Patterns of β-cell Autoantibody Appearance and Genetic Associations During the First Years of Life

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Short running title: First autoantibody and heterogeneity in T1D

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2 Tables, 2 Figures
Abstract

We analyzed demographic and genetic differences between children with various diabetes-associated autoantibodies reflecting the autoimmune process. In a prospective birth cohort comprising children with HLA-conferred susceptibility to type 1 diabetes (T1D) the pattern of autoantibody appearance was analyzed in 520 children with advanced β-cell autoimmunity associated with high disease risk. In 315 cases a single biochemical autoantibody could be identified in the first positive sample, to insulin (IAA) in 180, to GAD (GADA) in 107 and to islet antigen-2 (IA-2A) in 28. The age at seroconversion differed significantly between the three groups ($P=0.003$). IAA as the first autoantibody showed a peak of appearance during the second year of life, whereas GADA as the first autoantibody peaked later between age 3 to 5. The risk associated insulin gene rs689 AA genotypes were more frequent in children with IAA as the first autoantibody compared to the other children ($P=0.002$). The primary autoantigen in the development of β-cell autoimmunity and T1D seems to strongly correlate with age and genetic factors indicating heterogeneity in the initiation of the disease process.
Immune destruction of the insulin producing β-cells leading to clinical type 1 diabetes (T1D) is often a long-lasting process. During this preclinical period antibody responses to several autoantigens can be detected. The number of detectable autoantibodies usually increases with time and correlates with the probability of disease development (1). It is not clear whether a primary autoantigen does exist and immune responses against other molecules represent secondary antigen spreading or whether multiple molecules can be primary targets (2). Heterogeneity in the preferential age of appearance and HLA associations has been described for various autoantibodies (3-6). The early appearance and association with younger age at diagnosis of insulin autoantibodies (IAA) together with the essential role of the insulin molecule in the NOD mouse model have implied (prepro)insulin as the primary antigen (7). Antibody responses to all major antigens nevertheless accumulate with increasing age during the follow-up.

Natural history studies in high-risk children with sequential blood sampling at short intervals provide a unique possibility to analyze the appearance of autoimmunity in detail and help to resolve the question whether one or multiple primary antigens exist. In the current study we analyzed in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study children who developed advanced β-cell autoimmunity meaning multiple autoantibodies and high disease risk and correlated the specificity of the primary autoantibody with demographic and genetic parameters.
RESEARCH DESIGN AND METHODS

The DIPP study protocol includes a follow-up with sequential blood sampling and autoantibody measurements from birth at 3 to 6 month intervals until to the age of 12 month and 6 to 12 month intervals thereafter in children with HLA-conferred susceptibility to T1D (Supplementary Table and Figure) (8; 9). Islet-cell antibodies (ICA) were measured using immunofluorescence and antibodies against insulin, GAD65 (GADA) and islet antigen 2 (IA-2A) with specific radiobinding assays using cut-off rates based on the 99th percentile in more than 350 non-diabetic subjects (10). Disease sensitivities and specificities of the assays in our laboratory according to the 2002-2010 DASP workshops were 44-50% and 96-99% for IAA, 82-92% and 94-97% for GADA, and 64-72% and 97-100% for IA-2A, respectively. For the current study we selected children with “advanced autoimmunity” defined as at least two consecutive samples positive for two biochemically defined autoantibodies (IAA, GADA and/or IA-2A), two consecutive samples positive for one biochemical autoantibody and development of clinical diabetes or repeated positivity for ICA and IAA, which combination also has been associated with high risk of T1D in the DIPP study (10). This resulted in a cohort of 520 children out of whom a single biochemically defined autoantibody was detected before the development of multiple autoantibodies in 315 children. Cases with ICA appearing simultaneously with IAA as the first autoantibody were included as this group (N=66) was similar to that with IAA alone in respect for age and T1D progression. Accordingly we distinguished three groups of children, those with IAA (N=180, 109 male), GADA (N=107, 55 male) or IA-2A (N=28, 18 male) as their first biochemical autoantibody.
Genotyping methods. The major Caucasian HLA-DR/DQ haplotypes were defined using panels of sequence-specific oligonucleotide probes as described earlier (11). *INS* rs689 and *PTPN* rs2476601 were genotyped using the Sequenom® platform in the Genome Center of the University of Eastern Finland, Kuopio, or TaqMan® SNP genotyping array.

Statistical analysis. Data was analyzed using SPSS 19.0 Software (IBM SPSS Inc, Chicago, IL, USA). Kaplan-Meier log-rank test was used for comparing progression to clinical disease and Kruskall-Wallis test for comparing age distribution at the time of autoantibody seroconversion. Frequencies of genotypes were compared with Pearson Chi-square test and Fisher Exact test when appropriate.

