10 was ICA and GAD_{65} antibody-negative both at baseline and in 1989 which was 1.8 years before and 11.9 years after clinical onset of IDDM, respectively. The positive control individual had no family history of IDDM. She did not consent to an additional serum sampling to test whether the GAD_{65} antibodies had persisted over several years.

Longitudinal analyses of ICA and GAD antibodies in two subjects: Two girls (subjects number 3 and 4) also participated in a cohort study for general health risk factors. Each developed IDDM during the period of this study. Both were reexamined every 12 months. ICA and GAD_{65} antibody titres were retrospectively analysed and compared in the sequential sera (Fig. 2). Subject 3 remained weakly positive for GAD_{65} antibodies throughout the 11-year observation period, which included 5 years following the clinical onset of IDDM. Subject 4 showed a continual decrease from a high GAD_{65} antibody immunoreactivity during the 13-year observation period. She was weakly positive in the last sample, which was obtained 9 years following clinical onset of IDDM. A similar pattern was observed for ICA.

Table 1. Summary of autoantibody and HLA-analyses in individuals which were either positive for islet cell cytoplasmic antibodies (ICA) at baseline and/or developed diabetes during an average of 11.5 year observation period

Subject number/sex	Age at baseline	Age at diagnosis	Interval (years)	HLA		ICA		GAD_{65} autoantibodies[a]	
				DR	DQw	1975–77	1989	1975–77	1989
ICCA + → diabetes									
1M	6.1	12.4	6.3	2,3	2,6	2	Neg	2.9	Neg
2M	7.5	12.4	4.9	3	2	32	Neg	4.0	Neg
3F	9.2	16.3	7.1	3,4	2,8	16	8	1.8	1.6
4F	15.4	19.6	4.2	3,4	2,8	4	Neg	9.9	0.6[b]
ICCA + → healthy									
5M	7.4			3,4	2,8	32	16	14.9	13.9
6F	7.6			2	6	4	Neg	Neg	Neg
7M	14.7			1, w6	5,6	32	64	Neg	Neg
8M	18.2			1, w6	5,6	16	32	14.2	12.0
ICA − → diabetes									
9 M	5.1	12.3	7.5	NA	NA	Neg	NA	Neg	NA
10 M	12.4	14.2	1.8	4, w6	6,8	Neg	Neg	Neg	Neg
11 M	12.7	16.2	3.5	1,5	5,7	Neg	Neg	0.5[b]	3.2

[a] The quantitative values are based on Phosphor-Imager analyses except for those marked by [b], which are densitometric values

Sensitivity of individual and combined assays to predict IDDM: The sensitivity of a combined antibody assay positive for both ICA and GAD_{65} antibodies in a single sample to predict IDDM in the seven children who had a clinical onset during the follow-up period was 57 % (4 of 7; CL: 18–90 %) or the same as for ICA alone. The sensitivity of a GAD_{65} antibody assay alone to predict IDDM in the seven children was 71 % (5 of 7; CL: 29–96 %).

The sensitivity of a combined assay positive for both ICA and IAA in a single sample to predict IDDM in the seven children, who had a clinical onset during the follow-up period was 14 % (1 of 7; CL: 1–53 %) or the same as the sensitivity of IAA antibodies alone and several fold lower than the sensitivity of the ICA and GAD_{65} antibody assays alone.

Predictive value of ICA combined with GAD_{65} autoantibodies: The lack of GAD_{65} antibody data for all but 111 of the 2.805 children constituting this study base excludes the calculation of a positive and a negative predictive value and a specificity for GAD_{65} antibodies alone [36, 37]. However, these parameters can be calculated for a combined ICA and GAD_{65} antibody assay. Thus, the probability of a child acquiring IDDM within 10 years if both autoantibodies were positive at baseline (positive predictive value) was 67 % (4 of 6; CL: 22–96 %) as compared to 50 % (4 of 8; CL: 16–84 %) for ICA alone. The probability of absence of clinical IDDM among subjects not positive for both ICA and GAD_{65} antibodies (negative predictive value) was 99.89 % (2796 of 2799; CL: 99.68–99.98) or the same as for ICA alone (2794 of 2797=99.89 %; CL: 99.69–99.98). The specificity of a test positive for both ICA and GAD_{65} antibodies to predict IDDM within 10 years was 99.93 % (2796 of 2798; CL: 99.74–99.99) as compared to 99.86 % (2794 of 2798; CL: 99.63–99.96) for ICA alone.

Predictive value of ICA combined with IAA: The positive predictive value for a combined ICA and IAA assay was 100 % (1 of 1), which was higher than for a combined assay for ICA and GAD_{65} antibodies. Similarly the specificity of the combined assay was 100 % (2798 of 2798; CL: 99.86–100.00 %). The probability of absence of clinical IDDM among subjects not positive for both ICA and IAA was 99.79 % (2798 of 2804; CL: 99.53–99.92 %) compared to the negative predictive value for ICA alone and ICA combined with GAD_{65} antibodies of 99.89 %.

