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The effect of HLA class II, insulin and *CTLA4* gene regions on the development of humoral beta cell autoimmunity

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Abstract *Aims/hypothesis:* The aim of this study was to explore the contribution of genetic factors to the emergence of beta-cell-specific humoral autoimmunity. *Subjects and methods:* We analysed the effect of HLA class II, insulin (*INS*; -23 HphI variant) and cytotoxic T-lymphocyte-associated protein 4 (*CTLA4* [+49 and CT60]) genes on the appearance of beta-cell-specific autoantibodies in a large population-based birth cohort recruited in Finland. Infants carrying increased risk HLA *DQB1* genotypes were monitored for the appearance of autoantibodies (islet cell autoantibodies [ICA], insulin autoantibodies [IAA], glutamic acid decarboxylase autoantibodies [GADA] and islet antigen 2 antibodies [IA-2A]). Those who developed beta-

cell-specific autoantibodies were studied ($n=574$, mean follow-up time: 4.9 years; range 0.5–9.3). *Results:* IAA emerged at a higher rate in children with the -23 HphI AA *INS* genotype than in those carrying AT or TT variants (hazard ratio 2.1, 95% CI 1.4–2.9, $p<0.001$). This effect of the *INS* locus was present in both HLA *DQB1* risk groups. The appearance of IAA showed a strong association also with the HLA *DRB1*0401* allele (hazard ratio 13.1, 95% CI 1.8–93.4, $p<0.001$). The development of IA-2A was also somewhat accelerated by the *DRB1*0401* variant ($p=0.03$). Isolated ICA positivity was independent of the HLA and *INS* genotypes. None of the humoral immune markers showed association with the *CTLA4* gene. *Conclusions/interpretation:* The *INS* and the *DRB1* loci appear to contribute to the pathogenesis of type 1 diabetes by initiating/modifying insulin-specific autoimmunity. The emergence of IAA represents a crucial step in the development of beta cell autoimmunity in young children, in whom the appearance of GADA and IA-2A is linked to IAA.

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Abbreviations CTLA4: cytotoxic T-lymphocyte-associated protein 4/IDDM12 · DIPP: Type I Diabetes Prediction and Prevention project · GADA: glutamic acid decarboxylase autoantibodies · IAA: insulin autoantibodies · IA-2A: islet antigen 2 antibodies · ICA: islet cell antibodies · *INS*: insulin gene region/IDDM2 locus · JDFU: Juvenile Diabetes Foundation Units · RU: relative units · VNTR: variable number of tandem repeats

Introduction

Type 1 diabetes develops in genetically susceptible individuals as a result of immune-mediated damage of the pancreatic beta cells. Although cellular immunity is perceived to play a central role in the effector phase of islet

destruction, a combination of humoral markers (islet cell autoantibodies [ICA], insulin autoantibodies [IAA], glutamic acid decarboxylase autoantibodies [GADA] and islet antigen 2 antibodies [IA-2A]) has proved to be a useful tool for monitoring the development of islet autoimmunity and for prediction of progression to clinical disease [1–4]. Although the association of certain HLA haplotypes with the appearance of IAA, GADA and IA-2A seems to be consistent in various studies [5–8], it is still unclear at what stage of the autoimmune process the HLA genes exert their effect.

Although the HLA gene region accounts for approximately half of the genetic disease susceptibility, a number of other ‘minor’ loci also contribute [9–11]. The insulin gene (*INS*; *IDDM2*) and the cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) gene regions (*IDDM12*) are considered as confirmed non-HLA disease susceptibility loci [12–16]. Short (class I) alleles of the insulin gene 5′ variable number of tandem repeats (VNTR) are associated with increased type 1 diabetes susceptibility fitting an allele dosage model, while long class III alleles confer protection [17]. In the *CTLA4* region, a number of variants, like the +49A/G, have shown disease association in various ethnic groups, while the CT60 polymorphism was identified as a candidate causative disease variant [14–16, 18].