RESULTS

The high probability of progression to clinical diabetes among children with advanced β-cell autoimmunity regardless of the specificity of the autoantibody initiating the process is demonstrated in Figure 1. The significant heterogeneity between the three groups (*P*=0.021, Kaplan-Meier test) was due to the more rapid progression in children with IA-2A as their first autoantibody. No difference was seen between children with either IAA or GADA as the first autoantibody (*P*=0.490, Kaplan-Meier test).

When analyzing the age at the appearance of the first biochemical autoantibody the groups with various first autoantibodies differed clearly from each other (Figure 2,
left panel). IAA as the first autoantibody usually appeared very early with a sharp peak at the age between 1 and 2 years (median age 1.49 yrs, interquartile range 1.01-2.99 yrs) whereas the appearance of GADA as the first autoantibody was considerably more even and peaked between the age of 3 to 5 years and only slowly decreased thereafter (median age 4.04 yrs, interquartile range 2.57-6.80 yrs). IA12A as the first autoantibody was relatively rare with a pattern intermediate between IAA and GADA (median age 3.03 yrs, interquartile range 1.52-4.28). The difference in the age distribution between the three groups was highly significant ($P=0.003$, Kruskall-Wallis test). Secondary antibodies revealed totally opposite patterns, which masks the differences regarding primary autoantibodies if both are combined (Figure 2, right panel). GADA as the secondary antibody usually follow very rapidly the primary antibody response being most often IAA, and the peak also occurs before the age of 2 years. Secondary IAA are mostly following GADA and thus the age distribution is relatively even over a wide age range.

The three groups with different primary autoantibodies were then compared for the frequency of HLA-DR/DQ genotypes and genotypes of the two most important risk-associated non-HLA loci INS and PTPN22. Children positive for the HLA-DR3-DQ2 haplotype without HLA-DR4-DQ8 were rare in the study group because of the HLA screening criteria and we could not find significant differences in HLA genotypes when all three groups were compared and the number of comparisons was taken into account. However, all three DR3-DQ2 homozygous children were in the group with GADA as the first autoantibody whereas children with IA-2A as the first autoantibody were less often positive for the DR3-DQ2 halotype (4/28, 14.3%) than others (95/287, 33.1%). These differences reached nominal significance at the $P=0.05$
Table 1 demonstrates significant differences in the distribution of INS rs689 genotypes between groups with different first autoantibody. The frequencies of INS rs689 risk genotype A/A and the A allele were increased among children with IAA as the first autoantibody. There were no significant differences among PTPN22 rs2476601 genotypes. The association with INS gene polymorphism was not observed for IAA as the secondary autoantibody. The risk-associated rs689 AA genotype was seen in only 44/67 (65.7%) of the children with IAA as the secondary autoantibody, which is significantly lower than the proportion of 82.2% seen in children with IAA as the primary autoantibody (P=0.0054).

DISCUSSION

The appearance of various diabetes-associated autoantibodies in children at genetic risk has traditionally been described by curves showing a continuously increasing prevalence of each autoantibody with IAA starting to appear as the first and IA-2A as the last one (12; 13). When the rate of appearance has been assessed more closely, a peak in the appearance of all biochemical autoantibodies was demonstrated already before the age of 2 years whereafter the rate of new seroconversions decreased (14; 15).

In the current study we focused on examining the specificity of the first autoantibody to appear and analyzed secondary autoantibodies separately. This revealed clear
differences in the timing of autoantibody appearance, which remain unnoticed when combining primary and secondary autoantibody responses. We observed that IAA as the first autoantibody is appearing very early, median age 1.5, but IAA emerges evenly over many years as secondary autoantibody. The development of GADA as the first autoantibody is instead distributed rather evenly over the preschool age but as secondary peak early similarly to IAA. The kinetics of secondary autoantibodies is related to the rapid antigen spreading during the progression of the autoimmune response, and the conspicuous difference in the first autoantibody responses can accordingly be detected only in a follow-up program with short enough intervals in early childhood. The short interval of only 3 months between samples collected from the before the age of 2 years allowed us to distinguish the specificity of the primary autoantibody in most cases who developed advanced autoimmunity.

