

Humoral Responses to Islet Antigen-2 and Zinc Transporter 8 Are Attenuated in Patients Carrying *HLA-A*24* Alleles at the Onset of Type 1 Diabetes

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The *HLA-A*24* allele has shown negative associations with autoantibodies to islet antigen-2 (IA-2) and zinc transporter 8 (ZnT8) in patients with established type 1 diabetes. Understanding how this HLA class I allele affects humoral islet autoimmunity gives new insights into disease pathogenesis. We therefore investigated the epitope specificity of associations between *HLA-A*24* and islet autoantibodies at disease onset. *HLA-A*24* genotype and autoantibody responses to insulin (IAA), glutamate decarboxylase (GADA), IA-2, IA-2 β , and ZnT8 were analyzed in samples collected from patients with recent-onset type 1 diabetes. After correction for age, sex, and HLA class II genotype, *HLA-A*24* was shown to be a negative determinant of IA-2A and ZnT8A. These effects were epitope specific. Antibodies targeting the protein tyrosine phosphatase domains of IA-2 and IA-2 β , but not the IA-2 juxtamembrane region, were less common in patients carrying *HLA-A*24* alleles. The prevalence of ZnT8A specific or cross-reactive with the ZnT8 tryptophan-325 polymorphic residue, but not those specific to arginine-325, was reduced in *HLA-A*24*-positive patients. No associations were found between *HLA-A*24* and IAA or GADA. Association of an HLA class I susceptibility allele with altered islet autoantibody phenotype at diagnosis suggests CD8 T-cell and/or natural killer cell-mediated killing modulates humoral autoimmune responses. *Diabetes* 62:2067–2071, 2013

Autoantibodies to insulin (IAA), glutamate decarboxylase (GADA), islet antigen-2 (IA-2A), and zinc transporter 8 (ZnT8A) can appear many years before the diagnosis of type 1 diabetes and are powerful markers for predicting disease. IAA are generally the first antibodies to be detected in children at high genetic risk, followed by GADA; IA-2A and ZnT8A usually appear later (1). IA-2A responses often spread from the juxtamembrane (JM) region to the protein tyrosine phosphatase (PTP) region of IA-2 and IA-2 β (2). ZnT8A epitopes are less well defined, but one major epitope includes the arginine-tryptophan polymorphism at position 325 (*SLC30A8* single nucleotide polymorphism [SNP] rs1326663), which strongly influences ZnT8A responses (3).

HLA class II alleles confer the greatest genetic susceptibility for type 1 diabetes (4) but are also important determinants of humoral islet autoimmunity. Increased

IAA and IA-2A prevalence at diagnosis is associated with *HLA-DRB1*04* haplotypes (1,5), whereas GADA are more common in patients carrying *HLA-DRB1*03* (6). Among IA-2A-positive patients, *HLA-DQB1*02* haplotypes were negatively associated with JM autoantibodies (JMA) (5), and *HLA-DR4-DQ8* haplotypes were positively associated with IA-2 β autoantibodies (IA-2 β A) (7). Associations between HLA class II alleles and ZnT8A, however, are less clear (8). HLA class I alleles also influence diabetes susceptibility and humoral autoimmunity. In patients with established diabetes, negative associations have been found between the diabetes susceptibility gene *HLA-A*24* and IA-2A and between ZnT8A and the SNP rs9258750, which is in linkage with *HLA-A*24*, but not between HLA class I alleles and GADA (6,9,10). However, most of the patients in these studies had diabetes duration ≥ 5 years at the time of autoantibody testing, and these analyses may not have accounted fully for falls in antibody levels after diagnosis. Furthermore, neither study investigated the potential effect of *HLA-A*24* on IA-2A epitope responses nor could they investigate potential associations of IAA with these alleles because IAA would be obscured by antibodies raised to exogenous insulin.

Our aim was therefore to investigate the influence of *HLA-A*24* on islet autoantibody responses, including those to insulin and epitopes of IA-2, in a cohort of patients from whom samples were available close to diagnosis. Determining the effect of this HLA class I diabetes susceptibility allele on humoral islet autoimmunity at diabetes onset will give insights into the interaction between cytotoxic (CD8) and helper (CD4) components of the mature autoimmune response in type 1 diabetes.