Our knowledge of the effect of the insulin and *CTLA4* gene regions on type 1 diabetes-associated humoral autoimmunity is limited and controversial. An early study observed no association between the *INS* VNTR genotype and insulin-specific cellular and humoral autoimmunity [19], while class I alleles of the VNTR were found to contribute to the emergence of IAA in a larger case-control comparison [7]. It has also been suggested that the *INS* locus confers increased disease risk only in subjects with moderate-risk HLA genotypes [20]. However, recent studies have suggested that the *INS* polymorphism could modify progression to clinical disease among individuals with both high-risk and low-risk HLA genotypes [21–23]. Data on the effect of the *CTLA4* gene region on beta cell autoimmunity are similarly ambiguous. The *CTLA4*-3′(AT)_n microsatellite and the +49A/G polymorphism showed no association with humoral autoimmunity in Swedish and Belgian cohorts, whereas IA-2A showed association with the G allele of the +49A/G polymorphism in a Japanese study [7, 24, 25].

In the Type 1 Diabetes Prediction and Prevention project (DIPP) currently underway in Finland, population-based genetic screening for type 1 diabetes-associated HLA *DQB1* genotypes is performed in newborn infants followed by continuous monitoring of beta-cell-specific humoral autoimmunity in the risk cohort [26]. The aim of this study was to analyse the effect of the HLA class II, *INS*-VNTR and *CTLA4* loci on the development of diabetes-associated autoantibodies in an early phase of the autoimmune process.

Subjects and methods

Study design

In the framework of the DIPP study, screening for HLA-conferred susceptibility to type 1 diabetes is performed in all infants born in three university hospitals in Finland, i.e. Turku, Tampere and Oulu [26]. All infants ($n > 9,000$ by May 2004) with risk HLA *DQB1* genotypes (*DQB1**02/*0302, *DQB1**0302/X, *DQA1**05-*DQB1**02/Y, where $x \neq$ *DQB1**02, *DQB1**0301, *DQB1**0602, $y \neq$ *DQA1**0201-*DQB1**02, *DQB1**0301, *0302, *0602 or *0603) were followed and sampled at 3–12-month intervals as described [26]. ICA were measured in all serum samples and, if positive, IAA, GADA and IA-2A were tested in all serum samples available from that ICA-positive subject. In this study, we analysed HLA class II, *INS* and *CTLA4* genotypes in relation to humoral islet autoimmunity. The local ethics committees had approved the study and informed consent was obtained from the parents of the participating subjects. All study subjects were of Finnish ethnic origin. The study was conducted according to the principles of the Declaration of Helsinki.

Study population

Antibody-positive children All children who had developed ICA by May 2004 ($n = 574$, 180 with the *DQB1**02/*DQB1**0302 high-risk, 368 with the *DQB1**0302/X and 26 with the *DQA1**05-*DQB1**02/Y moderate-risk genotypes, 53.5% boys) were tested for autoantibodies (IAA, GADA and IA-2A) and for genetic markers (HLA *DRB1*-*DQA1*-*DQB1*, *INS* -23 HphI as well as *CTLA4*+49 and CT60 polymorphisms). At the time of this analysis the mean ($\pm$ SD) follow-up time in the *DQB1**02/*DQB1**0302 and *DQB1**0302/X groups was 4.9 ± 2.2 years (range 0.5–9.3 years), while it was somewhat shorter in those carrying the *DQB1**02/y combinations (mean 3.9 ± 1.4 years, range 0.5–6.1 years). By May 2004, 74 of these children (12.9%) had progressed to clinical type 1 diabetes diagnosed according to the WHO criteria.

Healthy control children without antibodies The control group comprised ICA-negative healthy children, who were randomly selected, but matched for sex, age and place of birth, from the entire DIPP birth cohort ($n = 2,563$, 52.9% boys, mean age 3.6 ± 1.5 years). HLA *DQB1* typing was done in all controls according to the DIPP screening scheme [26]. *DQA1* typing was performed in individuals with *DQB1**02, while *DQB1**0302-positive children were *DRB1**04 subtyped. The *INS* and *CTLA4* gene polymorphisms were typed as described below. This group was used for comparisons in the analysis of genetic effects on the appearance of ICA.

Methods

Genotyping The methods and the resolutions for the HLA *DQB1* 'full house' for the *DQA1* and *DRB1*04* typing were described earlier [27–29]. The *INS* –23 HphI and the *CTLA4*+49 polymorphisms were analysed using PCR and a lanthanide-labelled oligonucleotide hybridisation method [27, 30]. (*CTLA4*+49 A probe: 5'-Terbium-TGGCTAC-CAGGA-3'; G probe: 5'-Terbium-GGCTGCCAGGA-3'; –23 HphI T probe: 5'-Europium-CTGTCTCCCA-3'; A probe: 5'-Terbium-CTGTCTCCCA-3'). CT60 was genotyped using minisequencing (SnuPe Kit; GE Healthcare—formerly Amersham Biosciences—Chalfont St Giles, UK) and a MegaBACE 1000 sequencer (GE Healthcare).