The major groups of children with either IAA or GADA as their first autoantibody were extremely similar in the survival analysis; around 60% of the children in both groups progressed to T1D after follow-up for less than 10 years, but the small group presenting with IA-2A as their first autoantibody showed an even more rapid progression. Remarkable differences in the peak age at the appearance of the IAA and GADA as the first autoantibody despite similar progression rate after the autoantibody appearance indicate differences in the phase of initial induction of autoimmunity. The specificity of the first autoantibody could be determined by genetic factors. \textit{INS} gene risk allele was associated with the appearance of IAA as the first autoantibody. The children with IAA had a frequency similar to that observed in Finnish children with T1D (16) whereas children with GADA or IA-2A as the first autoantibody resembled background controls. This specific association of the \textit{INS} gene polymorphism with
IAA is in line with earlier findings (17; 18). INS gene risk allele was, however, associated with IAA only as the first autoantibody, and not with the IAA appearing as the secondary autoantibodies. The genetic regulation of insulin-specific immune response seems to differ depending whether IAA is induced as the first or as the secondary autoantibody. INS gene polymorphism regulates the thymic expression of insulin (19), and thus its role is linked to the thymic immune-regulation, i.e. central tolerance and induction of natural regulatory T-cells. These factors may be more important in the generation of insulin-specific immune response in the children with early induction of IAA, whereas the development of IAA as the secondary autoantibody, during antigen-spreading, may rather be regulated by the mechanisms of peripheral tolerance. The specificity of the first autoantibody was also strongly dependent on the age, which suggest that the specificity of initial autoantibody may also reflect different environmental factors exerting their influence in early infancy and in later childhood.

Distinct HLA associations of various autoantibodies are also known. At the diagnosis of the disease the HLA-DR3-DQ2 haplotype is more often seen in patients positive for GADA whereas DR4-DQ8 shows preferential association with IAA and IA-2A (3-6;20). As a result of the inclusion criteria in the DIPP study there were few children positive for HLA-DR3-DQ2 without HLA-DR4-DQ8 but still it is conspicuous that all three homozygotes had GADA as their first autoantibody. The HLA-DR3-DQ2 was instead less often present in the small group with IA-2A as the first autoantibody. These findings are in accordance with the observations seen in studies at the diagnosis of T1D.
The follow-up was most intensive and the results thus most representative for β-cell autoimmunity launched early in childhood. Some bias for higher numbers of cases showing seroconversion at an early age is also caused by accumulation of dropouts during the follow-up but this does not affect the validity of the comparisons between the groups. Expanded follow-up series will be needed to characterize autoantibody development in T1D diagnosed in adulthood, which in most countries includes a majority of the patients. The so far best characterized autoantibodies were included in the analysis but further information might be available from the inclusion of additional autoantibody specificities, most obviously zinc transporter 8 antibodies (ZnT8A) and the recently described ECL-IAA. However, the contribution of ZnT8A to the overall risk estimation seems to be small among children who are positive for multiple other autoantibodies and ZnT8A rarely present as the first autoantibody to appear (21-23). ECL-IAA was found more sensitive than radioimmunoassay to detect persistent IAA associated with T1D development and 94% of children who progressed to T1D in DAISY study were positive at 18 months (24). Data from both the DAISY and our study emphasize the importance of IAA as an early marker of beta-cell autoimmunity in children.

In conclusion, these results show age, genetics and antigen-specificity related heterogeneity in the initiation of β-cell autoimmunity, which might be informative in the search of the environmental triggers and prevention of the disease.
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Author contributions

J.I. acquired and reviewed the research data used in the analysis, undertook the statistical analysis and interpretation of the results, and drafted the manuscript. A.H., A-P. L., J.L., R.V., O.S., and M.K. acquired the data. J.L., O.V., R.V. and M.K. were involved in the interpretation of the results and critically reviewed the manuscript.
M.K. and R.V. were responsible for the autoantibody analyses. DIPP study was designed by J.I., M.K. and O.S. J.I. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

No potential conflicts of interest relevant to this article were reported.

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FIGURE LEGENDS

Fig. 1. Progression to clinical T1D among children with advanced autoimmunity when divided according to the first biochemical autoantibody to appear (IAA dashed line, GADA solid line and IA-2A dotted line). The follow-up started from the appearance of the first biochemical autoantibody.

Fig. 2. The age distribution for the appearance of the first autoantibody in the group of children with advanced β-cell autoimmunity (left panel) compared to the age distribution for the secondary autoantibodies appearing after the first one (right panel). The clear differences seen in the first autoantibody is masked by secondary autoantibodies if combined.
### TABLE 1.

