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Autoantibodies associated with Type I diabetes mellitus persist after diagnosis in children

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Summary To study the persistence of Type I (insulin-dependent) diabetes mellitus associated autoantibodies and their relation to genetic risk markers and clinical characteristics of the disease after clinical manifestation, serum samples were obtained from 90 children and adolescents at diagnosis and 2, 5 and 10 years later. The samples were analysed for islet cell antibodies (ICA) by immunofluorescence and for antibodies to glutamic acid decarboxylase (GADA), intracellular portion of the protein tyrosine phosphatase related IA-2 antigen (IA-2A) and insulin autoantibodies by specific radiobinding assays. Of the subjects tested 79 % were positive for IA-2A at diagnosis, 62 % for GADA, 81 % for ICA and 28 % for insulin autoantibodies, but the prevalence of IA-2A, GADA and ICA decreased substantially as a function of increasing duration of the disease ($p < 0.05$ or less), their levels following the same pattern ($p < 0.001$ for all three autoantibodies). Two thirds of the subjects still tested positive for at least one autoantibody specificity after the first 10 years of the disease and 42 % had two or three antibodies detectable. An increase over the initial antibody concentrations after the diagnosis was seen more often for GADA than for ICA ($p < 0.001$) or IA-2A ($p < 0.05$). In conclusion, autoantibodies associated with Type I diabetes appear to persist longer than expected after manifestation of the clinical disease, possibly due to small scale continuous beta-cell regeneration after diagnosis or to structural and/or functional mimicry between exogenous proteins and beta-cell antigens or both. [Diabetologia (1998) 41: 1293–1297]

Keywords IA-2 antibodies, GAD antibodies, islet cell antibodies, insulin autoantibodies, clinical characteristics, HLA risk markers.

Type I (insulin-dependent) diabetes mellitus is closely related to both cellular and humoral immune responses to insulin-producing beta cells [1]. Prediabetic patients often have autoantibodies against islet antigens [1] that are perceived as markers of beta-cell damage and at the time of diagnosis almost all children and adolescents with diabetes test antibody positive [2]. Little is known, however, about the persistence of these autoantibodies after clinical diagnosis. Islet cell antibodies (ICA) and antibodies to the 65 kDa isoform of glutamic acid decarboxylase (GADA) have been observed to persist after the clinical diagnosis of Type I diabetes, and with highly fluctuating concentrations for GADA [3, 4]. Antibodies to the protein tyrosine phosphatase related IA-2 antigen (IA-2A) have been detected in cross-sectional analyses of patients with long-term Type I diabetes [5]. We studied the persistence of ICA, GADA and IA-2A and their relationship to clinical characteristics and genetic risk markers in a longitudinal 10-year study in 90 children and adolescents with Type I diabetes.

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Abbreviations: GAD65, The 65 kDa form of glutamic acid decarboxylase; GADA, antibodies to GAD65; IA-2A, antibodies to the intracellular portion of the protein (amino acids 605–979) tyrosine-phosphatase related IA-2 antigen; IAA, insulin autoantibodies; ICA, islet cell antibodies; RU, relative unit.

Subjects and methods

Subjects. The group comprised 90 children and adolescents (36 girls) admitted to the Department of Paediatrics, University of Oulu, from 1 January 1983 to 31 December 1986 for initial treatment of Type I diabetes. Blood samples were taken at diagnosis and 2, 5 and 10 years later or at some of these times. There were 52 (58 %) patients with all four samples available, 27 (30 %) with three samples and 11 (12 %) with two samples. These 90 subjects represent 95 % of all children and adolescents under the age of 16 years in whom Type I diabetes was diagnosed during the period concerned in a hospital recruitment area with a total population of approximately 300,000. The mean age of the patients at diagnosis was 8.2 years (SD 3.9 years, range 0.9–15.6 years). Clinical remission was defined as a state occurring shortly after diagnosis in which the daily insulin dose was less than 0.5 $U \cdot kg^{-1} \cdot day^{-1}$ and HbA_1 lower than 9.2 % (mean + 3 SD for non-diabetic subjects). The serum samples were stored at –20 °C until analysed. All samples from the same patient were assayed for autoantibodies simultaneously. The protocol was approved by the local ethics committee, and the subjects or their parents or both gave informed consent to participation in the study.