HLA-tissue typing: HLA DR and DQ haplotypes are shown in Table 1. Subjects 1–5 and 10 were HLA DR3 and/or DR4 positive. All the HLA DR4-positive subjects had the DQw8 (DQB1*0302) allele which is present in 95 % of DR4-positive IDDM patients vs 50 % of DR4-positive control subjects [6, 38]. Subjects 7, 8 and 11 had the DR1, DQw5 (DQB1*0501) haplotype, which is associated with a weakly increased risk of IDDM [38]. Subject 6 was positive for the HLA-DR2, DQw6 (DQB1*0602) haplotype, which is negatively associated with IDDM [39]. Subject number 9 was not available for HLA-typing. The positive control individual did not consent to HLA-typing.

Discussion

We have assessed the predictive value of a combined assay for ICA and GAD_{65} antibodies in a single serum sample and with an average follow-up of 11.5 years in a low-risk childhood population. The low prevalence of ICA in the 2805 individuals analysed in this study (0.29 %) has been attributed to the high specificity and sensitivity of the pancreas used for the ICA analyses [30]. Other studies have reported values of 0.35 % [40], 1.05 % [41], 2.8 % [42], 3.0 % [43], and 4.1 % [44] respectively in childhood populations. The results presented here suggest that even compared to unusually specific ICA measurements alone, ICA analyses supplemented with GAD_{65} autoantibody analysis are likely to have increased specificity in predicting IDDM. Of a normal low-risk population of 2805 children who represented the study base for these analyses, four children would be incorrectly predicted to become diabetic in a 10-year follow-up by a single positive test for CF-ICA alone. This number was decreased to two subjects by requiring both CF-ICA positivity and GAD_{65} autoantibody positivity. Although analysis of GAD_{65} antibodies in 2805 sera was not possible by the current methodology, the presence of GAD_{65} antibodies in 1 of 100 individuals randomly selected from among the ICA-negative subjects, who did not develop IDDM, confirms earlier data on the prevalence of these antibodies in healthy individuals [45]. A prevalence of 1 % is however 3–4 fold higher than the cumulative incidence of IDDM in The Netherlands suggesting that a positive test for GAD_{65} antibodies alone, measured by current methods, is not sufficient to accurately predict the clinical onset of disease within a period of 11.5 years.

This study has confirmed the early appearance of GAD_{65} autoantibodies in a non-familial study. The antibodies were detected in five individuals in a single serum sample obtained 3.5–7 years before clinical onset of IDDM. None of these subjects had a first-degree family member with IDDM or non-IDDM. Amongst the three CF-ICA-negative individuals who developed IDDM, one was positive for GAD_{65} antibodies at baseline and in 1989, which was 3.5 years before and 9.5 years after clinical onset, respectively. The increase in sensitivity of the single GAD_{65} antibody test compared to the double antibody test was however counterbalanced by a decrease in specificity. It is possible that the predictive value of a test for GAD_{65} autoantibodies alone can be increased by introducing

different GAD_{65} antibody specificities. Recent studies have identified conformational epitopes associated with IDDM [19], linear epitopes specific for patients with stiff-man syndrome [12], and a linear epitope associated with protection against disease [46] in the GAD_{65} molecule. Assays that distinguish GAD_{65} antibodies of different epitope specificity may therefore be the next candidates for predictive tests. Alternatively, a combination of a test for GAD_{65} antibodies for high sensitivity and early detection, and a test for antibodies that appear late in beta-cell destruction, and may be indicative of massive loss of beta cells, may provide a better predictive value. A candidate for the latter test are the 37 kDa antibodies described by Christie et al. [47]. Finally since beta-cell destruction in humans is likely to be T-cell mediated as in the animal models, [1] and antibodies may not have a direct pathogenic role, the predictive value of antibody tests may always have the limitations of an indirect parameter. It is certainly conceivable that an autoimmune response induced to an antigenic epitope in a particular HLA-background is dominated by cytotoxic T cells and massive beta-cell destruction whereas the response to other epitopes in the same molecule in the same or different HLA-haplotype is restricted to antibodies and compatible with an intact or only minor loss of beta-cell mass.

In the absence of a direct test for beta-cell destruction, a combination of a positive test for CF-ICA and GAD_{65} autoantibodies together with HLA-typing may provide the most accurate predictive test currently available. In this regard it is notable however that subject number 5, who has been positive for CF-ICA and GAD_{65} antibodies for more than 12 years, has the HLA-DR3, DQw2 and DR4,DQw8 haplotype, which confers the highest known relative risk of IDDM [7]. Thus, even this high risk MHC allele in conjunction with both CF-ICA and GAD_{65} autoantibodies is still not sufficient to rapidly induce the onset of IDDM.

