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Insulin autoantibodies are of less value compared with islet antibodies in the clinical diagnosis of autoimmune type 1 diabetes in children older than 3 yr of age.

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Borg H, Marcus C, Sjöblad S, Fernlund P, Sundkvist G. Insulin autoantibodies are of less value compared with islet antibodies in the clinical diagnosis of autoimmune type 1 diabetes in children older than 3 yr of age. Pediatric Diabetes 2002: 3: 149–154. © Blackwell Munksgaard, 2002

Abstract: Background: Insulin autoantibodies (IAA), antibodies against endogenous insulin, may be detected in type 1 diabetic children before the start of insulin treatment.
Objective: To relate IAA to islet antibodies (i.e., islet cell antibodies [ICA], and antibodies against two ICA-related islet antigens, glutamic acid decarboxylase 65 [GADA] and protein tyrosine phosphatase IA-2 [IA-2 A]) at diagnosis, and to endogenous β-cell function at follow-up after diagnosis in diabetic children.
Subjects: We investigated 74 children, aged 1–15 yr, at the diagnosis of diabetes and 1–10 yr later. Insulin treatment may induce antibody development against exogenous insulin. Patients with insulin treatment $\geq$ 1 wk (n = 5) were therefore excluded from the final analysis.
Methods: Radioligand-binding assays based on human recombinant antigen were used to measure IAA, GADA, and IA-2 A. ICA were determined with indirect immunofluorescence.
Results: IAA were detected at a significantly lower frequency (43%; p $\leq$ 0.001) than ICA (86%), GADA (72%), and IA-2 A (80%). In agreement, IAA measurements only marginally increased the frequency of positive autoimmune markers at diagnosis of diabetes (from 97 to 99% positive for at least one autoantibody). Preserved β-cell function (detectable fasting p-C-peptide levels) was found in only nine patients, who were older (13 $\pm$ 3 vs. 7 $\pm$ 6 yr, p = 0.002) and had fewer of the antibodies (IAA, GADA, IA-2 A, ICA) in high titer (> median) compared with 60 patients with undetectable p-C-peptide levels.
Conclusions: Insulin autoantibodies are of less clinical value compared with islet antibodies in the diagnosis of autoimmune type 1 diabetes in children.

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Insulin autoantibodies (IAA), i.e., antibodies against endogenous insulin, may be detected in type 1 diabetic patients before the start of insulin treatment (1). In prediabetic subjects, IAA predict future β-cell failure (2). The reported frequency of IAA is high in recently diagnosed diabetic children (65%) (3, 4), especially in young children (90%) (3, 5). Islet antibodies – islet cell antibodies (ICA) and antibodies against two ICA-related islet antigens, glutamic acid decarboxylase 65 (GADA) and protein tyrosine phosphatase IA-2 (IA-2 A) – are also detected at a high frequency

in diabetic children. ICA are detected most commonly (85%), while GADA and IA-2 A are found in about 70% at the diagnosis of diabetes (6). Islet antibodies may be detected many years after diagnosis in children with type 1 diabetes (7). In contrast, the assessment of IAA after diagnosis in insulin-treated diabetic children is complicated by development of insulin antibodies (IA) against exogenous insulin (8). Current assays cannot separate IAA from IA. Accordingly, it has been suggested that IAA measurements have to be performed before insulin

treatment is started (9, 10). In other studies, however, samples taken up to a maximum between 5 d and 1 wk after insulin treatment were accepted (11, 12). The major aim of the current study was to evaluate the frequency of IAA in relation to islet antibodies at diagnosis and remaining β-cell function at follow-up in diabetic children. In addition, we tried to clarify whether it is possible to evaluate IAA during the first week after the start of insulin treatment in diabetic children.

