Effect of HLA Class I and Class II Alleles on Progression from Autoantibody Positivity to Overt Type 1 Diabetes in Children with Risk-Associated Class II Genotypes

Short running title: HLA and progression of diabetes-associated autoimmunity

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**Objective**—Class II alleles define the main HLA effect on type 1 diabetes, but there is an independent effect of certain class I alleles. Class II and class I molecules are differently involved in the initiation and effector phases of the immune response, suggesting that class I alleles would be important determinants in the rate of β-cell destruction. To test this hypothesis we analyzed the role of HLA class I and class II gene polymorphisms in the progression from diabetes-associated autoimmunity to clinical disease.

**Research design and methods**—The effect of HLA-DR-DQ haplotypes and a panel of class I HLA-A and -B alleles on the progression from autoantibody seroconversion to clinical diabetes was studied in 249 children persistently positive for at least one biochemical diabetes-associated autoantibody in addition to ICA.

**Results**—The progression to clinical disease was separately analyzed after the appearance of the first and the second persistent biochemical autoantibody using Cox regression. Multivariate analysis demonstrated a significant protective effect of the A*03 allele (OR=0.61; P=0.042 after first, and OR 0.55; P=0.027 after the second autoantibody), whereas the B*39 allele had a promoting effect after seroconversion for the second autoantibody (OR 2.4; P=0.014). When children with the DR3/DR4 genotype were separately analyzed, HLA-B*39 had a strong effect (OR 6.6, P=0.004 and OR=7.5, P=0.007, after the appearance of the first and the second autoantibody, respectively). The protective effect of A*03 was seen only among children without the DR3/DR4 combination.

**Conclusions**—These results confirm that class I alleles affect the progression of diabetes-associated autoimmunity, and demonstrate interactions between class I and class II alleles.

The HLA gene region on the short arm of chromosome 6 is the most important of the multiple gene loci affecting susceptibility to type 1 diabetes (T1D). Disease onset is preceded by the presence of circulating autoantibodies as a marker of the ongoing autoimmune process, and the duration of this period is highly variable (1). There may be differences in the genetic and environmental factors affecting the initiation of the autoimmune response and the later course of β-cell autoimmunity, conceivably leading to clinical disease. This has also been suspected for genes within the HLA region where susceptibility to T1D is mainly defined by alleles of class II DR and DQ genes although evidence has also accumulated for the contribution of class I alleles in the A and B loci (2-5). It has been proposed that class II genes determine the initiation of autoimmunity, whereas class I genes define the progression of β-cell damage (6). This model is theoretically supported by the roles of class II and class I molecules in the immune response. This response is initiated when CD4+ T cells recognize antigens in the context of class II HLA molecules, whereas cytotoxic CD8+ T-cells respond to antigenic peptides presented by the class I molecules.

To further explore this hypothesis we analyzed the effect of common class II haplotypes and a panel of class I alleles on the rate of progression to clinical diabetes in a follow-up group of children with established
diabetes-associated autoimmunity. This cohort of 249 children was derived from the Finnish Diabetes Prediction and Prevention (DIPP) study. All the subjects had at least one persistently positive biochemically defined autoantibody in addition to islet cell autoantibodies (ICA); 136 (54.6%) of them developed T1D during the follow-up period.

