Anti-glutamate decarboxylase and other antibodies at the onset of childhood IDDM: a population-based study

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Summary Sera obtained at diagnosis from 273 children (0–14 years) with insulin-dependent diabetes mellitus (IDDM) were studied to compare different autoantibody levels. The subjects comprise 75 % of all incident cases in New South Wales, Australia, for a 2-year period (ascertainment > 99 % complete). Antibodies against glutamate decarboxylase were measured by radioimmunoprecipitation, insulin autoantibodies (on 176 sera collected within 4 days of initiation of insulin therapy) by radioimmunoassay, thyroid peroxidase and antigliadin IgA antibodies by enzyme-linked immunoassay, and anti-endomysial IgA and islet cell antibodies by indirect immunofluorescence. Reference ranges for anti-glutamate decarboxylase and insulin autoantibodies were determined in a group of non-diabetic children. Of the sera 69 % were positive for anti-glutamate decarboxylase, 65 % for insulin autoantibodies, 71 % for islet cell antibodies (≥ 20 Juvenile Diabetes Foundation units), 10 % for anti-thyroid peroxidase, 2.6 % for antigliadin and 3.0 % for anti-endomysial antibodies. Islet cell antibodies and insulin autoantibodies were both

negative in 13.7 % of the sera, while only 5.8 % were negative for all three of islet cell antibodies, insulin autoantibodies and anti-glutamate decarboxylase. There was a higher frequency of anti-glutamate decarboxylase among girls than boys (75 % vs 63 %, $p = 0.03$) and a negative correlation between the level of insulin autoantibodies and age at diagnosis ($r = -0.41$, $p < 0.0001$). A higher frequency of anti-thyroid peroxidase was found with increasing age ($p = 0.05$). Higher titres of islet cell antibodies were associated with a higher frequency of both anti-glutamate decarboxylase ($p < 0.0001$) and insulin autoantibodies ($p = 0.003$). Five children (1.8 %) with clear elevations of antigliadin and anti-endomysial antibodies were found to have asymptomatic coeliac disease by small bowel biopsy. [Diabetologia (1994) 37: 1113–1120]

Key words Autoantibodies, glutamate decarboxylase, islet cell antibodies, insulin, thyroid peroxidase, coeliac disease.

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; ICA, islet cell antibodies; IAA, insulin autoantibodies; anti-GAD, antibodies against glutamate decarboxylase; anti-TPO, antibodies against thyroid peroxidase; AGA, antigliadin IgA antibodies; AEA, anti-endomysial antibodies; JDF U, Juvenile Diabetes Foundation units; PBS, phosphate buffered saline.

Although insulin-dependent diabetes mellitus (IDDM) appears to be a T-cell mediated disease, autoantibodies may be useful markers, in genetically susceptible individuals, of the beta-cell destruction that precedes clinical presentation [1]. These include islet cell antibodies (ICA) and insulin autoantibodies (IAA), with the recent addition of a quantitative assay for antibodies against glutamate decarboxylase (anti-GAD) [2]. We aimed to determine the frequency and levels of these and other antibodies in a large population-based group of newly-diagnosed diabetic children. Anti-GAD, determined with a quantitative assay, have not previously been studied in such a

group and compared with other autoantibodies. Anti-thyroid peroxidase (anti-TPO), anti-gliadin IgA (AGA) and anti-endomysial (AEA) antibodies were also measured because of the association of IDDM with autoimmune thyroid disease and coeliac disease. This allowed the frequency of thyroid autoimmunity and asymptomatic coeliac disease to be determined among newly-diagnosed diabetic children.