RESEARCH DESIGN AND METHODS

Newly diagnosed patients. Patients were recruited between 1985 and 2002 as part of the Bart's-Oxford (BOX) study of childhood diabetes (11). Sera collected within 3 months of diagnosis (median 1 day [range -61 to 90]) and genetic samples for *HLA-A*24* testing were available from 589 of these patients (median age, 11.0 years [0.7–20.9]). GADA, IA-2A, and ZnT8A had already been tested in all 589 sera and IA-2 β A in 588 sera. IAA results were available for 405 sera collected before or within 2 weeks after diagnosis (12). JMA and PTP autoantibodies (PTPA) were tested in 460 IA-2A-positive patients and considered negative in IA-2A-negative patients. The BOX study was approved by local research ethics committees.

Autoantibody assays. Autoantibodies to insulin, full-length GAD65, the intracytoplasmic (606-979) or JM (609-631) regions of IA-2, IA-2 β (723-1015), and ZnT8 (268-369) were measured by radioimmunoassay, as previously described (12). PTPA were measured, using the same protocol, against IA-2 (687-979). ZnT8A were tested in two separate assays using labels made with plasmids encoding arginine (ZnT8R) or tryptophan (ZnT8W) at position 325, provided by Dr. Vito Lampasona (San Raffaele Scientific Institute, Milan, Italy). Results were expressed in units derived from standard curves, except those for JMA, which were expressed as an index. Assay thresholds were set at the 97.5th percentile of schoolchild sera; 2860 for IAA, GADA, and IA-2A; 523 for ZnT8A; and 270 for IA-2 β A, JMA, and PTPA.

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TABLE 1
The distribution of autoantibodies, HLA class II, and HLA class I A*24 in the 589 patients

Autoantibody/genotype	n (%)
IAA*	283 (70)
GADA	465 (79)
IA-2A	461 (78)
JMA†	228 (39)
PTPA†	410 (70)
IA-2βA†	407 (69)
ZnT8A	415 (71)
ZnT8RA	112 (19)
ZnT8RWA	260 (44)
ZnT8WA	43 (7)
HLA class II	
DR3-DQ2/DR4-DQ8	217 (37)
DR4-DQ8/DR4-DQ8	30 (5)
DR3-DQ2/DR3-DQ2	47 (8)
DR4-DQ8/X	139 (24)
DR3-DQ2/X	90 (15)
XX	66 (11)
HLA class I	
A*24/A*24	6 (1)
A*24/A*Y	103 (17)
A*Y/A*Y	480 (81)
rs9258750	
Allele 1/allele 1	398 (68)
Allele 1/allele 2	174 (30)
Allele 2/allele 2	17 (3)
rs13266634 in SLC30A8	
CC	275 (47)
CT	267 (45)
TT	47 (8)

*In 405 patients with serum samples taken by 2 weeks of diagnosis.
†In 588 patients with serum samples available.

Genotyping. HLA class I-A typing was performed on blood or mouth swab DNA with sequence-specific PCR using published primer sets (13). Patients were screened for HLA-A*24, and if HLA-A*24 was present, were fully typed to establish HLA class I-A genotype; HLA-A*24/A*24 or HLA-A*24/A*Y (where Y is any other HLA-A genotype). HLA-A*24 four-digit typing was not performed because 98% of Caucasians carry HLA-A*2402 (14). This cohort had already been typed for HLA class II DRB1, DQA1, and DQB1 using sequence-specific primers (4). Taqman SNP Genotyping Assays were used for analysis of rs13266634 (encoding the SLC30A8 ZnT8-R325 W polymorphism)

TABLE 2
Association of HLA-A*24 with autoantibodies before and after correction for age, sex, HLA class II genotype, and duration of disease at sampling

	No A*24 n (%)	A*24 n (%)	Uncorrected		Corrected	
			HLA-A*24 OR (95% CI)	P value	HLA-A*24 OR (95% CI)	P value
IA-2A	384/480 (80)	77/109 (70.6)	0.60 (0.38–0.96)	0.032	0.46 (0.28–0.78)	0.003
JMA	190/479 (39.7)	38/109 (34.9)	0.81 (0.53–1.26)	0.353	0.68 (0.43–1.07)	0.098
PTPA	349/479 (72.9)	61/109 (56.5)	0.47 (0.31–0.73)	0.001	0.42 (0.26–0.66)	<0.001
IA-2βA	347/479 (72.4)	61/109 (56.0)	0.47 (0.3–0.71)	<0.001	0.40 (0.25–0.63)	<0.001
ZnT8A	354/480 (73.8)	61/109 (56)	0.45 (0.29–0.69)	<0.001	0.42 (0.27–0.66)*	<0.001
ZnT8RA alone	89/480 (18.5)	23/109 (21.1)	1.17 (0.70–1.97)	0.539	1.15 (0.68–1.94)*	0.609
ZnT8RWA cross-reactive	226/480 (47.1)	34/109 (31.2)	0.51 (0.33–0.79)	0.005	0.51 (0.32–0.80)*	0.004
ZnT8WA alone	39/480 (8.1)	4/109 (3.7)	0.43 (0.15–1.23)	0.107	0.38 (0.13–1.11)*	0.076
IAA	233/326 (71.5)	50/79 (63.3)	0.69 (0.41–1.15)	0.155	0.66 (0.37–1.18)	0.159
GADA	380/480 (79.2)	85/109 (78)	0.93 (0.56–1.54)	0.784	0.96 (0.57–1.63)	0.888