Methods

Subjects

In 137 consecutive children with a diagnosis of diabetes mellitus during 1987–96 at Huddinge University Hospital and Lund University Hospital, Sweden, a first venous blood sample was taken as early as possible after the diagnosis of diabetes, irrespective of whether insulin treatment had been initiated. In the current prospective study, there are 74 patients (median age 7.5 ± 6 yr, range 1–15; 34 girls) who had a blood sample volume large enough for measurement of IAA taken at diagnosis of diabetes. A second follow-up sample (also assessed for fasting p-C-peptide) was taken 1–10 yr after the diagnosis (7). All patients were treated with human recombinant insulin. We also tested 40 controls (20 girls: median age 11 yr, range 7–12) randomly selected from a group of healthy schoolchildren (13).

The follow-up periods varied and patients were therefore divided into three different groups with comparable numbers: follow-up time 1–3 yr (n = 27), 4–6 yr (n = 26), and 7–10 yr (n = 21). The study was approved by the ethics committee at the Karolinska Institute, Stockholm, Sweden.

Assay methods

Insulin antibodies were determined by a radioligand-binding assay based on ^{125}I-labeled human recombinant insulin (RSR Ltd, Cardiff, UK) (14). In this assay, a test sample (20 μL) and ^{125}I-insulin were incubated overnight at 4°C to form immunocomplexes. Anti-human IgG was then added and the mix was incubated for 2 h at 4°C. After addition of assay buffer and precipitation enhancer, centrifugation, and decantation, the radioactivity of the sediment was counted in a gamma-counter. Read from a standard curve constructed in the same run with calibrators assayed in duplicate, results are presented in units/mL (u/mL) arbitrarily defined by the manufacturer. A value above 1.0 u/mL was considered abnormal (as suggested by the manufacturer). One of the 40 healthy controls (2.5%) was IAA-positive (1.1 u/mL). The reproducibility of the assay was determined from the results of two control samples included in triplicate in each run. The estimated total assay CV was 2.1% at 5.4 μ/mL and 4.2% at 31.9 μ/mL. GADA and IA-2 A were determined by radioligand binding assays based on ^{35}S-methionine-labeled human recombinant in vitro transcribed-translated GAD 65 and IA-2, respectively (6, 15). The GADA and IA-2 A results are presented as indexes calculated according to the formula $100 \times (u - n)/(p - n)$, where u = counts per minute (CPM) of the unknown sample, n = CPM of the negative control, and p = CPM of the positive control. A GADA index above 4.6 and an IA-2 A index above 1.0 were considered abnormal (above 97.5 percentile of 99 healthy control children) (6). In the Diabetes Autoantibody Standardization Program (DASP), the GADA assay was performed with 80% sensitivity and 96% specificity and the IA-2 A assay with 58% sensitivity and 100% specificity (50 patients aged 9–30 yr and 50 healthy donors aged 18–28 yr tested). ICA were determined by a prolonged immunofluorescence assay and expressed in JDF units (16). The detection limit (cutoff for abnormality) was 2–4 JDF units for used pancreata. In the latest Diabetes Autoantibody Proficiency Program (no. 13, 20 samples tested), the ICA assay was performed with 100% sensitivity and 100% specificity. The results from the Diabetes Autoantibody Proficiency Programs are indicative only of assay performance.

Fasting plasma C-peptide (p-C-peptide) was used as a measure of endogenous β-cell function in the complete material. The detection limit was 0.10 nmol/L for the assay used (17).

Statistics

The non-parametric Mann–Whitney u-test was used to evaluate differences between groups, the Wilcoxon signed rank test for paired differences, Spearman's test for correlations, and Fisher's exact test for frequency differences. $p < 0.05$ was considered significant. Data are presented as median ± interquartile range), if not otherwise stated.

Results

Antibodies after ≤ 6 d of insulin treatment

Among patients tested ≤ 6 d after initiation of insulin treatment, there were no differences in IA frequencies or levels between those tested before (n = 7) and after (n = 62) the start of insulin treatment (Fig. 1).