Autoantibody assays The antibody assay parameters that apply have been described in detail [4]. The detection limit for ICA was 2.5 JDFU (sensitivity 100%, specificity 98%). IAA titres were quantified with a microassay [31, 32]. GADA and IA-2A were measured using specific radioligand assays [33, 34]. The cut-off limits for IAA, GADA and IA-2A positivity were 1.56, 5.36 and 0.43 RU, respectively, representing the 99th percentiles in a series comprising more than 370 non-diabetic Finnish children and adolescents. The disease sensitivities of the IAA, GADA and IA-2A assays were 44, 82, and 62%, respectively, while the specificity values were 98, 98 and 100%, respectively, based on the 2002 Diabetes Autoantibody Standardisation Programme (DASP) Workshop.

Statistical analysis Kaplan–Meier survival analysis was applied to evaluate genetic effects on the appearance of various autoantibodies. Differences between the survival

curves were tested with log-rank test. Cox regression was employed to dissect the effect of various factors (antibody status, genotypes) on autoantibody-free survival. Longitudinal data of all antibody measurements that were available for the cohort of antibody-positive children were analysed using a linear mixed model where age and group were used as fixed factors and subject as a random factor to account for correlation between repeated measurements. Logarithmic transformation of the antibody titres was applied to normalise their distribution. In the comparison of HLA, *INS* and *CTLA4* genotype frequencies between ICA-positive children and ICA-negative controls, chi-square statistics with Yates' correction were used. To compare the highest autoantibody titres detected during the whole follow-up period in children carrying different genotypes, the Kruskal–Wallis and the Mann–Whitney *U*-tests were used. In multiple comparisons, Bonferroni corrections were used (shown as p_c); p values lower than 0.05 were considered statistically significant. All statistical analyses were done using SPSS for Windows (version 11.0.1; SPSS, Chicago, IL, USA).

Results

HLA *DRB1*–*DQA1*–*DQB1* and the appearance of autoantibodies

The results of the Kaplan–Meier survival analysis on the emergence of autoantibodies in the various *DQB1* genotype groups are shown in Table 1. The median values for the antibody-free survival could not be calculated in most cases, since the cumulative antibody frequencies were usu-

Table 1 Cumulative frequencies of autoantibodies and antibody-free survival in children carrying various HLA *DQB1* and *DRB1* genotypes

Autoantibody analysed	HLA genotype or <i>DRB1*04</i> allele	Cumulative frequencies of antibody (%)	Antibody-free survival in years (mean [95% CI])	p value ^a
IAA	<i>DQB1*02/*0302</i>	54.2	5.7 (5.1–6.2)	0.012
	<i>DQB1*0302/X^b</i>	40.5	6.5 (6.1–6.9)	
	<i>DQA1*05–DQB1*02/Y^c</i>	16.6	5.3 (4.6–6.0)	
GADA	<i>DQB1*02/*0302</i>	50.6	6.0 (5.5–6.6)	0.011
	<i>DQB1*0302/X</i>	37.9	7.0 (6.6–7.3)	
	<i>DQA1*05–DQB1*02/Y</i>	34.1	4.7 (4.0–5.6)	
IA-2A	<i>DQB1*02/*0302</i>	60.6	6.4 (5.9–6.9)	0.017
	<i>DQB1*0302/X</i>	37.2	7.0 (6.7–7.4)	
	<i>DQA1*05–DQB1*02/Y</i>	10.2	5.7 (5.3–6.1)	
IAA	<i>DRB1*0401^d</i>	52.1	5.6 (5.2–6.0)	<0.0001
	<i>DRB1*0404</i>	37.0	7.1 (6.5–7.5)	
	<i>DRB1*0403</i>	6.1	8.2 (7.7–8.8)	
GADA	<i>DRB1*0401</i>	46.5	6.2 (5.8–6.6)	0.0007
	<i>DRB1*0404</i>	29.3	7.3 (6.8–7.8)	
	<i>DRB1*0403</i>	10.1	7.8 (7.0–8.7)	
IA-2A	<i>DRB1*0401</i>	50.3	6.3 (5.9–6.7)	<0.0001
	<i>DRB1*0404</i>	36.2	7.7 (7.2–8.2)	
	<i>DRB1*0403</i>	0.0	–	