RESEARCH DESIGN AND METHODS
The newborn infants were recruited to the DIPP study in three university hospitals in Finland: Turku, Oulu and Tampere. After initial screening for HLA-DQ-associated genetic risk, the follow-up group was sampled at 3-12 month intervals and serum tested for ICA. Originally, newborns positive for HLA-DQB1*0302 and without DQB1*0301 or DQB1*0602/3 alleles were selected for the study but later on also those with DQB1*0302/DQB1*0603 and boys with DQA1*05-DQB1*02 without DQA1*0201, DQB1*0301 or DQB1*0602/3 were accepted (7). If ICA were found to be positive, all samples available from that individual were tested for biochemically-defined autoantibodies, i.e. insulin autoantibodies (IAA), antibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA) and to the protein tyrosine phosphatase related IA-2 molecule (IA-2A). All ICA-positive children who tested persistently positive (at least two consecutive positive samples taken at an interval of 3 months or longer) for at least one biochemically-defined autoantibody besides ICA and whose sample was available for further genotyping (N=249) were selected for the study. Of them, 195 were persistently positive for two or three biochemically-defined autoantibodies. Altogether 201 of the children (80.7%) tested positive for IAA, 184 (73.1%) for GADA, and 176 (70.6%) for IA-2A. The lack of persistent autoantibody positivity was accepted only when the autoantibody was detected for the first time at the time of the diagnosis of diabetes, as no further samples were available after the diagnosis.

The median age of the children at the time of seroconversion to positivity for the first biochemically-defined autoantibody was 1.8 years (range 0.3 – 9.9 yrs) and 2.0 (range 0.8-9.9) for the second biochemically defined autoantibody. Median follow-up time from the first biochemically-defined autoantibody was 6.4 years (range 0.5-12.5 yrs) in the 113 children remaining non-diabetic and 2.8 years (range 0.0-10.8 years) in the 136 children progressing to T1D. Median follow-up time from the appearance of the second biochemically-defined autoantibody was 6.1 years (range 1.8-12.0 yrs) in the 78 children remaining unaffected and 2.5 years (range 0.0-5.8 years) in the 117 children progressing to T1D. The diagnosis of T1D was based on the WHO criteria (8).

Genotyping methods. HLA class II typing was performed as described earlier using a panel of lanthanide-labeled oligonucleotide probes (9; 10). HLA-DQB1 alleles were analyzed as the first step and DQA1 and DRB1 alleles thereafter as needed for the haplotype deduction. The typing protocol defined the presence of common European HLA-DR-DQ haplotypes with special reference to those associated with diabetes risk (11). The high risk genotypes formed by HLA-(DR3)-DQA1*05-DQB1*02 and HLA-DRB1*0401-DQB1*0302 or HLA-DRB1*0404-DQB1*0302 haplotypes were combined and named the DR3/DR4 genotype. All 249 children were successfully typed for the presence of class II haplotypes. The common HLA-A and -B alleles in the Finnish population were typed using allele-specific amplification and detection of amplification products on agarose gels (12). The defined alleles include those commonly detected in T1D-associated HLA-DR3 and DR4 positive haplotypes (5; 13). The presence of HLA-A*01, -A*02, -A*03, -A*24, -A*28, -A*32, -B*08, -B*27, -B*35, -B*39, -B*56, -B*60
statistical analysis. The effect of various HLA alleles, haplotypes and genotypes on the progression to clinical disease was tested applying Cox regression analysis. The PASW 18.0 statistical software (SPSS Inc., Chicago, IL) was used for the analyses.

