Immunogenetics of Type 1 diabetes and Celiac disease

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Next-Generation Sequencing Reveals That *HLA-DRB3*, *-DRB4*, and *-DRB5* May Be Associated With Islet Autoantibodies and Risk for Childhood Type 1 Diabetes

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The possible contribution of *HLA-DRB3*, *-DRB4*, and *-DRB5* alleles to type 1 diabetes risk and to insulin autoantibody (IAA), GAD65 (GAD autoantibody [GADA]), IA-2 antigen (IA-2A), or ZnT8 against either of the three amino acid variants R, W, or Q at position 325 (ZnT8RA, ZnT8WA, and ZnT8QA, respectively) at clinical diagnosis is unclear. Next-generation sequencing (NGS) was used to determine all *DRB* alleles in consecutively diagnosed patients ages 1–18 years with islet autoantibody–positive type 1 diabetes (n = 970) and control subjects (n = 448). *DRB3*, *DRB4*, or *DRB5* alleles were tested for an association with the risk of *DRB1* for autoantibodies, type 1 diabetes, or both. The association between type 1 diabetes and *DRB103:01:01 was affected by *DRB3**01:01:02 and *DRB3**02:02:01. These *DRB3* alleles were associated positively with GADA but negatively with ZnT8WA, IA-2A, and IAA. The negative association between type 1 diabetes and *DRB1**13:01:01 was affected by *DRB3**01:01:02 to increase the risk and by *DRB3**02:02:01 to maintain a negative association. *DRB4**01:03:01 was strongly associated with type 1 diabetes ($P = 10^{-36}$), yet its association was extensively affected by *DRB1* alleles from protective (*DRB1**04:03:01) to high (*DRB1**04:01:01) risk, but its association with *DRB1**04:05:01 decreased the risk. *HLA-DRB3*, *-DRB4*, and *-DRB5* affect type 1 diabetes risk and islet autoantibodies. *HLA* typing with NGS should prove useful to select participants for prevention or intervention trials.**

Genome-wide association studies through the Type 1 Diabetes Genetics Consortium (1–3) not only have confirmed the strongest association to single nucleotide polymorphisms in the *DR-DQ* region on chromosome 6 but also have reported an additional 50 non-*HLA* genetic loci of lesser association (1,4–6). Numerous studies confirmed the contribution of class II *DR* and *DQ* to type 1 diabetes risk and attempted to dissect the relative risk confirmed by these two closely located loci (7–10) and to extend it to the *HLA-DP* locus as well (11). Other genetic factors in linkage disequilibrium with *DR* and *DQ* may contribute to either enhancing or decreasing the risk through a possible association with islet autoantibodies at the time of not only clinical diagnosis (12–16) but also seroconversion (17,18).

DRB1 is present in all individuals. Allelic variants of *DRB1* are in linkage disequilibrium with either none or one of the genes *DRB3*, *DRB4*, and *DRB5*. In addition,

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there are several related pseudogenes (*DRB2*, *DRB6*, *DRB7*, *DRB8*, and *DRB9*). Through next-generation sequencing (NGS), an integrated genotyping system of exons 1–4 was developed to type all alleles of *DRB1*, *DRB3*, *DRB4*, and *DRB5* (19,20). Among individuals with the *DRB1**03:01 allele, the *DRB3**02:02 allele has been reported to show an independent risk for type 1 diabetes compared with the *DRB3**01:01 allele (21). The study concluded that the *DRB3**02:02 allele not only was a marker of high-risk *DRB1-DRB3* haplotypes but also increased the risk for *DRB1**03:01 haplotypes, particularly in individuals homozygous for *DRB1**03:01 (21).

We previously reported that *HLA-DR-DQ* genotypes may define various autoimmune phenotypes indicating strong differential associations with GAD65 (GAD antibody [GADA]), insulin autoantibody (IAA), IA-2 antigen (IA-2A), or the three variants (amino acids R, W, or Q on position 325) of ZnT8A (ZnT8RA, ZnT8WA, ZnT8QA, respectively) (14,22,23). In these studies, the analyses were limited to *HLA-DQ* alleles detected by allele-specific probes (24,25). A major limitation was that the *DRB3*, *DRB4*, and *DRB5* alleles located between the *DRA* and *DRB1* loci were not typed (26,27). In the current study, we used NGS of all *DRB* alleles (19,20) in consecutively diagnosed patients with type 1 diabetes with all islet autoantibodies analyzed (14,28,29) and in geographically matched control subjects (30). The main goal was to test the hypothesis that *DRB3*, *DRB4*, and *DRB5* alleles modify the risk conferred by *DRB1* for islet autoantibodies and type 1 diabetes.