OR, odds ratio. Age of diagnosis modeled as 0–4 years, 5–9 years, and 10–14 years; reference category ≥15 years. HLA class II modeled as DR-DQ genotype; reference category XX. *For ZnT8A, age was modeled as <5 years; reference category age ≥5 years.

and rs9258750 (in linkage with HLA-A*24 [10]). A restriction fragment–length polymorphism method was previously used to genotype 194 samples for rs13266634. Identical results were obtained in 105 patients typed using both methods. Allele distributions were in Hardy-Weinberg equilibrium.

Statistical analysis. The χ^2 test was used to compare autoantibody prevalence between patients according to HLA-A*24 or HLA class II haplotype and the Mann-Whitney U test to compare autoantibody levels. HLA class II genetic risk was analyzed as DR3-DQ2/DR4-DQ8, DR4-DQ8/DR4-DQ8, DR3-DQ2/DR3-DQ2, DR4-DQ8/X, DQ3-DQ2/X, and XX (where X was not DR3-DQ2 or DR4-DQ8). Logistic regression models were used to adjust for factors associated with islet autoantibodies (sex, age at diagnosis, DR-DQ genotype, and duration of disease at sampling). Age at diagnosis was modeled as four age bands (age younger than 5, 5–9, 10–14, and older than 15 years) or as age older and younger than 5 years. Analyses were performed using SPSS 16 software (SPSS Inc., Chicago, IL).

RESULTS

The distribution of islet autoantibodies, HLA class I A*24 alleles, and HLA class II DR3-DQ2 and DR4-DQ8 haplotypes in the patients is reported in Table 1. Of 589 patients, 109 carried at least one HLA-A*24 allele.

IA-2A was less common in patients carrying HLA-A*24 (71%) than in those without an HLA-A*24 allele (80%, $P = 0.003$ after adjustment; Table 2). IA-2A levels were also lower in IA-2A–positive patients carrying HLA-A*24 ($P < 0.001$, Fig. 1). rs9258750 was not associated with IA-2A ($P = 0.456$). PTPA and IA-2βA ($P < 0.001$ for both), but not JMA ($P = 0.098$), were negatively associated with HLA-A*24 (Table 2). IA-2A epitope responses were also strongly dependent on HLA class II genotype (Supplementary Fig. 1). IAA and GADA were not associated with HLA-A*24 (Table 2).

ZnT8A was less prevalent in patients carrying HLA-A*24 (56%) than in those without HLA-A*24 (74%, $P < 0.001$; Table 2). ZnT8As were also negatively associated with rs9258750 ($P = 0.026$), but this was explained by linkage to HLA-A*24 because rs9258750 did not add to models, including HLA-A*24 ($P = 0.662$). In contrast, the addition of HLA-A*24 significantly improved the model containing rs9258750 ($P = 0.005$). Autoantibodies that recognized ZnT8W or cross-reacted with both ZnT8 polymorphic variants (ZnT8RWA) were less common in patients carrying at least one HLA-A*24 allele (35 vs. 55%, $P < 0.001$ by χ^2), but the proportion of sera with antibodies specific to ZnT8R alone was similar in HLA-A*24–positive and –negative patients (21 vs. 19%, $P = 0.539$; Table 2). Levels of ZnT8RA