Autoantibody assays. IA-2A, ICA and insulin autoantibodies (IAA) were analysed as described previously [2]. We measured GADA with a radiobinding assay modified from that described by Bonifacio et al. [6]. The recombinant plasmid pGEM3 encoding the whole 65 kDa form of glutamic acid decarboxylase (GAD65) protein (a gift from E. Bonifacio, Milan, Italy) was multiplied in the *Escherichia coli JM 109* by standard techniques. The GAD65 protein was produced by in vitro transcription and translation of the purified plasmid using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, Wis., USA) in the presence of ^{35}S-methionine (Amersham, Little Chalfont, Bucks, UK). Unincorporated ^{35}S-methionine was removed by gel chromatography on a NAP-5 column (Pharmacia, Uppsala, Sweden). Serum samples of 2 µl were incubated overnight with 20,000 cpm of labelled GAD65. The next day 10 µl of Protein-A-Sepharose was added to isolate the immune complexes. After incubation for at least 1 h the samples were washed five times with approximately 750 µl of TBST [50 mmol/l Tris and 150 mmol/l NaCl (pH 7.4), containing 0.1 % Tween 20 (Fluka Chemie AG, Buchs, Switzerland)]. The activity of samples was measured with a scintillation counter (1450 Microbeta Trilux, Wallac, Turku, Finland) and the results were expressed in relative units (RU) based on a $\log_{10}/\log_{10}$ standard curve constructed on each plate using the cpm results of the dilution series of the plate in question (MultiCalc Wallac). The limit for positivity was set at the 99th percentile for 373 non-diabetic Finnish children and adolescents (5.35 RU). The disease sensitivity of the GADA assay was 69 % and its specificity 100 % based on 140 samples included in the 1995 Multiple Autoantibody Workshop (not published).

HLA typing. We typed HLA DR alleles by a standard two-stage microlymphocytotoxicity test as described earlier [2].

Other laboratory methods. Blood HbA_1 concentrations were determined electrophoretically, with a reference range of 5.5–8.4 % in non-diabetic children. Serum C peptide concentrations were analysed with a radioimmunoassay using commercial reagents that included antiserum M 1230 (Novo Research Institute, Bagsvaerd, Denmark). Proinsulin was separated from C peptide with polyethylene glycol before the assay. The detection limit was 0.02 nmol/l. Postprandial blood samples for the measurement of serum C peptide concentrations were obtained between 12.00 and 14.00 hours. These concentrations were considered to reflect residual beta-cell function, since a close correlation has been observed between concentrations of randomly sampled postprandial C peptide, serum C-peptide measured 60 min after a standardized breakfast and 24 h urinary C peptide excretion [7].

Data handling and statistical analysis. For statistical analysis the patients were divided into two groups by age (< 10 years and ≥ 10 years), into four groups by HLA DR phenotype [DR3/DR4 ($n = 28$), DR4/non-DR3 ($n = 41$), DR3/non-DR4 ($n = 16$) and other DR combinations ($n = 5$)] and into (a) two or (b) three groups based on the pattern for each antibody specificity [a. 1) at least the first sample antibody positive; 2) all samples negative, or b. 1) all samples antibody positive; 2) first sample positive, at least one negative sample later; and 3) all samples negative]. Two patients could not be categorized in terms of their GADA pattern, one in terms of ICA and one in terms of IA-2A. To evaluate differences between the groups in changes in antibody concentrations and clinical characteristics over time, ratios were formed between the values for each variable at two points in time (e. g. ICA_0/ICA_{2years}).

Variables with a skewed distribution (IA-2A, GADA, ICA, IAA, serum C peptide, remission and the respective ratios) were logarithmically transformed before statistical analysis. The data were evaluated statistically using the Chi-square test, the *t*-test for unpaired and paired analyses, one-way analysis of variance and one-way analysis of variance for repeated measures. The Bonferroni correction for multiple comparisons was done where appropriate. The results are expressed as proportions or geometric means (95 % confidence intervals), unless otherwise indicated.