Subjects and methods

Patients and sera. We established a register of incident cases of IDDM in the 0–14-year age-group in New South Wales (incidence rate 14.5 per 100,000 person years) and obtained a serum sample at diagnosis from new diabetic cases arising during a 2-year period (1990–1991). Case ascertainment for the register was over 99 % complete, using two independent methods to identify the cases (physician reporting and the National Diabetes Supply Scheme) [3, 4]. A serum sample was available from 273 (83.5 %) of 327 cases identified by physician reporting. Serum could not be obtained from 34 additional cases identified only by the National Diabetes Supply Scheme. The completeness of ascertainment by physician reporting alone was 90.1 %, estimated by the capture-recapture method [3, 4]. Therefore, serum was available from an estimated 75 % of all incident cases of IDDM in the population. Cases without a serum sample were not significantly different from those with a serum sample according to sex, age at diagnosis or ethnicity. The patients and/or their parents gave their informed consent and the study was approved by the Ethics Committee of the Royal Alexandra Hospital for Children.

The sera were collected as soon as possible after diagnosis (which was defined as the start of insulin therapy). The median delay between diagnosis and the collection of the serum was 3 days (interquartile range: 1–6 days) and 91 % of sera were obtained within 14 days of diagnosis. IAA levels from sera collected more than 4 days after diagnosis ($n = 89$) were excluded from the analysis. ICA and anti-GAD levels from sera collected more than 61 days after diagnosis ($n = 8$) were also excluded. The sera were stored at -20°C in aliquots, to avoid multiple thawing and refreezing of the samples, which may cause a reduction in ICA titre [5].

Epidemiological data including place of residence, ethnic background and family history of IDDM was collected with a questionnaire completed by the parents of the cases. For analysis, the place of residence was classified by Statistical Division [6] and ethnic background was classified as European or non-European.

Glutamate decarboxylase autoantibodies. Anti-glutamate decarboxylase (GAD) were measured on 261 samples collected within 61 days of diagnosis by a radioprecipitation assay, as previously described [2]. In brief, affinity purified porcine GAD was iodinated with ^{125}I . The GAD was enzymically active and known to contain both isoforms GAD67 and GAD65. After preabsorption with pooled normal sera, 100,000 cpm of [^{125}I]GAD was incubated overnight with test sera diluted 1:2 in Tris-Triton buffer (0.02 mol/l Tris, 0.15 mol/l NaCl, 0.5 % weight/volume TritonX-100, pH 7.4). [^{125}I]GAD-antibody complexes were precipitated by protein A-Sepharose and counted for radioactivity after three washes. The results were expressed as the percentage of the radioactivity precipitated by a positive reference serum, which thus had 100 units. The assay participated in the 1st International Diabetes

Workshop (IDW) anti-GAD standardisation workshop and achieved ratings of 100 % for specificity and sensitivity.

109 non-diabetic subjects, ranging in age from 0.5 to 19.1 years (median 10.6 years), were tested using this assay. These subjects were selected from patients attending an endocrine clinic, excluding those with autoimmune disease or a family history of diabetes. A third of the subjects were normal children referred for assessment of short stature, with no disease identified after detailed evaluation. The remainder of the children had non-autoimmune disorders that included congenital adrenal hyperplasia, post-irradiation growth hormone deficiency and dysmorphic syndromes. There was no difference in the anti-GAD levels between the group of normal children and the remainder. Among these non-diabetic subjects, anti-GAD levels ranged from 3 to 18 units, except for two outliers with values of 35 and 56 units (Fig. 1). Values greater than the 98th percentile (18 units) were considered positive.

Islet cell antibodies. ICA were measured on 259 samples collected within 61 days of diagnosis. The assay employed indirect immunofluorescence after prolonged incubation with 4 μm frozen sections of normal human pancreas from a single blood group type O donor. The sera to be tested were serially diluted in phosphate buffered saline (PBS) at pH 7.4 containing 4 % bovine serum albumin and aprotinin (Trasylol; Bayer, Leverkusen, Germany) 2500 kallikrein inhibitor units/ml [7]. The dilutions were randomised and 50 μl of each was incubated on pancreatic sections in a moisture chamber for 24 h at room temperature. The sections were then washed and incubated for 30 min with fluorescein tagged sheep anti-human IgG (Silenus, Melbourne, Australia) for 30 min. The sections were viewed under a Zeiss Axioscop 20 microscope with a 50 W mercury fluorescence burner and a Plan Neofluor 20/0.50 objective. Neither of two independent observers was aware of the identity or dilution of the sample being viewed. The assay was standardised through participation in the 5th IDW International Standardisation Workshop. One negative control serum and six positive control sera of known Juvenile Diabetes Foundation unit (JDF U) titres were included in each assay. For each assay a new standard line, drawn through the end-point titre of five of the positive controls, was used to convert the end-point titres of the tested sera into JDF U. An additional positive control of 23 JDF U was not used to derive the standard line, but was used to monitor the between-assay variation. The coefficient of variation of the $\log_2$ JDF U determined for this standard was 12 %. Thirty-nine non-diabetic control sera, including 31 children were tested and all were negative (< 20 JDF U). The detection limit of the assay was consistently at least 20 JDF U and sera with ≥ 20 JDF U were considered positive. Before analysis, the results were categorised as negative, 20–39 JDF U, 40–79 JDF U or ≥ 80 JDF U.