Both CF-ICA, IAA, and GAD_{65} antibodies were absent at baseline in two individuals who later developed IDDM. It is not known whether antibodies never developed in these individuals or whether antibody-positive periods were missed by the single sampling at baseline. Population studies employing regular sampling of sera over 10–20 years are clearly needed to assess the full value of antibody testing in predicting IDDM, and in particular to assess whether some individuals mount an autoimmune response which is restricted to T cells, or alternatively, experience a beta-cell destruction which is not of autoimmune aetiology.

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References

1. Cooke A (1990) An overview on possible mechanisms of destruction of the insulin-producing beta cell. Curr Top Microbiol Immunol 164: 125–142
2. Gorsuch A, Spencer K, Lister J et al. (1981) Evidence for a long prediabetic period in type 1 (insulin-dependent) diabetes mellitus. Lancet II: 1363–1365
3. Srikanta S, Ganda O, Rabizadeh A, Soeldner J, Eisenbarth G (1985) First-degree relatives of patients with type 1 diabetes mellitus. N Eng J Med 313: 461–464
4. Baekkeskov S, Landin M, Kristensen JK et al. (1987) Antibodies to a 64,000 Mr human islet cell antigen precede the clinical onset of insulin-dependent diabetes. J Clin Invest 79: 926–934
5. Bingley P, Bonifacio E, Gale E (1993) Can we really predict IDDM? Diabetes 42: 213–220
6. Nepom G (1993) Immunogenetics and IDDM. Diab Rev 1: 93–103
7. Deschamps I, Boitard C, Hors J et al. (1992) Life table analysis of the risk of type 1 (insulin dependent) diabetes in siblings according to islet cell antibodies and HLA-markers. An 8-year prospective study. Diabetologia 35: 951–957
8. Dahlquist G, Blom I, Holmgren G et al. (1985) The epidemiology of diabetes in Swedish children 0–14 years – a six year prospective study. Diabetologia 28: 802–808
9. Tuomilehto J, Lounamaa R, Tuomilehto-Wolf E et al. (1992) Epidemiology of childhood diabetes in Finland: background of a nationwide study of type 1 (insulin-dependent) diabetes mellitus. Diabetologia 35: 70–76
10. Greenbaum CJ, Palmer JP, Nagataki S et al. (1992) Improved specificity of ICA assays in the fourth international immunology of Diabetes Serum Exchange Workshop. Diabetes 41: 1570–1574
11. Palmer JP, Asplin CM, Clemens P et al. (1983) Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 222: 1337–1339
12. Baekkeskov S, Aanstoot HJ, Christgau S et al. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347: 151–156
13. Palmer J (1993) Predicting IDDM. Diabetes Rev 1: 104–115
14. Atkinson M, Maclaren NK, Riley WJ, Winter WE, Fisk DD, Spillar RP (1986) Are insulin autoantibodies markers for insulin-dependent diabetes mellitus? Diabetes 35: 894–898
15. Karjalainen J, Salmela P, Ilonen J, Surcel HM, Knip M (1989) A comparison of childhood and adult type-1 diabetes mellitus. N Engl J Med 320: 881–886
16. Atkinson MA, Maclaren NK, Scharp DW, Lacy PE, Riley WJ (1990) 64,000 Mr autoantibodies as predictors of insulin-dependent diabetes. Lancet 335: 1357–1360
17. Christgau S, Schierbeck H, Aanstoot HJ et al. (1991) Pancreatic beta 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 266: 21257–21264