IAA were found in 30 of 69 patients (43%), a frequency substantially lower compared with ICA (59 [86%]; $p < 0.0001$), GADA (50 [72%]; $p = 0.001$), and IA-2A (55 [80%]; $p < 0.0001$), respectively. The frequencies of all possible antibody combinations are shown in Table 1. IAA were detected in about half of the patients with two or three islet antibodies, and in
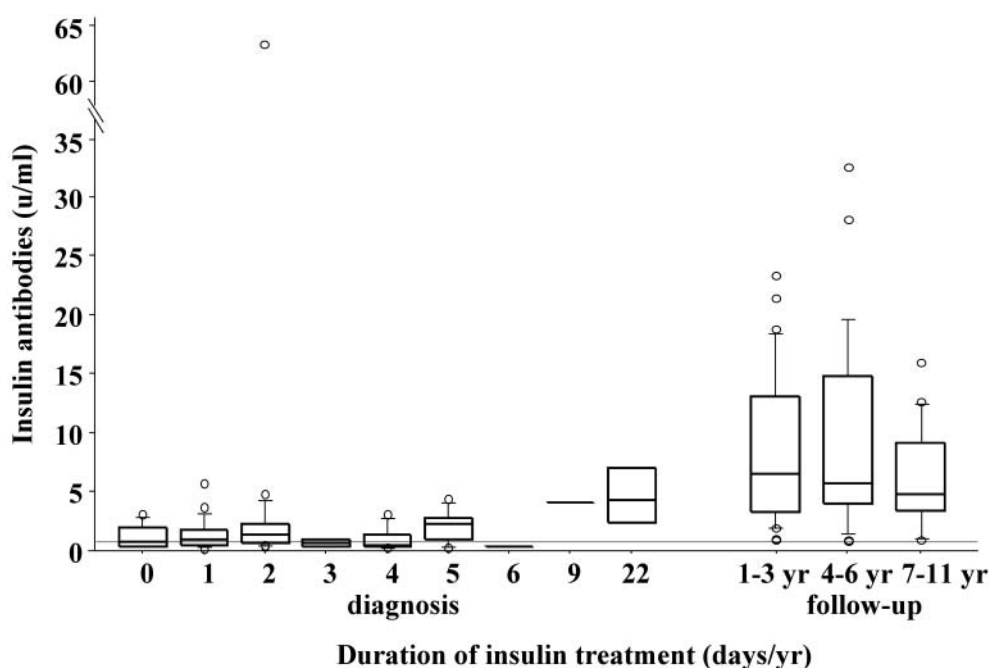
Fig. 1. Levels of insulin antibodies at diagnosis and at follow-up in diabetic children. At diagnosis, children with > 6 d of insulin treatment (n = 5) had higher antibody levels compared with those with ≤ 6 d of insulin treatment (n = 69, p = 0.01) and were excluded from the main study. At follow-up, antibody levels were significantly increased compared with those at diagnosis (p < 0.0001). Horizontal lines indicate the 10th, 25th, 50th, 75th and 90th percentiles. Dotted line indicates cutoff (1.0 μ/mL)

two of the three patients lacking islet autoantibodies, but in none of the patients with only one islet antibody. The two patients with only IAA had significantly lower IAA levels than IAA-positive patients who were positive for islet antibodies (1.4 ± 0.2 vs. 2.3 ± 2.0 u/mL, p = 0.04). In total, nine patients were single-antibody-positive (ICA, 3; GADA, 3 ; IA-2 A, 1; and IAA, 2), and 68 of 69 (99%) patients showed at least one of the four antibodies at diagnosis. There were no significant differences in IAA frequencies and levels between girls and boys.

Age

In contrast to islet antibodies, there was a clear association between IAA and age. IAA-positive patients were significantly younger than IAA-negative patients (6 ± 5 vs. 9 ± 6 yr; p = 0.004). In fact, all seven patients aged 1–3 yr were IAA-positive at diagnosis compared with only nine of 36 patients (25%) 8–15 yr of age (p = 0.0004; Table 2). Further, patients 1–3 yr of age had significantly higher IAA levels compared with IAA-positive patients 4–15 yr of age (3.8 ± 2.3 vs. 1.9 ± 1.5 u/mL, p = 0.01). Islet antibodies were found positive at diagnosis in all seven patients aged 1–3 yr and in 59 of 62 (95%) patients aged 4–15 yr at diagnosis.