Results

Antibody concentrations and prevalences. The concentrations of IA-2A, GADA and ICA decreased considerably as a function of increasing duration of the disease (Fig. 1) and the prevalences followed the same pattern. At diagnosis 79 % of subjects had IA-2A, after 2 years 74 %, after 5 years 66 % and after 10 years 58 % ($\chi^2_{df3} = 8.4$, $p < 0.05$), the corresponding proportions being 62 %, 53 %, 32 % and 25 % ($\chi^2_{df3} = 28.2$, $p < 0.001$) for GADA and 81 %, 60 %, 41 % and 30 % ($\chi^2_{df3} = 47.1$, $p < 0.001$) for ICA, leading to a decrease in the proportion of patients with three antibodies and an increase in the proportion of antibody negative subjects (Fig. 2). Even so, 67 % of the subjects still had at least one autoantibody detectable 10 years after diagnosis, while 42 % tested positive for two or more antibodies. An increase in circulating GADA concentrations over those at diagnosis was seen in 30 patients (33 %), the corresponding proportion for IA-2A being 17 % (15/89; $p < 0.05$), whereas none of the patients showed an increase over their initial ICA ($p < 0.001$). Striking variations could be observed in circulating GADA concentrations with time in a substantial proportion of the patients (Fig. 3).