RESULTS
We started by using the Cox regression univariate analysis to test the effect of all typed class I alleles and class II haplotypes on the progression to type 1 diabetes after the appearance of the first biochemically-defined autoantibody as well as after the appearance of at least two biochemically-defined autoantibodies. Those class I alleles with a significant effect (P<0.05) in the analysis carried out after the appearance of either one or two persistently positive biochemical autoantibodies were selected for multivariate analysis together with the HLA-DR3/DR4 genotype, which in the univariate analysis showed a significant association (P=0.010) with progress to disease when tested after the appearance of the first biochemical autoantibody. When the effect of the HLA-DR-DQ haplotypes was tested, DR(3)-DQA1*05-DQB1*02 was found to be associated with progression to T1D (P=0.015) and (DR1/10)-DQB1*0501 with protection against overt disease (P=0.03). Similar associations were also found when these two haplotypes were associated with DRB1*0401-DQB1*0302; these haplotype associations were most probably secondary to the DR3/DR4 genotype association because of the selection criteria used for the majority of the study participants. (DR3)-DQA1*05-DQB1*02 was found to be associated with progression to T1D (P=0.015) and (DR1/10)-DQB1*0501 with protection against overt disease (P=0.03). Similar associations were also found when these two haplotypes were associated with DRB1*0401-DQB1*0302; these haplotype associations were most probably secondary to the DR3/DR4 genotype association because of the selection criteria used for the majority of the study participants. (DR3)-DQA1*05-DQB1*02 was found to be associated with progression to T1D (P=0.015) and (DR1/10)-DQB1*0501 with protection against overt disease (P=0.03). 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DISCUSSION
Our results confirm the strong effect of two class I alleles on the progression of T1D-associated autoimmunity. The possibility that other linked genes in the MHC block are behind these findings cannot be excluded, but the binding of autoimmune epitopes efficiently presented to cytotoxic CD8 cells these by class I alleles is an attractive hypothesis for the operative mechanism. Although it is clearly demonstrated that class II HLA genotypes associated with T1D are also linked to positivity for multiple autoantibodies predicting the development of clinical disease (7; 14; 15), their additional
role in affecting the progression of β-cell autoimmunity cannot be excluded. Two studies based on the DPT-1 trial series have set out to analyze the effect of class II HLA alleles on the progression to T1D among autoantibody-positive first-degree relatives. Redondo and co-workers concluded that the strong class II genotype effects observed were mainly due to differences in the initial autoantibody profiles of the study subjects (16). The other DPT-1 study selected only subjects with ICA and IAA with initially preserved β-cell function and reported that the DQB1*0302 allele was associated with progression while DQB1*0301 was related to protection against T1D (17). The DPT-1 cohort comprised older individuals than our subjects and the timing of seroconversion could not be defined in the DPT-1 study.

In the present survey we were able to start the analysis from the initial detection of autoantibodies in children closely monitored from birth onward. After performing the analysis from the appearance of the second instead of the first biochemical antibody, the original tendency of the DR3/DR4 effect disappeared, indicating that the possible effect might reflect expansion of the autoimmune response to a more advanced type with more autoantibody specificities associated with a higher risk.

The strong linkage disequilibrium between alleles in class I and class II HLA loci emphasizes the importance of stratification for class II alleles when looking for class I effects. The major finding in our study, the strong effect of B*39 on the progression from β-cell autoimmunity to clinical disease, was only seen in subjects carrying the combination of both major class II risk haplotypes, the DR3/DR4 genotype. We have earlier observed HLA-B*39 to be common in T1D-associated DRB1*0404 haplotypes in Finland (5), and we have also been able to demonstrate its predisposing effect on T1D risk in DRB1*0404-DQB1*0302 positive subjects in Estonia and Russia (18). The presence of the HLA-B*39 allele in the DRB1*08-DQB1*0402 haplotype has also been observed found to be a risk factor for T1D (3). Extensive SNP analysis has recently confirmed the independent effect of class I alleles and especially the B*39 allele on T1D risk (2). In this study HLA-B*39 was also in most cases apparently found in DRB1*0404-positive haplotypes but interestingly the combination with DR3-positive haplotype was needed as the B*39 effect could not be seen in other genotypes with DRB1*0404. Among DR3/DR4 heterozygotes, 7 out of 11 B*39 positive samples were DRB1*0404 positive. HLA-B*39 might of course also be associated with the other haplotype in these cases but in a Finnish family trio analysis we detected B*39 in only 0.9% of 326 (DR3)-DQA1*05-DQB1*02 haplotypes transmitted to the diabetic child (unpublished data). This difference in B*39 effect between DR3/DR4 and other genotypes may suggest some specific interactions between class II and class I.

The genetic constitution of our study population, the Finnish population in general, and especially the follow-up cohort derived through HLA DQ-based genetic screening (7) must also be taken into account when interpreting the results. Almost all children included in the current analysis carried the DR4-DQB1*0302 haplotype with either DRB1*0401 or DRB1*0404. We are thus unable to distinguish between the findings associated with either DR3/DR4 genotype or DR3 positivity. Because of the genetic screening criteria applied for the study group we were unable to analyze the possible further effects of protective or neutral class II genotypes on the progression of β-cell autoimmunity.