All children tested repeatedly positive for ICA. Children carrying the antibody analysed in the comparisons could also carry additional molecular autoantibodies

^aLog-rank test, $df=2$ for all p values

^b $x \neq DQB1*02, *0301, *0602$

^c $y \neq DQB1*0302, *0301, *0603, *0602, DQA1*0201–DQB1*02$

^d*DRB1*04* subtypes carry *DQB1*0302* on the same haplotype, the second haplotype is x or *DR3/DR7–DQB1*02*

ally lower than 50%; therefore the mean values for antibody-free survival are shown in the tables. The cumulative frequencies of IAA were higher and antibody-free survival was shorter among children with the *DQB1*02/*0302* genotype compared with those in the *DQB1*0302/X* group. The lowest cumulative IAA frequency was observed in the *DQA1*05-DQB1*02/Y* group. Similarly, IA-2A appeared at a higher rate among children with the *DQB1*02/*0302* and *DQB1*0302/X* combinations than in those carrying the *DQA1*05-DQB1*02/Y*. Also GADA developed at the highest frequency in the *DQB1*02/*0302* group, while children with the *DQB1*0302/X* or *DQA1*05-DQB1*02/Y* combinations had similar cumulative frequencies of GADA.

A strong differential effect of the various *DRB1*04* alleles on the emergence of autoantibodies was detected in children carrying the *DR4-DQB1*0302* haplotype (Table 1). In the Cox regression, analysis, the unadjusted hazard ratios for developing IAA in the *DRB1*0401* and **0404* groups were 13.1, (95% CI: 1.8–93.4) and 6.8 (95% CI: 1.2–49.2, $p<0.001$; reference allele: *DRB1*0403*). The association of IAA with *DR4* subtypes remained significant in the Cox regression analysis also after adjusting for the GADA and IA-2A status (hazard ratios for **0401* and for **0404* were 5.0 [95% CI 2.1–36.2] and 3.3 [95% CI 2.1–23.9], respectively, $p=0.017$, $df=2$). Similarly, the effect of the *DRB1*04* alleles on the appearance of IA-2A remained significant after adjusting for the IAA and GADA status ($p=0.03$, $df=2$). No such independent *DRB1*04* effect was observed, however, on the appearance of GADA.

A quantitative effect of *DRB1*04* on IAA and IA-2A levels was detected when the peak antibody levels seen during follow-up were compared. Children with the *DRB1*0401* allele had higher antibody levels than those carrying the *DRB1*0404* allele ([median and interquartile range]: *DRB1*0401* group IAA: 16.7 [7.5–51.5] RU and IA-2A: 98.3 [28.5–120.8] RU *DRB1*0404* group IAA: 8.7 [4.8–12.1] RU, $p_c=0.02$; and IA-2A: 29.5 [2.0–99.7] RU, $p_c=0.039$, respectively).

To explore the effect of *DRB1*04* subtypes on the emergence of ICA, we compared the *DRB1*04* allele frequencies in children who tested positive for ICA alone with those observed among the antibody-negative controls. There was no difference between the two groups (children testing positive for ICA only: *DRB1*0401* 57.9%, **0404* 33.7%, **0403* 6.7% vs controls 59.0, 32.8 and 7.2%, respectively).

The insulin locus and the appearance of autoantibodies

Survival analysis of the effect of the insulin locus on the appearance of autoantibodies The cumulative frequency of IAA was higher and autoantibody-free survival was shorter in children with the *INS -23 HphI* AA genotype than in those carrying the AT or TT combinations (hazard ratio 2.1. [95% CI: 1.4–2.9] $p<0.0001$; Table 2, Fig. 1a). The effect of the *INS* locus on the development of IAA seemed even stronger in those children where IAA appeared as the first antibody (hazard ratio: 2.5 [95% CI 1.6–3.4] $p<0.00001$). Similarly, the cumulative frequency of IAA was increased also in –23 HphI AA carrying subjects who tested negative for GADA or IA-2A (Table 2, Fig. 1b). Accordingly, in the Cox regression model the effect of *INS* was significant after adjusting for the GADA and IA-2A carrier status (adjusted hazard ratio for the appearance of IAA in the –23HphI AA group was 1.7 [95% CI 1.2–2.4] reference: AT/TT genotype, $p=0.004$). The insulin locus had a clear effect on the development of IAA in all three *DQB1* genotype groups (Fig. 1c, Table 3). In the Cox regression the effect of –23HphI on IAA development adjusted for HLA *DQB1* effects was highly significant (hazard ratio for the AA combination 2.1 [95% CI 1.5–3.0], $p<0.0001$).