Antibodies after 1–3 wk on insulin treatment

Four of five patients (80%) tested after 1–3 wk of insulin treatment were IA-positive and had significantly

higher antibody levels than IA-positive patients tested within 1 wk after insulin treatment (4.2 ± 3.0 vs. 2.1 ± 2.0 u/mL; p = 0.01). Moreover, IA levels among these four patients were not significantly different from those at follow-up amongst the 69 included patients. This suggests that antibodies against exogenous insulin had developed in these four patients and therefore patients tested for IAA after the first week were not included in the comparison be-

Table 1. Frequency of insulin autoantibodies (IAA) in relation to the different combinations of islet antibodies (islet cell antibodies [ICA], glutamic acid decarboxylase 65 antibodies [GADA] and protein tyrosine phosphatase IA-2 antibodies [IA-2A]) in 69 children with recent onset diabetes. Superscripts indicate the number of different antibodies in the individual patient

Islet antibodies	Total (n)	IAA (n)	
		Present	Absent
ICA+ GADA+ IA-2 A+	39	18[a]	21[b]
ICA+ GADA+	5	3[b]	2[c]
ICA+ IA-2 A+	12	6[b]	6[c]
GADA+ IA-2 A+	3	1[b]	2[c]
ICA+	3	–	3[d]
GADA+	3	–	3[d]
IA-2 A+	1	–	1[d]
No islet antibodies	3	2[d]	1[e]
Total	69	30	39

[a]Four antibodies.
[b]Three antibodies.
[c]Two antibodies.
[d]One antibody.
[e]No antibodies.

Table 2. Frequency of insulin autoantibodies (IAA), islet cell antibodies (ICA), glutamic acid decarboxylase 65 antibodies (GADA) and protein tyrosine phosphatase IA-2 antibodies (IA-2A) at diagnosis of diabetes in relation to age at onset. Data presented as n (%)

Antibodies	Age at onset		
	1–3 yr (n = 7)	4–7 yr (n = 26)	8–15 yr (n = 36)
IAA+	7 (100)	14 (58)[a]	9 (25)[b,c]
ICA+	6 (86)	22 (84)	31 (86)
GADA+	5 (71)	18 (69)	27 (75)
IA-2 A+	7 (100)	19 (73)	29 (81)

Significantly lower compared with [a]1–3 yr, p = 0.03; [b]1–3 yr, p = 0.0004; [c]4–7 yr, p = 0.03.

tween antibody status at, or soon after, the diagnosis vs. at follow-up.

Insulin antibodies (IA) at follow-up

Insulin antibody concentrations at follow-up were significantly higher than IAA concentrations at diagnosis (6.4 ± 9.0 vs. 2.1 ± 2.0 u/mL; $p < 0.0001$). Moreover, IA were detected in significantly more patients at follow-up than IAA at diagnosis (63 [91%] vs. 30 [46%] of the 69 patients, $p < 0.0001$) (Fig. 1). There were no significant differences in IA frequencies or levels between those followed up after 7–10 yr and those with a shorter follow-up period. IA levels at follow-up in IA-positive patients were not correlated with age at onset ($r_s = -0.13$, $p = 0.58$).

β-cell function at follow-up

At follow-up, nine patients showed fasting p-C-peptide levels above the detection limit (0.10–0.52 nmol/L), whereas the remaining 60 had undetectable values (i.e., β-cell failure). The nine patients with preserved β-cell function were followed up within 5 yr after the diagnosis (9 of 9 vs. 39 of 60 [65%], $p = 0.049$), were significantly older (13 ± 3 vs. 7 ± 6 yr, $p = 0.002$), and had fewer of the antibodies (IAA, GADA, IA-2A, ICA) in high titer (> median) (0 ± 1 vs. 1 ± 2, $p = 0.02$) at diagnosis, compared with those with β-cell failure at follow-up. The number of patients without or with only one of the antibodies in high titer clearly differed between patients with preserved β-cell function and those with β-cell failure at follow-up (9 of 9 [100%] vs. 33 of 60 [55%], $p \leq 0.01$).