RESEARCH DESIGN AND METHODS

Study Design

We used a case-control study design to calculate odd ratios (ORs) for HLA alleles, haplotypes, and genotypes. Patients were from the nationwide Swedish Better Diabetes Diagnosis (BDD) study (14,22,28), which has involved ongoing participation since 2005 of all 42 pediatric clinics in Sweden. American Diabetes Association and World Health Organization criteria were used for the diagnosis of diabetes and to classify the disease (31). However, we included only patients who at the time of clinical diagnosis had one or several of the following autoantibodies against insulin: IAA; GADA; IA-2A; and ZnT8RA, ZnT8WA, or ZnT8QA (14,22,28). The Karolinska Institutet ethics board approved the BDD study (2004/1:9).

Study Population

Nine hundred seventy patients given a diagnosis of diabetes between 9 months and 18 years of age were sequentially enrolled in the BDD study (14,22,28). Four hundred forty-eight control subjects matched for age (1–18 years), sex, and place of residence were analyzed at the same time (30).

DNA Extraction

The Plasmid Maxiprep Kit (QIAGEN) was used to isolate DNA according to the manufacturer's instructions from frozen whole-blood samples of patients and control subjects.

HLA NGS Analysis

The NGS *HLA* typing approach used PCR-based amplification of *HLA* and sequencing with Illumina MiSeq technology as previously described in detail (19,20). Briefly, the laboratory steps comprise consecutive PCR reactions with bar coding incorporated into the PCRs for individual sample tracking followed by application to the MiSeq system. Robust assays for each target loci of all *HLA-DR* alleles were developed. The depth of genotyping was extended to *HLA-DRB3*, *-DRB4*, and *-DRB5* to include exons 2 and 3 for all *DR* alleles. The analytical tools to define haplotypes and genotypes were developed in collaboration with Scisco Genetics (Seattle, WA). To date, these tools have been tested with 100% accuracy on >2,000 control samples genotyped with the present NGS approach (19,20).

Islet Autoantibodies

GADA, IA-2A, IAA, and the three variants of ZnT8A (ZnT8RA, ZnT8WA, or ZnT8QA) were determined in quantitative radiobinding assays by using in-house standards to determine levels as previously described in detail (14,32).

Statistical Analysis

Because patients and control subjects were enrolled from a larger population study of type 1 diabetes, we used Epi Info 7.1.3.0 (http://wwwn.cdc.gov/epiinfo) for managing all epidemiological data, tracking all biological samples, and performing quality control analyses. For descriptive statistical data analysis, we used SPSS version 22 software (IBM Corporation, Chicago, IL) to calculate frequency distributions, visualizations, and two-group comparison tests of type 1 diabetes associations with pertinent epidemiological variables.

We performed allelic association analysis of multiallelic genes of *HLA-DRB1*, *-DRB3*, *-DRB4*, and *-DRB5*. Under Hardy-Weinberg equilibrium, the allelic analysis computes allelic frequencies among patients only, control subjects only, and the combination of both. Given excessive polymorphism, we chose to test allele-specific associations with type 1 diabetes by score test (33). The score test evaluates the allele-specific score under the null hypothesis with no allelic associations and is referred to here as H-score because it was developed specifically for testing haplotype associations. Under the null hypothesis, H-score has an asymptotic normal distribution, which is then used to compute the *P* value. To estimate allele-specific magnitudes of the type 1 diabetes association, we chose the reference allele that has comparable allelic frequencies between patients and control subjects and has relatively high allelic frequency. In comparison with this reference allele, we calculated the OR for every allele. The OR of the reference allele is 1, an OR <1 implies a protective allele, and an OR >1 is a risk allele. For all computations, we used the R function haplo.cc (http://cran.r-project.org/web/packages/haplo.stats/index.html). The function haplo.cc computes the haplotype-based association used for the haplotype analysis described next and assesses the allelic association by introducing a monomorphic locus as the second locus.

In the *HLA-DR* locus, alleles of *HLA-DRB1* are in linkage disequilibrium with alleles of either *HLA-DRB3* or *-DRB4* or *-DRB5*. Hence, by treating these subunits and their allelic variations as different alleles, analysis of *HLA-DRB1* and *-DRB3*, *-DRB4*, or *-DRB5* by haplo.cc produces estimates of frequencies for all haplotypes provided that expectation numbers of corresponding haplotypes are five or more copies in both patients and control subjects. Similarly, the function haplo.cc produces H-scores and *P* values for all included haplotypes. By following the same principle of choosing the reference haplotype, we computed haplotype-specific ORs. To facilitate interpretation of estimated haplotype-specific ORs, we created a two-dimensional table showing H-scores for specific pairs of alleles at *HLA-DRB1* and *-DRB3*, *-DRB4*, or *-DRB5*.