Similarly, the effect of the *INS* locus on development of IAA was present both in the *DRB1*0401* and **0404* subtype categories (Table 3) (hazard ratio for the –23 HphI AA genotype adjusted for the *DRB1*04*: 2.0 [95% CI 1.4–2.9], $p<0.0001$).

Table 2 Cumulative frequencies of autoantibodies and antibody-free survival in children carrying various insulin gene genotypes

Autoantibody analysed	Other autoantibodies	–23 HphI genotype	Cumulative frequencies of antibodies (%)	Antibody-free survival in years (mean [95% CI])	p value ^a
IAA	GADA+/-;	AA	49.6	5.9 (5.5–6.3)	<0.0001
	IA-2A+/-;	AT/TT	30.3	7.4 (6.9–7.9)	
	ICA+ (n=574)				
	GADA-; IA2A-;	AA	18.9	8.1 (7.7–8.5)	
GADA	ICA+ (n=352)	AT/TT	9.0	8.6 (8.2–8.9)	0.02
	IAA+; IA2A+/-;	AA	79.8	3.7 (3.2–4.2)	
	ICA+ (n=218)	AT/TT	77.9	3.8 (2.7–4.8)	
	IAA-; IA2A+/-;	AA	19.9	8.4 (8.1–8.7)	
IA-2A	ICA+ (n=356)	AT/TT	16.5	8.3 (7.9–8.7)	0.70
	IAA+; GADA+/-;	AA	82.1	3.8 (3.4–4.3)	
	ICA+ (n=218)	AT/TT	83.6	4.2 (3.2–4.2)	
	IAA-; GADA+/-;	AA	13.5	8.8 (8.5–9.0)	
	ICA+ (n=356)	AT/TT	19.0	8.7 (8.4–8.9)	0.72

^aLog-rank test

Fig. 1 Survival analysis of the effect of the insulin locus on the appearance of insulin autoantibodies (IAA). **a** Cumulative frequencies of IAA in children carrying different insulin gene -23 HphI genotypes. The cumulative frequency of IAA was higher in the AA group than in the AT/TT group ($p<0.0001$). **b** Cumulative frequencies of IAA in GADA- and IA-2A-negative children carrying different insulin gene -23 HphI genotypes. The cumulative frequency of IAA was higher in the AA group than in the AT/TT group ($p=0.02$). Key (**a**, **b**): solid line, subjects with the AA variant; dotted line, subjects carrying AT or TT combinations. **c** Cumulative frequencies of IAA in children carrying different HLA *DQB1-INS*(-23)HphI combined genotypes. Key: bold dashed line, children with HLA *DQB1*02*0302-INS*(-23)HphI-AA; bold dotted line, individuals carrying HLA *DQB1*02*0302-INS*(-23)HphI-AT/TT; solid line, subjects with the HLA *DQB1*0302/X-INS*(-23)HphI-AA; thin dotted line, the HLA *DQB1*0302/X-INS*(-23)HphI-AT/TT group. IAA frequencies were higher in the AA groups in both HLA categories ($p=0.0002$; $df=3$).

Cumulative frequencies of GADA and antibody-free survival showed differences between the *INS* -23 HphI AA and AT/TT genotype groups in the Kaplan–Meier analysis when the autoantibody data set was analysed unstratified (AA group 46.0% vs AT/TT group 31.8%, and 6.4 years [95% CI 6.1–6.8] vs 7.3 years [95% CI 6.7–7.8], respectively, log-rank 5.5, $p=0.02$; Fig. 2a). However, no effect of the *INS* polymorphism was detected on the appearance of GADA when children were stratified according to their IAA status (Table 2, Fig. 2b). Importantly, GADA appearance was strongly influenced by IAA status, as IAA-positive children developed GADA at a higher rate than IAA-negative children (hazard ratio 12.5 [95% CI 8.6–18.1], $p<0.0001$).