Genotypes of T1D risk associated SNP polymorphisms in INS and PTPN22 genes according to the first autoantibody to appear.

| First autoantibody | INS rs689 | GADA (N=107) | IA-2A (N=28) |
|--------------------|-----------|--------------|--------------|
|                    | A/A       | 148 (82.2%)  | 67 (62.6%)   | 19 (67.9%)  |
|                    | A/T       | 31 (17.2%)   | 36 (33.6%)   | 9 (32.1%)   |
|                    | T/T       | 1 (0.6%)     | 4 (3.7%)     | 0 (0.0%)    |
| **P=0.002, df=4**  |           |              |              |             |
| A                  | 327 (90.8%)| 170 (79.4%)  | 47 (83.8%)   |
| T                  | 33 (9.2%)  | 44 (20.6%)   | 9 (16.1%)    |
| **P=0.001, df=2**  |           |              |              |             |
| PTPN22 rs2476601   |           |              |              |             |
|                    | T/T       | 16 (8.9%)    | 4 (3.7%)     | 0 (0.0%)    |
|                    | C/T       | 45 (25.0%)   | 24 (22.4%)   | 7 (25.0%)   |
|                    | C/C       | 119 (66.1%)  | 79 (73.8%)   | 21 (75.0%)  |
| **P=0.226, df=4**  |           |              |              |             |
| T                  | 77 (21.4%)| 32 (15.0%)   | 7 (12.5%)    |
| C                  | 283 (78.6%)| 182 (85.0%)  | 49 (87.5%)   |
| **P=0.077, df=2**  |           |              |              |             |
Supplementary Figure 1. Situation of the Finnish Diabetes Prediction and Prevention (DIPP) study in the end of year 2012

Children screened for HLA-DR/DQ associated genetic risk
N=168,055

Children defined to be at genetic risk
N=14,876

Children beginning the regular follow-up
N=12,497

Children developing advanced autoimmunity
N=520

Children developing Type 1 diabetes
N=271
Supplementary Table 1. Genetic screening and follow-up scheme in various phases of the DIPP study. Three centers (Turku, Oulu and Tampere) with roughly similar recruitment.

| Eligibility criteria for HLA based genetic screening | Follow-up | Antibody measurements |
|-----------------------------------------------------|-----------|-----------------------|
| Nov 1994 – Mar 1997                                | Nov 1994 – Feb 2010 | In the Turku center 3 month intervals until age of 2 yrs; 6 month intervals thereafter. |
| DQB1*03:02 present; DQB1*03:01, DQB1*06:02/3 not present |           | ICA screening. If positive IAA, GADA and IA-2A from all samples |
| Apr 1997 – Aug 2004                                |           | Jan 2003 – |
| DQB1*03:02 present; DQB1*03:01, DQB1*06:02 not present | Sep 1995 – Feb 2010 | In the Oulu center 3, 6, 12, 18 and 24 months and yearly thereafter. |
| Boys with HLA-DQA1*05-DQB1*02 haplotype also eligible in one of the three centers (Turku) | Oct 1997 – Feb 2010 | Same in the Tampere center |
| Sep 2004 – Feb 2010                                |           | |
| DQA1*05-DQB1*02/DRB1*04 (not *04:03/6)-DQB1*03:02 present† | Oct 2004 – Feb 2010 | In all centers 3 month intervals after AAB positivity |
| DQA1*05-DQB1*02/DQA1*05-DQB1*02 present† |           | |
| DRB1*04 (not *04:03/6)-DQB1*03:02/ DRB1*04 (not *04:03/6) – DQB1*03:02 present† |           | |
| DQB1*04 (not *04:03/6)-DQB1*03:02/DRB1*08-DQB1*04 present† |           | |
| DRB1*04:01-DQB1*03:02 present; DQB1*05:03, DQB1*06:02, DQA1*02:01-DQB1*03:03 present |           | |
| DQA1*05-DQB1*02 / DQA1*03-DQB1*03:03 present |           | |
| Mar 2010 –                                        | Mar 2010 – | 3, 6, 9, 12, 24 months and yearly thereafter. All centers |
| DQA1*05-DQB1*02 / DRB1*04:01/2/4/5-DQB1*03:02 |           | |
| DQA1*05-DQB1*02 / DQA1*05-DQB1*02 |           | |
| DRB1*04:01/2/4/5-DQB1*03:02 / DRB1*04:01/2/4/5-DQB1*03:02 |           | |
| DRB1*04:01/2/5-DQB1*03:02 / DQB1*04 present |           | |
| DRB1*04:01/2/5-DQB1*03:02 / DQB1*05:01 present |           | |
| DRB1*04:01/2/5-DQB1*03:02 / DQB1*06:04 present |           | |
| DRB1*04:01/2/5-DQB1*03:02 / DQA1*02:01-DQB1*02 present |           | |
| DRB1*04:01/2/5-DQB1*03:02 / DQA1*03-DQB1*03:03 present |           | |
| DQA1*05-DQB1*02 / DQA1*03-DQB1*03:03 present |           | |

†Eligible for TEDDY study and primarily recruited for it