Insulin autoantibodies. IAA were measured on 176 samples collected within 4 days of the start of insulin therapy, using a competitive fluid phase radioassay [8]. The assay participated in the IAA proficiency test programme (N.K. Maclaren, Gainesville, Fla., USA) and achieved ratings of 100 % for sensitivity, specificity, consistency and validity. The sera were tested in duplicate. For each sample two aliquots of 150 μl were pre-incubated with, and two aliquots without, excess unlabelled recombinant human insulin (Actrapid HM, Novo Nordisk, Gentofte, Denmark) for 60 min at 4°C . ^{125}I labelled porcine insulin (Ventrex Laboratories, Portland, Me., USA) was then added to all tubes and incubated for 7 days at 4°C . Twelve randomly selected tubes were counted for total radioactivity. Antibody bound insulin was then precipitated with polyethylene glycol in Veronal buffer (pH 8.6) and the pellet

from each tube was counted. The percentage of the labelled insulin bound by the serum was then calculated for each tube and the difference was determined between the tubes pre-incubated with and without unlabelled insulin. This difference was multiplied by the amount of unlabelled insulin originally added to the tubes to give the amount of insulin bound by the serum, expressed in nU/ml of serum. A reference range was determined by testing 90 non-diabetic children with no evidence of autoimmune disease or family history of diabetes. The IAA levels in these subjects were normally distributed with a mean of 4.2 nU/ml and SD 9.2 nU/ml. Results greater than 3 SD above the mean (33 nU/ml) were considered positive.

Thyroid peroxidase autoantibodies. Autoantibodies to thyroid peroxidase (anti-TPO) were measured with an enzyme-linked immunosorbent assay (ELISA) (ELitest anti-TPO, Henning, Berlin, Germany) on 266 samples. Anti-TPO in the sera bind to native human thyroid peroxidase coated onto the wells of a microtitre plate and are detected with protein A conjugated to alkaline phosphatase. Among 50 non-diabetic children, anti-TPO levels were unmeasurable (< 50 U/ml) in all except four individuals (with normal thyroid function tests), who had levels of 54, 56, 59 and 92 U/ml. Results greater than 100 U/ml were considered positive, in agreement with the reference range for adults suggested by the manufacturer (0–100 U/ml).

Antigliadin antibodies. Antigliadin IgA antibodies (AGA) by ELISA (Gluten IgA-EIA; Pharmacia Diagnostics, Uppsala, Sweden) [9] were measured on all sera. AGA bind to gliadin coated onto a microtitre well and are detected with a rabbit anti-human IgA conjugated to β -galactosidase. The reference range is 0–24 units [10].

Anti-endomysial antibodies. Anti-endomysial IgA antibodies (AEA) were measured on 271 sera by indirect immunofluorescence on frozen sections of monkey oesophagus (Inova Diagnostics; San Diego, Calif., USA). Sera were diluted 1:5 in PBS at pH 7.4 and 25 μ l was incubated on the tissue sections in a moisture chamber for 30 min. The sections were washed twice with PBS and incubated for 30 min with 25 μ l of fluorescein-tagged goat anti-human IgA (Kallestad, Austin, Tex., USA) diluted 1:8 in PBS. After washing again, the sections were observed with a fluorescence microscope (Leitz Ortholux II with a 150 W xenon fluorescence burner and Plan Apo 25/0.65 objective) and scored as positive or negative [11]. Positive sera were later serially diluted to determine the end-point titre. Ten control children were all negative. Positive and negative control sera were included in each assay.