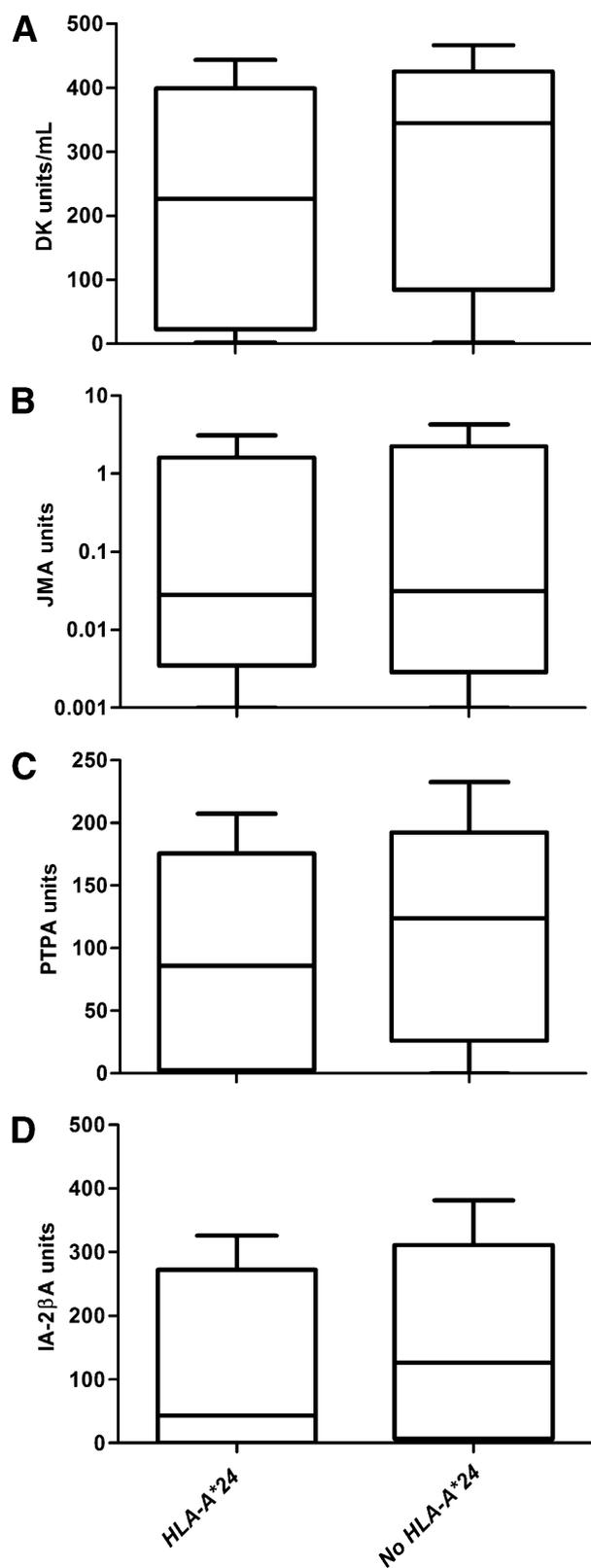


FIG. 1. Box plots show the differences in autoantibody epitope levels among 460 IA-2A-positive patients according to *HLA-A*24* genotype ($n = 77$ with *HLA-A*24* and $n = 383$ without *HLA-A*24*). IA-2A ($P < 0.001$) (A), JMA ($P = 0.661$) (B), PTPA ($P = 0.022$) (C), and IA-2 β A ($P = 0.003$) (D). The horizontal line in the middle of each box indicates the median; the top and bottom borders of the boxes represent the upper and lower quartiles, respectively, and the whiskers represent lower or upper quartile ± 1.5 times the interquartile range.

in ZnT8RA-positive patients and levels of ZnT8WA in ZnT8WA-positive patients showed no associations with *HLA-A*24* (data not shown). As expected, the *SLC30A8* SNP rs13266634 was associated with ZnT8A epitope specificity (Supplementary Table 1), but the distribution of rs13266634 genotypes was similar in patients with and without *HLA-A*24* (Supplementary Table 2).

DISCUSSION

IA-2A and ZnT8A were less common at clinical onset of type 1 diabetes in patients carrying at least one *HLA-A*24* allele, even after correction for age at onset and HLA class II genetic risk. Furthermore, the effects were epitope-specific; PTPA and IA-2 β A prevalence and levels showed strong negative associations with *HLA-A*24*, whereas JMA were not affected significantly. Antibodies to ZnT8W and ZnT8RW, but not those to ZnT8R alone, were reduced in patients carrying *HLA-A*24*. IAA and GADA showed no significant association with the presence of *HLA-A*24*.