Similarly, in the unstratified data set the emergence of IA-2A appeared to be influenced by the -23 HphI variant (cumulative frequency in the AA group: 43.6 vs 35.9% in the AT/TT group; median antibody-free survival: 6.7 years [95% CI 6.3–7.1] vs 7.7 years [95% CI 7.2–8.1], respectively; log-rank 9.8, $p=0.002$; Fig. 2c). However, no effect of the *INS* locus was observed when the cohort was stratified according to the IAA status (Table 2, Fig. 2d). Emer-

Table 3 Cumulative frequencies of insulin autoantibodies (IAA) and IAA-free survival in children carrying various HLA *DQB1* and *DRB1* and insulin gene genotypes

HLA genotype	-23 HphI genotype	Cumulative frequency of IAA (%)	IAA free survival in years (mean [95% CI])	p value ^a
<i>DQB1*02/*0302</i>	AA	60.1	4.2 (2.3–6.2)	<0.0001 ($df=5$)
	AT/TT	39.1	7.0 (5.9–8.0)	
<i>DQB1*0302/X^b</i>	AA	45.4	6.1 (5.6–6.6)	7.4 (6.8–8.0)
	AT/TT	24.7	7.4 (6.8–8.0)	
<i>DQB1*02/Y^c</i>	AA	19.2	5.1 (4.2–6.1)	3.8 (3.2–4.4)
	AT/TT	14.3	3.8 (3.2–4.4)	
<i>DRB1*0401^d</i>	AA	57.4	4.5 (3.2–5.8)	<0.0001 ($df=3$)
	AT/TT	39.4	6.5 (5.8–7.2)	
<i>DRB1*0404</i>	AA	42.8	6.6 (5.9–7.3)	
	AT/TT	16.7	8.5 (7.8–9.2)	

^aLog-rank test

^b $x \neq DQB1*02, *0301, *0602$

^c $y \neq DQB1*0302, *0301, *0603, *0602, DQA1*0201-DQB1*02$

^d*DRB1*04* subtypes carry *DQB1*0302* on the same haplotype, the second haplotype is *x* or *DR3/DR7-DQB1*02*

Fig. 2 Survival analysis of the effect of the insulin locus on the appearance of GADA and IA-2A. *Solid lines*, subjects with the AA variant; *dotted lines*, individuals carrying AT or TT combinations. **a** Cumulative frequencies of GADA in children carrying different insulin gene -23 HphI genotypes—unstratified data set. GADA frequencies were higher in the AA group ($p=0.02$). Cumulative frequencies (**b**) of GADA in IAA-negative children with different -23 HphI genotypes ($p=0.70$). Cumulative frequencies (**c**) of IA-2A in children carrying different insulin gene -23 HphI genotypes—unstratified data set. IA-2A frequencies were higher in the AA group ($p=0.002$). **d** Cumulative frequencies of IA-2A in IAA-negative children carrying different insulin gene -23 HphI genotypes ($p=0.72$)

gence of GADA was higher in IAA positive children than in those without IAA (hazard ratio 20.9 [95% CI 13.1–33.0] $p<0.0001$).

INS effects on IAA levels The peak IAA levels detected during follow-up were compared between groups of children carrying different *INS* genotype. Subjects with the -23 HphI AA variant had higher IAA levels than those with AT or TT (AA: 15.3 [6.9–46.6] RU; AT: 13.2 [4.0–36.8] RU; TT: 7.5 [4.6–18.5] RU, $p_c=0.02$, $df=2$; Fig. 3a). Similarly, IAA titres were observed to be higher in both HLA categories in children with the -23 HphI AA genotype compared with those who had AT or TT (*DQB1**02/*0302-HphI AA 20.5 [6.9–54.3] RU versus AT/TT 13.1 [5.7–38.3] RU; *DQB1**0302/X-HphI AA 13.2 [6.1–52.6] RU vs AT/TT 8.7 [3.4–22.8] RU, $p_c=0.007$, $df=3$; correction factor=2; Fig. 3b). In the linear mixed model analysis we used all antibody measurements from each child to estimate the difference in the antibody status between the various genotype groups. The *INS* locus showed a clear gene dosage effect on IAA status. The IAA levels in subjects carrying -23 HphI AA and AT variants were higher

than in those with TT (estimated average difference between AA and TT: 7.3 [95% CI 5.7–10.6] RU; between AA and AT/TT: 2.4 [95% CI: 0.4–5.1] RU; $p=0.0005$).