Statistical analysis

None of the autoantibody levels was normally distributed, except for the IAA levels in the control group. IAA levels in the cases normalised after log transformation. Statistical analysis was with Spearman's rank correlation, the chi-squared test, the Mantel-Haenszel chi-squared test and Wilcoxon's rank sum test. For the log-transformed IAA data, linear regression and Pearson's correlation were used. Before log-transformation, negative and zero values of IAA were assigned the value of one to avoid losing information from these data points. Probability values greater than 0.05 were regarded as not statistically significant.

Fig. 1. The levels of antibodies to glutamate decarboxylase (anti-GAD) in 109 non-diabetic children ($\triangle$) and 261 newly-diagnosed diabetic children ($\blacktriangle$). The dashed line represents the 98th percentile for anti-GAD among the non-diabetic children (18 units)

Results

Frequency and levels of autoantibodies. Overall, anti-GAD (> 18 units) were detected in 69 % of cases, ICA (≥ 20 JDF U) in 71 % and IAA (≥ 33 nU/ml) in 65 %. The median level of anti-GAD was 47 units (interquartile range: 15 to 90) (Fig. 1). The titre of ICA was 20 to 39 JDF U in 22 % of the cases, 40 to 79 JDF U in 35 %, and ≥ 80 JDF U in 14 %. The median level of IAA was 83 nU/ml (interquartile range: 23 to 279).

Ten percent of the cases (13 male, 14 female) were anti-TPO positive (> 100 U/ml) and among positive cases the median level of anti-TPO was 1369 (interquartile range: 509–3317). None of the patients had any symptoms of thyroid disease. Thyroid function tests were performed on 21 of 27 cases positive for anti-TPO and two were hypothyroid, with an elevated level of thyroid stimulating hormone and a decreased level of free thyroxine.

AGA were positive in 2.6 % of the cases and AEA in 3.0 %. For five patients (1.8 %), ranging in age from 7.3 to 13.9 years, there was a clear elevation of both AGA (range: 43–225 units) and AEA levels (titre of 1:160). Although asymptomatic, all five were later found to have abnormalities on small bowel biopsy diagnostic of coeliac disease and were treated with a gluten-free diet. Another three patients had a borderline elevation of AGA (highest 27 units) or equivocal AEA. Three of these had antibody levels repeated at a later time point, and all were negative.

Associations with age and gender. Figure 2 shows the percentage of the cases positive for autoantibodies by age at diagnosis. IAA were more frequent in

Fig. 2. The percentage of newly-diagnosed diabetic children positive for autoantibodies, according to age at diagnosis. (ICA, islet cell antibodies [diagonal lines]; IAA, insulin autoantibodies [cross-hatch]; anti-GAD, glutamate decarboxylase autoantibodies [solid black]; anti-TPO, thyroid peroxidase autoantibodies [checkered]; AGA, antigliadin IgA antibodies [white]; AEA, antiendomysial antibodies [horizontal lines]). The number of patients analysed is given below each bar. IAA levels on sera collected more than 4 days after the start of insulin therapy were excluded from analysis

Fig. 3. The insulin autoantibody level (IAA), on a logarithmic scale, plotted against the age at diagnosis in 176 newly-diagnosed diabetic children. The dashed line represents the cut-off for IAA positivity (33 nU/ml) and the solid line represents the linear regression of $\log_{10}$ IAA on age at diagnosis ($R^2 = 0.17$, $p < 0.0001$)

younger age groups (Mantel-Haenszel χ^2 for trend = 18.14, $p < 0.0001$) and there was a negative correlation between the level of IAA ($\log_{10}$ -transformed) and age at diagnosis ($r = -0.41$, $p < 0.0001$, Pearson correlation) (Fig. 3). There was no significant correlation between the level of anti-GAD and age at diagnosis ($r = 0.05$, $p = 0.4$). A higher frequency of anti-TPO was found with increasing age (Mantel-Haenszel χ^2 for trend = 3.84, $p = 0.05$). There was no significant association between the frequency of ICA, anti-GAD, AGA or AEA and age at diagnosis.