18. Seißler J, Hering B, Richter W et al. (1992) Antibodies to the M_r 64,000 (64k) protein in islet cell antibody positive non-diabetic individuals indicate high risk for impaired beta cell function. Diabetologia 32: 550–554
19. Richter W, Shi Y, Baekkeskov S (1993) Autoreactive epitopes defined by diabetes-associated human monoclonal antibodies are localized in the middle and C-terminal domains of the smaller form of glutamate decarboxylase. Proc Natl Acad Sci USA 90: 2832–2836
20. Atkinson MA, Kaufman DL, Newman D, Tobin AJ, Maclaren NK (1993) Islet cell cytoplasmic autoantibody reactivity to glutamate decarboxylase in insulin-dependent diabetes. J Clin Invest 91: 350–356
21. Christie M, Landin OM, Sundkvist G, Dahlquist G, Lernmark A, Baekkeskov S (1988) Antibodies to a M_r-64,000 islet cell protein in Swedish children with newly diagnosed type 1 (insulin-dependent) diabetes. Diabetologia 31: 5597–5602
22. Richter W, Eiermann TH, Endl J et al. (1993) Human monoclonal islet specific autoantibodies share features of islet cell and 64 kDa antibodies. Diabetologia 36: 785–790
23. Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ (1991) Two genes encode distinct glutamate decarboxylases. Neuron 7: 91–100
24. Kim J, Richter W, Aanstoot H et al. (1994) Differential expression of GAD65 and GAD67 in human, rat and mouse pancreatic islets. Diabetes 42: 1799–1808
25. Petersen J, Russel S, Marshall M et al. (1993) Differential expression of glutamic acid decarboxylase in rat and human islets. Diabetes 42: 484–495
26. Hagopian W, Michelsen B, Karlsen A et al. (1993) Autoantibodies in IDDM primarily recognize the 65,000-Mr rather than the 67,000-Mr isoform of glutamic acid decarboxylase. Diabetes 42: 631–636
27. Velloso LA, Kämpe O, Hallberg A, Christmanson L, Betsholtz C, Karlson FA (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
28. Riley WJ, Atkinson MA, Schatz DA, Maclaren NK (1990) Comparison of islet autoantibodies in 'pre-diabetes' and recommendations for screening. J Autoimmunity 1: 47–51
29. Bingley P, Bonifacio E, Shattock M et al. (1993) Can islet cell antibodies predict IDDM in the general population? Diabetes Care 16: 45–50
30. Bruining GJ, Molenaar JL, Grobbee DE et al. (1989) Ten year follow-up study of islet-cell antibodies and childhood diabetes. Lancet I: 1100–1103
31. Baekkeskov S, Warnock G, Christie M, Rajotte RV, Larsen PM, Fey S (1989) Revelation of specificity of 64K autoantibodies in IDDM serums by high-resolution 2-D gel electrophoresis. Unambiguous identification of 64K target antigen. Diabetes 38: 1133–1141
32. Kozak M (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44: 283–292
33. Bordier C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J Biolog Chem 256: 1604–1607
34. Giphart M, Roep B, Drabbels J et al. (1992) Relative contribution of HLA-DQA and DQB alleles to predisposition to insulin dependent diabetes mellitus. Hum Immunol 34: 142–146
35. Dean BM, Becker F, McNally JM et al. (1986) Insulin autoantibodies in the prediabetic period: correlation with islet cell antibodies and development of diabetes. Diabetologia 29: 339–342
36. Vecchio T (1966) The predictive value of a single diagnostic test in unselected populations. N Engl J Med 274: 1171–1173
37. Dawkins R (1986) Sensitivity of autoantibody testing. In: Rose N, MacKay I (ed) The autoimmune diseases. New York: Academic Press, pp 669–693
38. Svejgaard A, Ryder LP (1989) HLA and insulin dependent diabetes: an overview. Genetic Epidemiology 6: 1–14
39. Thomson G, Robinson W, Kuhner M et al. (1988) Genetic heterogeneity modes of inheritance, and risk estimates for a joint study of Caucasians with insulin-dependent diabetes mellitus. Am J Hum Genet 43: 799–816
40. Bergua M, Sole J, Marion G, Perez M (1987) Prevalence of islet cell antibodies, insulin antibodies, and hyperglycemia in 2291 schoolchildren. Diabetologia 30: 724–726
41. Boehm BO, Manfras B, Seißler J et al. (1991) Epidemiology and immunogenetic background of ICA positive non-diabetic schoolchildren: Ulm-Frankfurt population study. Diabetes 40: 1435–1439
42. Bingley P, Bonifacio E, Shattock M et al. (1993) Can islet cell antibodies predict IDDM in the general population? Diabetes Care 16: 45–50
43. Landin-Olsson M, Palmer J, Lernmark Å et al. (1992) Predictive value of islet cell and insulin autoantibodies for type 1 (insulin-dependent) diabetes mellitus in a population-based study of newly diagnosed diabetic and matched control children. Diabetologia 35: 1068–1073
44. Karjalainen J (1990) Islet cell antibodies as predictive markers for IDDM in children with high background incidence of disease. Diabetes 39: 1144–1150
45. Sigurdsson E, Baekkeskov S (1990) The 64-kDa beta cell membrane autoantigen and other target molecules of humoral autoimmunity in insulin-dependent diabetes mellitus. Curr Top Microbiol Immunol 164: 143–168
46. Ujihara N, Daw D, Gianani R, Liping Y, Powers AC (1993) Identification of distinct GAD autoantibody subtypes and a GAD protein epitope. Autoimmunity 15 [Suppl] 68 (Abstract)
47. Christie MR, Tun RYM, Lo SSS et al. (1991) Antibodies to GAD and tryptic fragments of the islet 64 K antigen as distinct markers for development of IDDM: studies with identical twins. Diabetes 41: 782–787