RESULTS

DRB1 Alleles

The NGS for typing *HLA-DRB1* in Swedish patients with type 1 diabetes and control subjects identified 25 different *DRB1* alleles. By using *DRB1**13:02:01 as the reference (equal frequency in patients and control subjects), only seven other alleles showed no association with type 1 diabetes (Table 1). The H-score of the five *DRB1* alleles that were positively associated with type 1 diabetes could be ranked as *DRB1**04:01:01 > *03:01:01 > *04:05:01 > *04:04:01 > *04:02:01 (Table 1). Significant negative associations were observed for a total of 13 different *HLA-DRB1* alleles. The H-score ranked the top six negatively associated alleles as *15:01:01 > *07:01:01 > *13:01:01 > *11:01:01 > *11:04:01 > *14:54:01. An unadjusted *P* value of 0.01 for multiple comparisons was used as a threshold value for marking positive or negative associations, even though multiple alleles at this gene locus are tested on their associations with type 1 diabetes. Correcting multiple comparisons by number of alleles would have led to excessively conservative results because alleles are functional, and estimated log ORs are highly correlated. Correction was not attempted for results interpretation.

DRB3, *DRB4*, and *DRB5* Alleles

The NGS for typing the *HLA-DRB3*, *-DRB4*, and *-DRB5* alleles revealed eight alleles (Table 2). Subjects lacking any *HLA-DRB3*, *-DRB4*, and *-DRB5* alleles (nonamplified, indicating the presence of a pseudogene) are also indicated as ID9. Using *DRB3**03:01:01 as the reference (equal frequency in patients and control subjects), significant risk was conferred by *DRB4**01:03:01 and *DRB3**01:01:02

Table 1—Estimated allelic frequencies in pooled samples, control subjects only, and patient cases only and computed H-scores and their associated *P* values and estimated ORs for *HLA-DRB1* among 970 patients and 448 control subjects

		Haplotype Frequency					
ID	DRB1	Pooled	Control	Case	H-score	*P* value	OR
1	*13:02:01	0.044	0.044	0.045	0.140	8.88E-01	1.000
2	*01:01:01	0.087	0.094	0.084	−0.840	4.01E-01	0.841
3	*01:02:01	0.007	0.010	0.006	−1.280	2.01E-01	0.527
4	*01:03	0.003	0.008	0.001	−2.974	2.94E-03	0.182
5	*03:01:01	0.204	0.103	0.252	9.270	1.87E-20	2.301
6	*04:01:01	0.279	0.103	0.361	14.718	4.94E-49	5.114
7	*04:02:01	0.011	0.004	0.014	2.210	2.71E-02	6.135
8	*04:03:01	0.004	0.009	0.001	−3.287	1.01E-03	0.153
9	*04:04:01	0.067	0.047	0.076	2.832	4.63E-03	2.077
10	*04:05:01	0.010	0.002	0.014	2.907	3.65E-03	8.162
11	*04:07:01	0.004	0.009	0.001	−3.287	1.01E-03	0.117
12	*04:08:01	0.004	0.004	0.004	−0.117	9.07E-01	1.041
13	*07:01:01	0.048	0.099	0.024	−8.552	1.21E-17	0.259
14	*08:01:01	0.039	0.042	0.038	−0.575	5.66E-01	0.736
15	*09:01:02	0.014	0.011	0.016	0.910	3.63E-01	1.939
16	*10:01:01	0.005	0.008	0.003	−1.717	8.59E-02	0.290
17	*11:01:01	0.022	0.050	0.009	−6.804	1.01E-11	0.183
18	*11:04:01	0.006	0.017	0.001	−4.755	1.98E-06	0.114
19	*12:01:01	0.012	0.022	0.007	−3.330	8.67E-04	0.547
20	*13:01:01	0.039	0.077	0.021	−7.360	1.83E-13	0.218
21	*13:03:01	0.005	0.010	0.003	−2.626	8.64E-03	0.278
22	*14:54:01	0.006	0.016	0.002	−4.506	6.59E-06	0.123
23	*15:01:01	0.051	0.150	0.005	−15.834	1.83E-56	0.042
24	*15:02:01	0.004	0.009	0.002	−2.929	3.40E-03	0.223
25	*16:01:01	0.009	0.015	0.007	−2.015	4.39E-02	0.541

Red is a positive and green a negative association of statistical significance.