Discussion

In the current study, IAA were detected at a clearly lower frequency (43%) than islet antibodies (ICA, 86%; GADA, 72%; IA-2 A, 80%) in children with recently diagnosed diabetes. In contrast to islet antibodies, IAA were most frequent and found at highest levels in young children. In fact, all patients aged 1–3 yr at diagnosis were IAA-positive, as compared with 58% of those aged 4–7 yr and 25% of those aged

8–15 yr. Although human recombinant insulin was used in all patients, insulin treatment increased the frequency of IA at follow-up; 91% of the children were IA-positive at follow-up. Unlike IAA, IA at follow-up did not correlate with age at onset, and the levels of IA correlated with neither IAA positivity nor IAA levels.

As confirmed in the current study, in children with recently diagnosed diabetes, the frequency of IAA is age-dependent, with the highest frequency in young children (70–100%) (3, 10, 18). On the other hand, the IAA frequency was clearly lower in children aged 8–15 yr. The overall IAA frequency in all children in this study was, however, clearly lower than in previous studies in other countries (3, 19, 20) but the same as in a previous Swedish study (11), indicating that there may be geographic differences in the frequency of IAA in diabetic children. The possibility of low sensitivity of the IAA assays should also be considered. It is known that different IAA assays show less concordance than different GADA and IA-2 A assays (21). In the current study, compared with islet antibodies occurring in 95% of the children aged 4–15 yr at diagnosis of diabetes, IAA did not add any substantial clinical information regarding autoimmunity. Therefore, IAA seem to be of little value in the clinical classification of type 1 vs. type 2 diabetes in children older than 3 yr of age. The differential diagnosis between type 1 and type 2 diabetes in children is of increasing importance due to the rising incidence of type 2 diabetes (22, 23). The limited value of IAA measurements in the clinical context is further supported by the observation that in the two patients with isolated IAA positivily, IAA levels were just above cutoff, indicating that IAA may be false-positive. At follow-up, preserved β-cell function was only observed in a few of the oldest children with short disease duration of diabetes, and in most of these cases p-C-peptide levels were low. This favors the assumption that all evaluated children had type 1 diabetes. All patients with preserved β-cell failure at follow-up had none or only one of the autoantibodies in high titer at diagnosis; however, this was also found in most patients (33 of 60 [55%]) with β-cell failure at follow-up. Nevertheless, the higher number

of the antibodies in high titer (antibody level > median) in those with β-cell failure at follow–up supports an association between antibody levels and the severity of the type 1 diabetic process, as previously described in patients with adult-onset diabetes (24, 25). Although this study infers that IAA are of limited value in the clinical classification of diabetes in children older than 3 yr of age, IAA measurements, of course, are important in studies on the pathogenesis of type 1 diabetes.

Our study confirms that there is a development of IA induced by insulin treatment (8). Nevertheless, our study supports previous observations that samples taken up to 5–7 d after insulin treatment has been started may be used for IAA determination (11, 12). There were no significant differences in IA frequencies or levels before and within 6 d after the start of insulin treatment. Figure 1 indicates, however, that already on day 5, IA levels may have been slightly increased ($p = 0.18$), compared with those on days 0–4. In contrast, patients treated with insulin for more than 1 wk showed as high IA levels in the first sample as the IA levels in the patients followed up. Most likely, IAA-positive patients treated with insulin for more than 1 wk had developed antibodies against exogenous insulin. Our observation, however, that patients with a follow-up time of 7–10 yr did not show higher IA levels than those followed up for 1–3 yr indicates that the booster effect of exogenous insulin may be low. Our finding that the increment in IA was similar in IAA-negative and IAA-positive patients confirms a previous study (9). Hence, it appears that antibodies to exogenous insulin develop independently from the autoimmune reaction against endogenous insulin.

In conclusion, the frequency of IAA at the diagnosis of diabetes in children was clearly lower (43%) than islet antibodies (ICA, 86%; GADA, 72%; IA-2 A, 80%). Although IAA were detected in two of three children negative for islet antibodies at diagnosis of diabetes, IAA measurements only marginally increased the frequency of autoimmune markers in children with recent onset diabetes.

Acknowledgements

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