The *CTLA4* gene polymorphisms and the appearance of autoantibodies

No effect of the *CTLA4*+49 or CT60 polymorphisms on the emergence of islet-specific autoantibodies was observed in this study (data not shown).

Discussion

We analysed the effect of the insulin, the *CTLA4* and the HLA *DRB1*–*DQA1*–*DQB1* genes on the development of humoral beta cell autoimmunity in a large population-derived Finnish birth cohort with increased HLA *DQB1*-conferred genetic risk for type 1 diabetes (the DIPP study) [26]. The selection criteria for the study cohort comprising ICA-positive children limited our possibilities to analyse

Fig. 3 Insulin autoantibody (IAA) levels in children carrying various insulin gene (–23) HphI genotypes. The box plots represent the medians (thick solid line) and the interquartile ranges, while error bars mark the lowest and highest values that are not outliers. **a** IAA levels were higher in children with the *IDDM2* –23 HphI AA

risk genotype than in those with AT or TT ($p_c=0.005$, $df=2$). **b** Both in the *DQB1**02/*0302 and *DQB1**0302/*X* groups IAA levels were higher in children with the –23 HphI AA genotype than in those carrying AT or TT variants ($p_c=0.007$, $df=3$; correction factor=2)

thoroughly the HLA effects, since most study subjects carried the *DR4*–*DQB1**0302 haplotype, however, the contribution of the insulin and *CTLA4* loci to the disease-specific autoimmune phenomena could be fully explored. We also analysed the effect of *DRB1**04 alleles on the *DR4*–*DQB1**0302 haplotypes, since they confer different degrees of disease susceptibility in the Finnish population, as the *DRB1**0401 allele is associated with higher risk than the *DRB1**0404 variant and the *DRB1**0403 allele is protective [28, 35]. At the *INS* locus the –23 HphI polymorphism was used as genetic risk marker, where the A alleles are in strong linkage disequilibrium with the 5' *INS* VNTR class I susceptibility variants and the T alleles are linked with the protective class III alleles [12, 21]. The well-characterised *CTLA4*+49 and CT60 polymorphisms were chosen as disease markers at the *IDDM2* locus [14, 16].

This is the first population-based study demonstrating that the *INS* locus affects the emergence of humoral beta cell autoimmunity and it provides strong evidence that *INS* directly influences humoral insulin-specific autoimmunity. A number of findings support this observation. Firstly, IAA emerged at a higher rate in children carrying the –23HphI AA genotype compared with those who had AT or TT variants. This effect appeared to be even more pronounced in those infants who developed IAA as their first antibody reactivity. These observations strongly support the importance of the *INS* locus in the initiation of the autoimmune process. Secondly, a gene dosage effect of the *INS* locus on the appearance of IAA was detected, as the autoantibody titre was proportional to the number of *INS* susceptibility alleles. Importantly, the *INS* locus influenced

the development of IAA in all three HLA risk groups. Although the *INS* locus is associated with disease risk, the effect of *INS* on the emergence of IAA seems to be a genuine one, since IAA as such are not associated with particularly high diabetes risk [1, 4, 36].

The association of IAA with the risk *INS* genotype is supported by observations made in two other recent studies on patients with newly diagnosed type 1 diabetes and on first-degree relatives [7, 22]. Recently, a more preserved beta cell function in recent-onset cases was found in individuals with the *INS* VNTR class III allele compared with those carrying class I alleles [37]. These data are in line with the hypothesis proposing that the *INS*-VNTR has a role in the regulation of insulin gene transcription in the thymus and modulation of insulin-specific self tolerance [38, 39].

A positive association between IA–2A and GADA with the *INS* risk genotype was also implicated by the analysis of the unstratified data set; however, it is explained by the fact that the majority (81.7%) of IA–2A and GADA-positive children also had IAA. The lack of a primary association between IA–2A or GADA and *INS* was clearly shown by the Cox regression and also by the survival analysis in the data set stratified according to the IAA status (see Fig. 2). These data points to the existence of two different pathways that result in the emergence of GADA and IA–2A. In the '*INS*-dependent' pathway, insulin autoimmunity plays a central role in the initiation of the disease process, while the appearance of GADA and IA–2A is linked to the presence of IAA, probably by antigen spreading. In IAA-negative individuals the initiation of

beta cell autoimmunity and development of GADA and IA-2A are likely controlled by factors other than the *INS* locus.