Table 2—Estimated allelic frequencies in pooled samples, control subjects only, and patient cases only and computed H-scores and their associated *P* values and estimated ORs for *HLA-DRB3*, *-DRB4*, and *-DRB5* (*DRB345*) among 970 patients and 448 control subjects

		Haplotype Frequency					
ID	DRB345	Pooled	Control	Case	H-score	*P* value	OR
1	DRB3*03:01:01	0.044	0.044	0.045	0.140	8.88E-01	1.000
2	DRB3*01:01:02	0.196	0.146	0.220	4.774	1.81E-06	1.352
3	DRB3*02:02:01	0.105	0.166	0.077	−6.862	6.80E-12	0.552
4	DRB4*01:01:01	0.021	0.031	0.016	−2.538	1.12E-02	0.587
5	DRB4*01:03:01	0.419	0.261	0.492	12.576	2.84E-36	2.190
6	DRB5*01:01:01	0.052	0.153	0.006	−15.860	1.20E-56	0.039
7	DRB5*01:02	0.003	0.007	0.001	−2.633	8.45E-03	0.132
8	DRB5*02:02	0.010	0.015	0.007	−1.845	6.50E-02	0.609
9	Nonamplified	0.146	0.171	0.135	−2.544	1.10E-02	0.805

Red is a positive and green a negative association of statistical significance.

(Table 2). The remaining alleles, except *DRB5**02:02 (neutral), were negatively associated with type 1 diabetes: *DRB5**01:01:01 > *DRB3**02:02:01 > *DRB5**01:02 > *DRB4**01:01:01.

HLA-DRB1, *-DRB3*, *-DRB4*, and *-DRB5* Haplotypes

Twenty-eight *HLA-DRB1*, *-DRB3*, *-DRB4*, and *-DRB5* haplotypes were identified in the 1,418 subjects (Table 3). Nonamplified *DRB3*, *DRB4*, and *DRB5* were found on

Table 3—Estimated haplotypic frequencies in pooled samples, control subjects only, and patient cases only and computed H-scores and their associated *P* values and estimated ORs for *HLA-DRB1* and *DRB3*, *DRB4*, and *DRB5* (*DRB345*) among 970 patient and 448 control subjects

			Haplotype Frequency					
ID	DRB1	DRB345	Pooled	Control	Case	H-score	*P* value	OR
1	*13:02:01	DRB3*03:01:01	0.044	0.044	0.045	0.140	8.88E-01	1.000
2	*01:01:01	Nonamplified	0.087	0.093	0.084	−0.743	4.58E-01	0.879
3	*01:02:01	Nonamplified	0.007	0.010	0.006	−1.280	2.01E-01	0.534
4	*01:03	Nonamplified	0.003	0.008	0.001	−2.974	2.94E-03	0.174
5	*03:01:01	DRB3*01:01:02	0.165	0.087	0.202	7.855	3.99E-15	2.132
6	*03:01:01	DRB3*02:02:01	0.039	0.016	0.049	4.034	5.48E-05	3.674
7	*04:01:01	DRB4*01:03:01	0.277	0.101	0.359	14.686	7.97E-49	5.335
8	*04:02:01	DRB4*01:03:01	0.011	0.004	0.014	2.210	2.71E-02	6.864
9	*04:03:01	DRB4*01:03:01	0.003	0.009	0.001	−3.692	2.23E-04	0.069
10	*04:04:01	DRB4*01:03:01	0.065	0.046	0.074	2.756	5.85E-03	2.159
11	*04:05:01	DRB4*01:03:01	0.010	0.002	0.013	2.746	6.04E-03	8.020
12	*04:07:01	DRB4*01:03:01	0.004	0.009	0.001	−3.287	1.01E-03	0.126
13	*04:08:01	DRB4*01:03:01	0.004	0.004	0.004	−0.117	9.07E-01	1.158
14	*07:01:01	DRB4*01:01:01	0.017	0.029	0.011	−3.250	1.16E-03	0.473
15	*07:01:01	DRB4*01:03:01	0.031	0.071	0.013	−8.133	4.19E-16	0.195
16	*08:01:01	Nonamplified	0.039	0.041	0.038	−0.433	6.65E-01	0.752
17	*09:01:02	DRB4*01:03:01	0.013	0.011	0.015	0.757	4.49E-01	2.196
18	*10:01:01	Nonamplified	0.005	0.008	0.003	−1.717	8.59E-02	0.293
19	*11:01:01	DRB3*02:02:01	0.022	0.048	0.009	−6.530	6.56E-11	0.208
20	*11:04:01	DRB3*02:02:01	0.006	0.017	0.001	−4.755	1.98E-06	0.116
21	*12:01:01	DRB3*02:02:01	0.012	0.022	0.007	−3.330	8.67E-04	0.559
22	*13:01:01	DRB3*01:01:02	0.023	0.042	0.014	−4.860	1.17E-06	0.266
23	*13:01:01	DRB3*02:02:01	0.015	0.034	0.006	−5.642	1.68E-08	0.182
24	*13:03:01	DRB3*01:01:02	0.005	0.010	0.003	−2.626	8.64E-03	0.277
25	*14:54:01	DRB3*02:02:01	0.006	0.016	0.002	−4.506	6.59E-06	0.126
26	*15:01:01	DRB5*01:01:01	0.051	0.148	0.005	−15.755	6.35E-56	0.045
27	*15:02:01	DRB5*01:02	0.003	0.007	0.001	−2.633	8.45E-03	0.119
28	*16:01:01	DRB5*02:02	0.009	0.015	0.007	−2.015	4.39E-02	0.504

Red is a positive and green a negative association of statistical significance.