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Table 1. Cox regression analysis of HLA effects on progression to clinical diabetes in children with diabetes-associated autoantibodies.

Follow-up from appearance of first biochemically-defined autoantibody (Stratified according to age at appearance of first biochemically defined autoantibody)

| Variable    | P value | HR for diabetes progression (95% CI) |
|-------------|---------|-------------------------------------|
| HLA-A*03    | 0.042   | 0.611 (0.380-0.982)                 |
| HLA-A*24    | 0.191   | 1.422 (0.839-2.411)                 |
| HLA-B*39    | 0.159   | 1.549 (0.843-2.844)                 |
| HLA-DR3/4   | 0.070   | 1.482 (0.968-2.269)                 |

Follow-up from appearance of second biochemically-defined autoantibody (Stratified according to age at appearance of second biochemically-defined autoantibody)

| Variable    | P value | HR for diabetes progression (95% CI) |
|-------------|---------|-------------------------------------|
| HLA-A*03    | 0.027   | 0.552 (0.326-0.933)                 |
| HLA-A*24    | 0.598   | 1.176 (0.644-2.146)                 |
| HLA-B*39    | 0.014   | 2.401 (1.196-4.823)                 |
| HLA-DR3/4   | 0.809   | 1.063 (0.647-1.746)                 |

DR3/4 = (DR3)-DQA1*05-DQB1*02/DRB1*0401/4-DQB1*0302
Table 2. Cox regression analysis of HLA class I effects on progression to clinical diabetes in children with diabetes-associated autoantibodies when categorized according to presence of DR3/DR4 class II combination

| Variable  | P value | HR for diabetes progression (95% CI) | P value | HR for diabetes progression (95% CI) |
|-----------|---------|-------------------------------------|---------|-------------------------------------|
| HLA-A*03  | 0.015   | 0.490 (0.276-0.870)                 | 0.741   | 1.142 (0.518-2.518)                 |
| HLA-A*24  | 0.096   | 1.643 (0.915-2.948)                 | 0.142   | 0.372 (0.099-1.391)                 |
| HLA-B*39  | 0.660   | 1.208 (0.520-2.805)                 | 0.004   | 6.564 (1.801-23.926)                |

Follow-up from appearance of second biochemically-defined autoantibody (Stratified according to age at appearance of second biochemically-defined autoantibody)

| Variable  | P value | HR for diabetes progression (95% CI) | P value | HR for diabetes progression (95% CI) |
|-----------|---------|-------------------------------------|---------|-------------------------------------|
| HLA-A*03  | 0.003   | 0.388 (0.208-0.724)                 | 0.554   | 1.310 (0.535-3.206)                 |
| HLA-A*24  | 0.252   | 1.455 (0.766-2.765)                 | 0.117   | 0.297 (0.065-1.356)                 |
| HLA-B*39  | 0.013   | 3.160 (1.279-7.806)                 | 0.007   | 7.454 (1.740-31.928)                |
LEGENDS FOR THE FIGURES

Fig. 1. The effect of the HLA-B*39 allele on the progression to type 1 diabetes after seroconversion to persistent positivity for ICA and at least one biochemically-characterized autoantibody in children with the HLA-DR3/DR4 combination (A), and children with other class II genotypes (B). HLA-B*39-positive children indicated by solid line and B*39-negative by dashed line. Kaplan-Meier analysis demonstrated a highly significant difference between the B*39 positive (N=11) and B*39 negative (N=53) groups among children carrying the DR3/DR4 combination ($P=0.00007$, Log Rank test) but no difference was seen between the HLA-B*39 positive (n=17) and negative children (n=127) who did not carry the high-risk HLA class II combination ($P=0.768$, Log Rank test). The panels below the Figure show the number of subjects followed at each time point.