Fig. 4. Comparison of the glutamate decarboxylase autoantibody levels in 129 male and 132 female newly-diagnosed diabetic children. The dashed line represents the cut-off for anti-glutamate decarboxylase autoantibody (GAD) positivity (18 units). Each box plot shows the median (a), 25th (b) and 75th (c) centiles for anti-GAD. The error bars show the 10th and 90th centiles. Data points below the 10th or above the 90th centile are shown individually

Figure 4 shows a sex difference in anti-GAD that was not found for the other antibodies. The levels of anti-GAD were higher among girls (median 57.5 units, interquartile range: 18.5 to 96) than boys (median 34 units, interquartile range: 13 to 82) ($p = 0.02$, Wilcoxon test). Similarly, 75% of the girls were anti-GAD positive compared with 63% of the boys ($p = 0.03$).

Associations with family history, season, geographical area and ethnic background. A history of IDDM in a first degree relative was present in 6.9% of cases. There were no significant differences in the frequencies of any of the autoantibodies between cases with and without an affected first degree relative. There were no significant differences with respect to season of onset, geographical area of residence at the time of diagnosis or ethnic background.

Associations between autoantibodies. Higher titres of ICA were associated with a greater frequency of anti-GAD (Mantel-Haenszel χ^2 for trend = 19.24, $p < 0.0001$) and higher levels of anti-GAD (Fig. 5). Figure 6 shows a similar but less prominent association between IAA and ICA (Mantel-Haenszel χ^2 for trend = 8.64, $p = 0.003$). However, there was no significant association between the levels of anti-GAD and IAA ($r = 0.06$, $p = 0.5$). Among cases positive for anti-GAD, 12.4% (22 of 177) were positive for

Fig. 5. The relationship between the glutamate decarboxylase antibody level (anti-GAD) and the islet cell antibody titre (ICA) in 257 newly-diagnosed diabetic children. The dashed line represents the cut-off for anti-GAD positivity (18 units). Each box plot shows the median (a), 25th (b) and 75th (c) centiles for anti-GAD. The error bars show the 10th and 90th centiles. Data points below the 10th or above the 90th centile are shown individually

Fig. 6. The relationship between the insulin autoantibody level (IAA) and the islet cell antibody titre (ICA) in 175 newly-diagnosed diabetic children. The dashed line represents the cut-off for IAA positivity (33 nU/ml). Each box plot shows the median (a), 25th (b) and 75th (c) centiles for anti-GAD. The error bars show the 10th and 90th centiles. Data points below the 10th or above the 90th centile are shown individually

Fig. 7. The percentage of newly-diagnosed diabetic children positive for each combination of islet cell antibodies (ICA), insulin autoantibodies (IAA) and glutamate decarboxylase autoantibodies (anti-GAD). The results are shown for 173 children with measurements of all three autoantibodies

anti-TPO, compared with only 4.9 % (4 of 77) among cases negative for anti-GAD, although this difference was not statistically significant ($p = 0.06$).

Results for both ICA and IAA were available for 175 cases and, of these, 24 (13.7 %) were negative for both markers. In comparison, results for all three autoantibodies (ICA, IAA and anti-GAD) were available for 173 cases (Fig. 7) and only 10 (5.8 %) were negative for all three markers. These triple negative cases were not significantly different from the remainder in terms of age, sex or the presence of other antibodies (anti-TPO, AGA and AEA).

Discussion

We studied newly-diagnosed diabetic children from a large population-based register and found that 69 % were positive for anti-GAD, 71 % for ICA, and 65 % for IAA. Only 5.8 % were negative for all three markers. The addition of anti-GAD as a serological marker to the combination of ICA and IAA more than halved the number of autoantibody negative children.