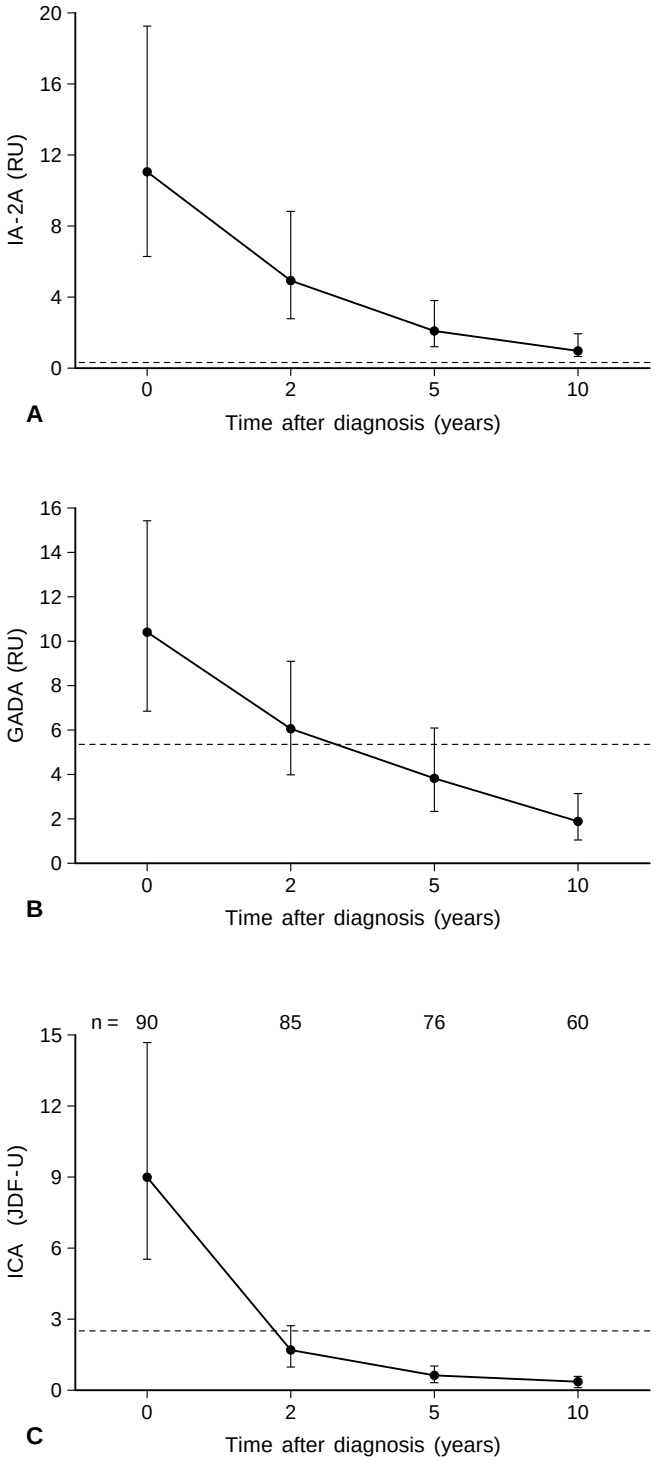

Fig. 1 A–C. Concentrations of IA-2A (**A**), GADA (**B**) and ICA (**C**) decreased during the first 10 years after Type I diabetes was diagnosed. The values are geometric means, 95 % confidence intervals. The dotted lines indicate the cut-off limits for antibody positivity. Statistical analyses, including Bonferroni correction for multiple analyses: **A**. IA-2A; 0 vs 2 years $p < 0.001$, 0 vs 5 years $p < 0.001$, 0 vs 10 years $p < 0.001$, 2 vs 5 years $p < 0.001$, 2 vs 10 years $p < 0.001$ and 5 vs 10 years $p < 0.01$. **B**. GADA 0 vs 2 years $p < 0.001$, 0 vs 5 years $p < 0.001$, 0 vs 10 years $p < 0.001$, 2 vs 5 years $p < 0.001$ and 2 vs 10 years $p < 0.05$. **C**. ICA; 0 vs 2 years $p < 0.001$, 0 vs 5 years $p < 0.001$, 0 vs 10 years $p < 0.001$, 2 vs 5 years $p < 0.001$ and 2 vs 10 years $p < 0.001$. JDF, Juvenile Diabetes Foundation

Persistence of antibodies in relation to age, sex and HLA risk markers. Over the first 2 years after diagnosis GADA decreased more sharply in the patients under the age of 10 years than in older patients ($p < 0.05$), but there was no significant effect of sex on the changes in GADA concentrations. The decrease in IA-2A and ICA concentrations did not differ between the patients under and over 10 years of age or between the boys and girls (data not shown). No relation was seen between the decrease in IA-2A, GADA and ICA concentrations after clinical presentation and HLA DR phenotype (data not shown).

Relation between autoantibody positivity and clinical characteristics of Type I diabetes. The decrease in serum C peptide from 5 to 10 years was steeper in the patients who remained IA-2A positive than in those who converted to seronegativity for IA-2A or had no detectable IA-2 A on any occasion ($p < 0.05$). No differences could be observed in changes in HbA_1 values or daily insulin doses after diagnosis between the subjects who were persistently positive for IA-2A and the other patients, and the duration of clinical remission was the same in both. The patients who remained GADA positive throughout showed no increase in HbA_1 levels between 2 and 5 years, whereas the other patients had an increase in HbA_1 values during this period ($p < 0.05$). There were no differences in the changes observed in serum C peptide concentrations, daily insulin doses or the duration of clinical remission between the two groups. The changes seen in serum C peptide concentrations or HbA_1 levels after the clinical manifestation of Type I diabetes were not associated with the persistence of ICA positivity. The daily insulin dose increased more conspicuously during the first 2 years after diagnosis in the patients who remained ICA negative than in those who were positive for ICA at least initially ($p < 0.05$) but there was no difference in the duration of clinical remission between these two groups.

Discussion

The concentrations of all autoantibodies (IA-2A, GADA and ICA) decreased in general as a function of longer duration of Type I diabetes, leading to low average concentrations after 10 years. The most conspicuous individual variation was shown by GADA, in that a considerable proportion of the patients had higher GADA concentrations on one or more occasions during the follow-up than at diagnosis. In addition, we found two subjects who converted from GADA negativity to positivity after having had the disease for several years. In contrast, ICA and IA-2A concentrations showed a more uniform pattern of decrease over time in most patients. These findings

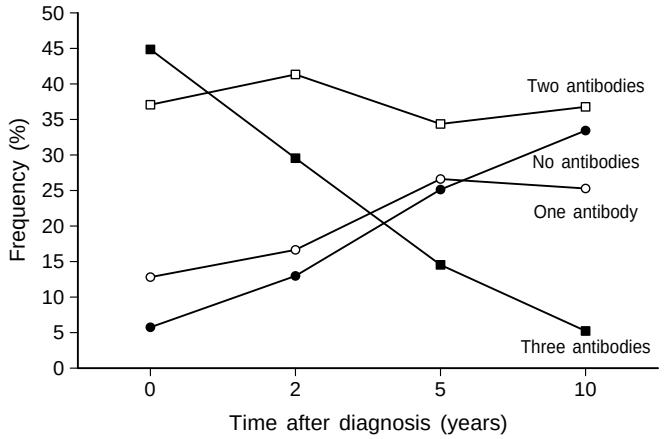

Fig. 2. The frequency of patients testing positive for three antibody specificities (IA-2A, GADA, ICA) decreased during the first 10 years of Type I diabetes ($p < 0.001$) and the proportion of patients with no antibodies increased ($p < 0.001$)