Table 4—Estimated H-scores for all haplotypes between *HLA-DRB1* and *-DRB3*, *-DRB4*, and *-DRB5*, with their marginal H-scores listed by rows (*HLA-DRB1*) and by columns (*HLA-DRB3*, *-DRB4*, and *-DRB5*)

	DRB3			DRB4		DRB5				
DRB1	*03:01:01	*01:01:02	*02:02:01	*01:01:01	*01:03:01	*01:01:01	*01:02	*02:02	Nonamplified	H-score
*13:02:01	0.140									0.140
*01:01:01									−0.743	−0.840
*01:02:01									−1.280	−1.280
*01:03									−2.974	−2.974
*03:01:01		7.855	4.034							9.270
*04:01:01					14.686					14.718
*04:02:01					2.210					2.210
*04:03:01					−3.692					−3.287
*04:04:01					2.756					2.832
*04:05:01					2.746					2.907
*04:07:01					−3.287					−3.287
*04:08:01					−0.117					−0.117
*07:01:01				−3.250	−8.133					−8.552
*08:01:01									−0.433	−0.575
*09:01:02					0.757					0.910
*10:01:01									−1.717	−1.717
*11:01:01			−6.530							−6.804
*11:04:01			−4.755							−4.755
*12:01:01			−3.330							−3.330
*13:01:01		−4.860	−5.642							−7.360
*13:03:01		−2.626								−2.626
*14:54:01						−4.506				−4.506
*15:01:01						−15.755				−15.834
*15:02:01							−2.633			−2.929
*16:01:01								−2.015		−2.015
H-score	0.140	4.774	−6.862	−2.538	12.576	−15.860	−2.633	−1.845	−2.544	

All H-scores are rounded to their integers, and their absolute values >2 are deemed significant. Red is a positive and green a negative association of statistical significance.

the *DRB1**01:01:01-, *DRB1**01:03-, *DRB1**01:02:01-, *DRB1**08:01:01-, and *DRB1**10:01:01-containing haplotypes. Only the *DRB1**01:03-containing haplotype showed a negative association (OR 0.174, P = 0.00293) in relation to the *DRB1**13:02:01-*DRB3**03:01:01 reference haplotype (OR 1.0).

Positive associations were found in six haplotypes, with *DRB1**04:01:01-*DRB4**01:03:01 showing the highest H-score (Table 3). *DRB4**01:03:01 may be present in six different haplotypes containing either *DRB1**04:01:01 (risk, haplotype 7), *DRB1**04:02:01 (risk, haplotype 8), *DRB1**04:04:01 (risk, haplotype 10), *DRB1**04:05:01 (risk, haplotype 11), *DRB1**04:03:01 (protection, haplotype 9), or *DRB1**04:07:01 (protection, haplotype 12). Taken together, *DRB4**01:03:01 may be present on four haplotypes conferring risk and two haplotypes showing a negative association, therefore having a potential protective effect.

Haplotypic Interactions Between *DRB1* and *DRB3*, *DRB4*, and *DRB5*

To gain an intuitive insight into haplotypic modification of *DRB1* by *DRB3*, *DRB4*, and *DRB5* alleles, all H-scores (rounded to the integer for easy visualization) are shown in a two-way diagram with rows of *DRB1* alleles and columns of *DRB3*, *DRB4*, and *DRB5* alleles (Table 4). As shown, the presence of a single horizontal and vertical cross (i.e., *DRB1**01:03 with nonamplified pseudogenes *DRB1**15:02:01-*DRB5**01:02 and *DRB1**16:01:01-*DRB5**02:02) implies that the association is haplotype specific; hence, one cannot differentiate effects of two alleles. The association between type 1 diabetes and *DRB1**03:01:01 (H-score 9) was found to be affected by *DRB3**01:01:02 (H-score 8) and *DRB3**02:02:01 (H-score 4). Similarly, the associations between type 1 diabetes and *DRB1**07:01:01 (H-score −9) and *DRB1**13:01:01 (H-score −7) were modified by *DRB3*, *DRB4*, and *DRB5*. *DRB1**07:01:01 was modified by both *DRB4**01:01:01 (H-score −3) and *DRB4**01:03:01 (H-score 13); hence, two *DRB4* alleles with opposite risks when analyzed individually results in a negative association on the *DRB1**07:01:01 haplotype. *DRB1**13:01:01 was modified by both *DRB3**01:01:02 (H-score 5) and *DRB3**02:02:01 (H-score −7), which also showed opposite risks when analyzed individually, but when present in the *DRB1**13:01:01 haplotype, the H-score of −7 indicated protection.