Other studies have reported varying frequencies of ICA in newly-diagnosed diabetic children, ranging from 54 % to 94 % [5, 7, 12–15]. This may be partly explained by differences in sensitivity between laboratories and by inter-laboratory variation in ICA measurements, despite improvement after standardi-

sation workshops [16–19]. A limiting factor may be variation in the pancreatic substrate, as major differences have been reported in the results (standardised in JDF U) obtained with different pancreases in the same assay [20]. The radioimmunoprecipitation assay for anti-GAD used in this study does not have these limitations [2].

The ICA assay is likely to measure antibodies against several different islet antigens. Our finding of a strong association between ICA titres and anti-GAD levels is consistent with evidence that GAD is an ICA antigen [21–23]. We also found an association between ICA titre and IAA level. Insulin does not stain on frozen pancreatic sections but IAA cross-react with proinsulin [24], which can be immunostained on frozen sections [25]. The other autoantigen(s) accounting for the remainder of ICA staining remain to be identified and biochemically characterised. A ganglioside [26] and other recently discovered autoantigens [27–30] are candidates.

Vardi et al. [8, 13] found IAA in 50–69 % of newly diagnosed patients aged under 32 years, similar to the prevalence of 65 % that we found in newly-diagnosed children. We confirmed previous findings that IAA occur more frequently, and at higher levels, in younger children [13, 15, 31, 32]. In agreement with other studies, we found no association between ICA titre and age at diagnosis [7, 12, 13], although a lower frequency of ICA has been reported among older adult patients with newly-diagnosed IDDM, especially in men [33]. There were no significant age differences in the frequency or level of anti-GAD among the children we studied.

Other studies, using different assay formats and involving smaller numbers of recently-diagnosed patients of all ages, have reported frequencies of anti-GAD varying from 25 to 70 % [14, 34–37], compared with the frequency of 69 % that we found in children. This may reflect differences in assay sensitivity. We found a higher frequency and higher levels of anti-GAD in females than in males, in agreement with two other studies [34, 38]. Martino et al. [34] found a significantly higher frequency of anti-TPO among anti-GAD positive than among anti-GAD negative adults with newly-diagnosed IDDM and suggested that this may represent a subgroup associated with polyendocrine autoimmunity. There was a similar association in the children we studied, although this was not statistically significant.

Immune-mediated diseases that may coexist with IDDM include thyroiditis and coeliac disease. We found that 10 % of newly-diagnosed diabetic children were positive for anti-TPO, a sensitive and specific marker of thyroid autoimmunity [39, 40]. There was a significantly higher frequency in older than in younger children. Studies in adult IDDM patients without clinical or biochemical evidence of thyroid dysfunction have reported positive levels in 18 to

24 % [39, 40]. A minority of children with anti-TPO in our study had evidence of thyroid dysfunction, but the presence of this antibody indicates a high risk for future hypothyroidism [41, 42]. Coeliac disease was present in at least 1.8 % of the children we studied. Other studies, not restricted to newly-diagnosed patients, have found prevalence rates ranging from 1.5 to 3.5 % [43–48]. Antigliadin IgA is of greater value than antigliadin IgG as a screening test for coeliac disease [44, 45, 49]. However, antigliadin IgA may be falsely negative in patients with IgA deficiency, which occurs in approximately 2.5 % of children with IDDM [50] and with increased frequency in coeliac disease. Both antigliadin IgA (AGA) and anti-endomysial IgA antibodies (AEA) have been reported to have high sensitivity and specificity [9, 51]. We found that AGA was of equal value to AEA when screening diabetic children, and it has the advantage of an ELISA format, suitable for handling large numbers of samples.

In conclusion, we found a high frequency of anti-GAD in children with newly-diagnosed IDDM. The addition of anti-GAD as a serological marker to the combination of ICA and IAA more than halved the number of autoantibody negative children and only 5.8 % were negative for all three serological markers. There was a higher frequency of anti-GAD in girls than in boys, a higher frequency of IAA in younger patients and a higher frequency of anti-TPO in older patients. Asymptomatic coeliac disease occurred in at least 1.8 % of newly-diagnosed diabetic children.

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