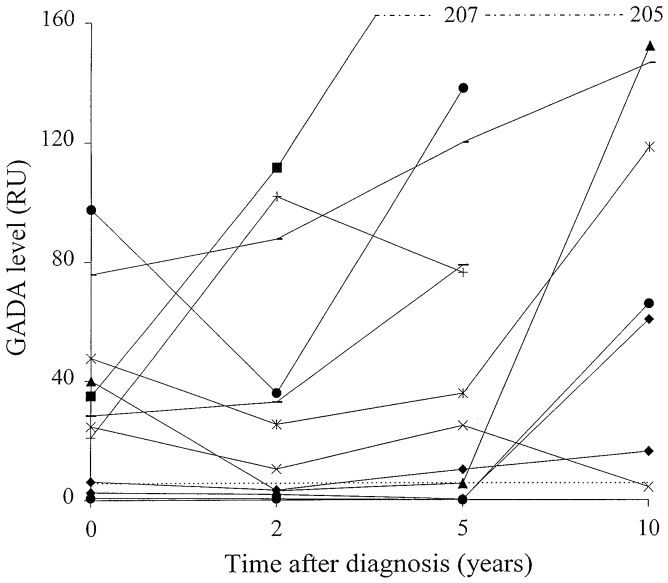

Fig. 3. The eleven patients with the most conspicuous fluctuations in GADA concentrations over their first 10 years of clinical Type I diabetes. The dotted line indicates the cut-off limit for GADA positivity

suggest that GADA behave in many patients with Type I diabetes in a similar manner to most organ specific autoantibodies in other autoimmune diseases which are characterized by substantial fluctuations in the humoral immune response over a long period after clinical manifestation [8].

Our results indicate that disease-associated antibodies persist longer than expected in Type I diabetes. After a disease duration of 10 years one third of these young patients still had ICA, one fourth of them tested positive for GADA, and more than half of them had detectable IA-2A. Although the frequencies of all autoantibodies nevertheless fell as a function of the duration of Type I diabetes, two thirds of the patients still had at least one disease-associated autoantibody at the end of our follow-up and about 40% tested positive for two or more antibodies. Thus, the previous assumption of a rapid disappearance of autoantibodies in Type I diabetes may not be correct [9].

The persistence of autoantibody positivity showed a weak relation with endogenous insulin secretion and clinical characteristics at and after the diagnosis of overt Type I diabetes. Beta-cell function, as measured by serum C peptide, decreased steeper in patients who were persistently IA-2A positive than in other groups, a phenomenon which was reported previously to be associated with persistent ICA positivity [10]. In contrast, the reduced increase in daily insulin requirement over the first 2 years in subjects persistently positive for ICA suggests higher beta-cell function or increased insulin sensitivity or both in these patients. Our study results do not provide any clinical indications for monitoring disease associated autoantibodies after the diagnosis of Type I diabetes. We could not find any relation between the HLA DR phenotypes and changes in antibody concentrations over the first 10 years of clinical Type I diabetes, indicating that the humoral immune response is not regulated by the HLA defined genetic susceptibility, but possibly by other genetic elements or environmental factors.

Why do autoantibodies associated with Type I diabetes persist? We have seen in some patients that the autoantibody level remains the same or even increases despite a substantial reduction in beta-cell function. Although IA-2 and GAD are not beta-cell selective, it is unlikely that other cells of the body would release enough antigen to keep up a humoral immune response in patients with Type I diabetes. One option is that exogenous proteins possessing structural or functional homology with GAD, IA-2 or other beta-cell molecules or both account for the persistence of antibodies against these beta-cell antigens. In addition, there could be a continuous, but small scale regeneration of beta cells in the pancreas of Type I diabetic patients which is sufficient to maintain an immune response without having any clinical impact [11].

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