On the other hand, marginal associations of *DRB3*, *DRB4*, and *DRB5* alleles are extensively modified by *DRB1* alleles (Table 4). For example, *DRB4**01:03:01 was marginally associated with type 1 diabetes (H-score 13), yet its association was extensively modified by *DRB1* alleles from protective (H-score −8) to high risk (H-score 15).

Haplotypic Interactions Between *DRB1*, *DRB3*, *DRB4*, and *DRB5* and Islet Autoantibodies in Patients

Levels of islet autoantibodies were determined at the time of clinical diagnosis. Levels, expressed in arbitrary units using in-house reference sera (14) or the World Health Organization standard for GADA and IA-2A (34), were transformed with cubic root to correct the excessive skewed distributions (Table 5). The most common haplotype, *DRB1**04:01:01-*DRB4**01:03:01 (in 36% of the patients) was used as the reference. To ease interpretation, Table 6 lists the integer part of z scores, indicating a positive association (red), negative association (green), or no statistical association (no color) (absolute values of z scores <2).

Table 5—Haplotypic association analysis of *DRB1* and *DRB3* or *DRB4* (*DRB345*) haplotypes with five islet autoantibodies (IAA, GADA, IA-2A, ZnT8RA, ZnT8WA, ZnT8QA) among all 448 patients, with the most common haplotype *DRB104:01:01-*DRB4**01:03:01 as the reference**

ID	DRB1	DRB345	Hap	GADA				ZnT8WA				IAA				IA-2A				ZnT8QA			
			Freq	log OR	SE	T-Stat	P-value	log OR	SE	T-Stat	P-value	log OR	SE	T-Stat	P-value	log OR	SE	T-Stat	P-value	log OR	SE	T-Stat	P-value
R	*04:01:01	DRB4*01:03:01	0.359	0.013	0.157	0.079	0.937	0.383	0.156	2.461	0.014	-0.475	0.162	-2.934	0.003	3.413	0.274	12.475	0.000	-0.427	0.161	-2.660	0.008
1	*01:01:01		0.084	0.031	0.197	0.159	0.874	-0.257	0.194	-1.327	0.185	-0.607	0.219	-2.765	0.006	-1.376	0.265	-5.197	0.000	-0.237	0.205	-1.155	0.248
2	*03:01:01	DRB3*01:01:02	0.202	0.664	0.155	4.279	0.000	-0.231	0.145	-1.596	0.111	-0.201	0.155	-1.297	0.195	-1.599	0.206	-7.774	0.000	-0.336	0.155	-2.161	0.031
3	*03:01:01	DRB3*02:02:01	0.049	0.890	0.256	3.479	0.001	-0.542	0.220	-2.465	0.014	-0.285	0.237	-1.202	0.230	-2.042	0.261	-7.830	0.000	-0.444	0.245	-1.811	0.070
4	*04:02:01	DRB4*01:03:01	0.014	-0.073	0.398	-0.182	0.855	-0.265	0.392	-0.676	0.499	-0.222	0.426	-0.522	0.602	-0.598	0.523	-1.143	0.253	-0.014	0.405	-0.036	0.971
5	*04:04:01	DRB4*01:03:01	0.074	0.408	0.194	2.107	0.035	0.009	0.181	0.047	0.962	-0.289	0.199	-1.457	0.145	-1.034	0.232	-4.454	0.000	-0.128	0.192	-0.666	0.506
6	*04:05:01	DRB4*01:03:01	0.013	-0.663	0.421	-1.574	0.116	-0.910	0.441	-2.064	0.039	-0.362	0.458	-0.790	0.430	0.488	0.757	0.644	0.520	-1.847	0.745	-2.478	0.013
7	*07:01:01	DRB4*01:01:01	0.011	1.412	0.568	2.487	0.013	-0.632	0.450	-1.406	0.160	-0.163	0.473	-0.344	0.731	-0.797	0.594	-1.342	0.180	-0.027	0.459	-0.059	0.953
8	*07:01:01	DRB4*01:03:01	0.013	0.501	0.463	1.081	0.280	0.099	0.442	0.225	0.822	0.015	0.454	0.033	0.974	-0.711	0.680	-1.046	0.296	0.191	0.443	0.430	0.667
9	*08:01:01		0.038	-0.272	0.268	-1.014	0.311	-0.453	0.266	-1.702	0.089	0.163	0.272	0.598	0.550	-1.648	0.351	-4.699	0.000	-0.444	0.293	-1.516	0.130
10	*09:01:02	DRB4*01:03:01	0.015	0.555	0.423	1.312	0.190	-0.408	0.394	-1.035	0.301	0.785	0.394	1.995	0.046	-0.539	0.572	-0.942	0.346	-0.179	0.421	-0.425	0.671
11	*13:01:01	DRB3*01:01:02	0.014	0.086	0.419	0.205	0.838	-0.570	0.417	-1.367	0.172	-0.120	0.439	-0.272	0.785	-1.558	0.563	-2.769	0.006	-0.376	0.452	-0.833	0.405
12	*13:02:01	DRB3*03:01:01	0.045	-0.288	0.241	-1.195	0.232	0.347	0.248	1.401	0.162	-0.307	0.260	-1.179	0.239	-1.702	0.301	-5.654	0.000	0.339	0.241	1.408	0.160
O	Rare		0.070	0.405	0.209	1.936	0.053	-0.315	0.198	-1.591	0.112	-0.171	0.212	-0.809	0.419	-1.098	0.265	-4.145	0.000	0.007	0.205	0.034	0.973