A strength of this study is that the patients were well characterized, with detailed genetic data available. Our regression model showed that the influence of *HLA-A*24* was not explained by linkage to HLA class II *DRB1*03-DQ2* or *DRB1*04-DQ8* haplotypes. Furthermore, we were able to test for all major islet autoantibodies, including the major IA-2A-related epitopes and both of the main polymorphic variants of ZnT8A. In contrast to previous studies, the sera from our cohort were collected within a few months of diagnosis, which removed the confounding effect of disease duration on antibody levels (15,16). Our power to test for associations between *HLA-A*24* and IAA was limited, however, because fewer patients had been sampled within the 14-day window from diagnosis allowed for IAA testing. A negative association between *HLA-A*24* and IAA may be revealed in larger cohorts.

In agreement with previous studies, we found a negative association of IA-2A prevalence with *HLA-A*24* (6,9). We showed that this negative association was reflected in the level of the IA-2A response and was largely explained by reduced reactivity to epitopes in the PTP regions of IA-2 and IA-2 β . In contrast to Howson et al. (10), however, we found that *HLA-A*24* was the primary influence on ZnT8A prevalence rather than the linked locus identified by rs9258750. This suggests that a common mechanism may explain the reduced prevalence of ZnT8A and IA-2A. Because *HLA-A* is a major determinant of peptide presentation to cytotoxic CD8 T cells and can also modulate natural killer cell activity (17), the *HLA-A*24* variant itself may mediate the observed effects, even though HLA class II CD4 T-helper cells are more commonly associated with controlling antibody responses (18).

One potential explanation for reduced autoantibody responses could be a loss of antigenic stimulus caused by more complete destruction of β -cells by diabetes onset. Consistent with this mechanism, long-term residual β -cell function was shown to be lower in patients with long-standing type 1 diabetes carrying *HLA-A*24* (19). Stimulated C-peptide close to diagnosis was lower in patients homozygous for the T allele encoding ZnT8-325W, but no correlation was found between loss of ZnT8A and residual C-peptide during the early years of clinical disease (15,16). The relationship between residual antigenic stimulus and autoantibody responses therefore remains unclear.

Another possibility is that if β -cell killing is more rapid in *HLA-A*24*-positive individuals, inter- and intramolecular

spreading of the autoimmune response could be attenuated. IA-2A often appears after IAA and GADA, and like ZnT8A, may identify a new phase in disease progression. If autoreactive CD8 T cells are less potent at killing target cells than their antiviral counterparts (20), IA-2A and ZnT8A may play a more important role in amplifying and maintaining T-cell responses in individuals with weaker HLA class I genetic susceptibility (21). *HLA-A*24* is associated with younger type 1 diabetes onset (22), but early studies suggesting that diabetes progression may be quicker in individuals carrying this allele (18,19) have not yet been confirmed (23).

We are unable to explain why the influence of *HLA-A*24* on ZnT8A is dependent on the polymorphic 325 residue. Differential expression of ZnT8R and ZnT8W could affect major histocompatibility complex class I presentation of these antigens to CD8 T cells in the thymus or in the periphery (8). The C-allele (encoding ZnT8-325R) confers a minor risk of type 2 diabetes and has been associated with reduced insulin secretion (3), but no direct evidence that this polymorphism alters ZnT8 expression is available. Alternatively, the polymorphism at position 325 could alter the T-cell epitope repertoire (8), thereby modifying the influence of *HLA-A*24* on humoral autoimmunity (24), but so far, no association has been found between the breadth of the ZnT8 CD4 T-cell responses in patients and ZnT8A (25).

IA-2A and ZnT8A responses were attenuated in patients with *HLA-A*24* at the onset of type 1 diabetes in an epitope-specific manner. Most patients carrying *HLA-A*24* were still positive for these autoantibodies, but the influence of this class I allele on antibody prevalence and levels was comparable to that observed previously with some class II alleles (5). Large prospective studies are required to show whether these reduced responses were caused by lack of spreading or loss of autoantibodies during the prodrome and whether this correlates with β -cell destruction. Determining how *HLA-A*24* alters humoral islet autoimmunity will be important to our understanding of the interactions between CD4, CD8, and natural killer cells in disease pathogenesis and the potential contribution of islet autoantibodies to β -cell destruction. This may help to explain disease heterogeneity and identify pathways amenable to therapeutic modulations that halt disease progression.

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A.E.L. and A.J.K.W. researched data, contributed to discussion, and wrote the manuscript. K.M.G. researched data, contributed to discussion, and reviewed and edited the manuscript. R.J.A. and J.C.G. researched data and reviewed and edited the manuscript. P.J.B. researched data, contributed to discussion, reviewed and edited the manuscript, and coordinated the BOX study. A.J.K.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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