In the analysis on the relation of HLA class II and beta-cell-specific autoantibodies, the association of IAA and IA-2A with the *DR4-DQB1*0302* haplotype was supported by our data as both antibodies developed at a higher rate in children carrying this haplotype compared with the *DQA1*05-DQB1*02/Y* group. In addition, children carrying the high-risk *DQB1*02/*0302* genotype had higher positive seroconversion rates for these antibodies than those with the *DQB1*0302/X* combination. In the *DQA1*05-DQB1*02/Y* group, GADA were the most frequent autoantibody reactivity detected. These observations are in line with findings by ourselves and other groups that indicate that the two major disease-associated HLA class II haplotypes could mediate alternative, although probably related pathways in the emergence of beta-cell-specific autoimmunity [5–8].

Importantly, we detected a differential effect of *DRB1*04* alleles present on the *DR4-DQB1*0302* haplotypes on the development of diabetes-related autoantibodies, where the *DRB1*0401* allele showed a considerably stronger effect on the emergence of IAA than the *DRB1*0404* variant, while the *DRB1*0403* allele almost completely blocked the appearance of antibodies. The absence of antibody response in children with *DRB1*0403* could be related to the peptide binding specificity of the encoded DR molecule that has been shown to share three-dimensional and electro-chemical properties with particular HLA-DQ heterodimers protective from type 1 diabetes [40]. In the Cox regression analysis, no effect of the *DRB1*04* subtypes was seen on the emergence of GADA, and the association of IA-2A with *DRB1*0401* was only marginally significant. We also showed here that the effect of the *INS* locus is independent of the *DRB1*04* variant. There are only limited published data on this issue. A previous Swedish study suggested that IA-2A were associated with *DRB1*0401* [41], but otherwise possible links between the autoantibodies and the *DRB1*04* alleles have remained unexplored. Our data indicate that the *DRB1*0401* variant—which confers the highest disease risk in the Finnish population [35]—could play an important role in the initiation of the insulin-specific immune processes. In IAA-positive subjects the development of GADA and IA-2A appear to be linked to IAA, therefore, this genetic variant could indirectly influence GAD and IA-2-related autoimmunity.

In the analysis of genetic effects on the appearance of ICA, survival analysis was not feasible to explore the contribution of *DRB1*04* haplotypes as those data were not available for the whole follow-up cohort (>9,000 children). Therefore we compared frequencies of these haplotypes between children testing positive for ICA only (most of whom had ICA titres below 20 JDFU) and autoantibody-negative control children. No difference was seen in these comparisons. This and others' findings indicate that low ICA titres show no association with HLA, whereas children

with multiple autoantibodies or high titres of ICA show a HLA genotype distribution resembling that seen in patients with type 1 diabetes [42–45]. This implies that the appearance of low-titre ICA alone may represent harmless immune phenomena unrelated to the diabetes-specific disease process. It is important to note, that HLA seem to influence the progression of the autoimmune process, as the protective *DQB1*0602* allele prevents development of diabetes in ICA-positive subjects [46, 47].

In this study we could not see any effect of the *CTLA4+49* and *CT60* polymorphisms on type 1 diabetes-specific humoral autoimmunity. The findings were similarly negative in the Belgian and Swedish surveys, where the *CTLA4* 3' UTR (AT)_n microsatellite was used as genetic marker [7, 24]. However, IA-2A and *CTLA4+49* A/G were reported to be associated with type 1 diabetes in Japanese patients, but this observation might be secondary to the increased prevalence of autoimmune thyroid disease among the patients included in that study [25]. Our findings indicate that the two polymorphisms in the *CTLA4* region studied have no major role in type 1 diabetes-related humoral autoimmunity, the statistical power of the analysis being more than 90% to detect a hazard ratio of 1.6; $\alpha=0.05$. It has to be noted, that the effect of *CTLA4* on type 1 diabetes susceptibility is very weak in the Finnish population (OR<1.3 for the +49GG genotype; unpublished) and in that range the study had limited statistical power.

In conclusion, we propose that the insulin locus plays a central role in the initiation of the autoimmune process in IAA-positive individuals, while the appearance of GADA and IA-2A is a secondary phenomenon. In addition, the HLA *DRB1*0401* variant also seems to have a role in the regulation of insulin autoimmunity. In IAA-negative individuals the development of GADA and IA-2A and progression to clinical disease is likely controlled by factors other than the *INS* locus.

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