The yellow highlights indicate differences in haplotypic associations for ZnT8RA and ZnT8QA whether the DRB3 is either *01:01:02 or *02:02:01 on the DRB1*03:01:01 haplotype. Similarly, GADA and IA-2A vary dependent on the DRB4 subtype on the DRB1*07:01:01 haplotype. Association statistics are coefficients, SEs, *z* scores, and *P* values. Hap freq, haplotype frequency; *t* stat, *t* statistic.

At the significance level of 0.05, all haplotypes of *HLA-DRB1*, *-DRB3*, *-DRB4*, and *-DRB5* have various patterns of associations with the six different autoantibodies. In particular, *DRB3**01:01:02 and *DRB3**02:02:01 regulate autoantibody association of *DRB1**03:01:01 through specific autoantibodies ZnT8QA and ZnT8RA, respectively. On the other hand, *DRB4**01:01:01 and *DRB4**01:03:01 affected autoantibody associations of *DRB1**07:01:01 through a positive regulation with GADA, while weakly regulating IA-2A with a marginal H-score of −1.60.

DISCUSSION

Use of the NGS to type patients with newly diagnosed type 1 diabetes and control subjects resulted in the following principal findings. First, among the 25 *HLA-DRB1* alleles, only 4 (*DRB1**03:01:01, *DRB1**04:01:01, *DRB1**04:04:01, and *DRB1**04:05:01) were positively associated with type 1 diabetes. The H-score was used as a way to rank the relative degree of protection. Second, NGS detected nine alleles of *DRB3*, *DRB4*, and *DRB5*, including chromosomes with only nonamplified loci. Only *DRB4**01:03:01 and *DRB3**01:01:02 were positively associated; the remaining five alleles were negatively associated with the disease. Most importantly, *DRB4* was dichotomized in that *DRB4**01:03:01 was positively but *DRB4**01:01:01 was negatively associated with type 1 diabetes. Similarly, *DRB3**01:01:02 was positively but *DRB3**02:02:01 was negatively associated with the disease. Because either one of these two alleles may be present on a haplotype containing *DRB1**03:01:01, it cannot be excluded that the risk of this allele for type 1 diabetes is affected by the *DRB3* alleles to either increase or decrease the risk. Third, in dissecting the extended *DRB1-DRB3-DRB4-DRB5* haplotypes (28 haplotypes were identified), a major finding was that the two *DRB1**03:01:01-containing haplotypes remained positively associated with diabetes whether either *DRB3**01:01:02 or *DRB3**02:02:01 was present (although the positively associated *DRB3**01:01:02 showed a *P* value

Table 6—Patterns of haplotypic associations with autoantibodies (with *z* scores)

ID	DRB1	DRB345	IAA	GADA	IA-2A	ZnT8RA	ZnT8WA	ZnT8QA
R	*04:01:01	DRB4*01:03:01	Reference haplotype					
1	*01:01:01		−4		−4			
2	*03:01:01	DRB3*01:01:02	−2	4	−8		−2	−3
3	*03:01:01	DRB3*02:02:01	−2	3	−7	−2	−2	
4	*04:02:01	DRB4*01:03:01			−2			
5	*04:04:01	DRB4*01:03:01	−3	2	−4			
6	*04:05:01	DRB4*01:03:01		−2			−3	−4
7	*07:01:01	DRB4*01:01:01		2				
8	*07:01:01	DRB4*01:03:01			−2			
9	*08:01:01				−4	−2	−2	−2
10	*09:01:02	DRB4*01:03:01			−2			
11	*13:01:01	DRB3*01:01:02			−2			
12	*13:02:01	DRB3*03:01:01	−2		−3	2		
O	Rare				−4			

The yellow highlights indicate differences in haplotypic associations for ZnT8RA and ZnT8QA whether the DRB3 is either *01:01:02 or *02:02:01 on the DRB1*03:01:01 haplotype. Similarly, GADA and IA-2A vary dependent on the DRB4 subtype on the DRB1*07:01:01 haplotype. Green indicates negative associations; red, positive associations; and blank, null associations.

for risk that was three times higher than for the *DRB3**02:02:01-containing haplotype).

More importantly, the marginal associations of the *DRB3*, *DRB4*, and *DRB5* alleles were extensively affected by *DRB1* alleles. The analyses of the different extended *DRB1-DRB3-DRB4-DRB5* haplotypes strongly suggest that the risk for type 1 diabetes cannot be assigned to a single *DRB1* allele but that *DRB3*, *DRB4*, and *DRB5* in linkage disequilibrium have to be taken into account when dissecting the role of *HLA-DR* in type 1 diabetes.

The NGS offered considerable strength to the study. The method used allows extended *DRB1-DRB3-DRB4-DRB5* haplotypes to be computed without information on descent. The sequencing of coding regions of exon 1–4 of both *DRB1* and *DRB3*, *DRB4*, and *DRB5* allowed the detection of all functional genes, whereas pseudogenes were not amplified. Despite the presence of pseudogenes in the *DRB3-DRB4-DRB5* region, it was possible to compute complete haplotypes also in subjects with pseudogenes such as *DRB1**01:01:01, *DRB1**01:02:01, *DRB1**08:01:01, and *DRB1**10:01:01, respectively. The methods for NGS of *HLA* alleles vary between approaches and methodologies; however, the present method is a strength because it allows high-resolution typing of alleles, which until now have been understudied. In addition, due to reduced costs of PCR, novel instrumentations and approaches in bioinformatics make NGS *HLA* typing affordable and precise.

The current study population is unique because it represents consecutive patients with newly diagnosed type 1 diabetes in Sweden (22,28). The patients in the current study were all born to parents who were born in Sweden, as was the case for their grandparents (28). According to Swedish pediatric diabetes guidelines, all patients with type 1 diabetes <18 years old are seen by diabetes specialists in one of the pediatric diabetes clinics in Sweden. A potential weakness to this study is that we were not able for reasons of funding to analyze a control group of equal number as the patients. However, the current control subjects were selected to represent the geographical location of the patients (30).

To our knowledge, there is only one previous publication on NGS of *DRB* genes (21), which studied 143 control subjects and 337 patients of a much larger cohort and reported that both *DRB3**01:01 and *DRB3**02:02 alleles showed an increased risk for type 1 diabetes. In particular, the authors suggested that on *DRB1**03:01 haplotypes, the *DRB3**02:02 allele contributes to type 1 diabetes risk (21). The current analysis of patients with newly diagnosed type 1 diabetes and control subjects shows results consistent with this conclusion. However, the current results differ because we found that *DRB3**02:02 was negatively associated with type 1 diabetes (Table 2), and when considered with *DRB1**03:01:01, we found that *DRB3**02:02:01 reduced the risk for type 1 diabetes compared with the *DRB1**03:01:01-*DRB3**01:01:02 haplotype (Table 3).

The *DRB1**03:01:01-containing haplotypes may carry either the *DRB3**02:02:01 or the *DRB3**02:02:01 alleles. It was recently demonstrated that children homozygous for *DR*3/3 (*DRB1**03:01:01/*DRB1**03:01:01) in the TEDDY (The Environmental Determinants of Diabetes in the Young) study had an increased risk for developing GADA as their first islet autoantibody (17). Further studies are therefore needed to determine whether *DRB3**02:02:01 affects the risk for GADA as the first islet autoantibody.

The negative association of *DRB1**01:03 with type 1 diabetes was independent of *DRB3*, *DRB4*, and *DRB5* because it resides on a haplotype unable to express any of these *DR* subtypes. The interpretation is that the *DRB1* protein heterodimer confers protection probably by inducing immunological tolerance.

A paucity of studies of other autoimmune diseases have investigated the possible contribution of *DRB1-DRB3-DRB4-DRB5* haplotypes. Thrombotic thrombocytopenic purpura was reported to be positively associated with *DRB3* and negatively associated with *DRB4*, although the *DRB3* and *DRB4* subtyping was at low resolution to conclude whether the association was due to linkage disequilibrium to either *DR3* or *DR4* (35). The meaning of the results in the current study is that *DRB3*, *DRB4*, and *DRB5* may affect not only the risk for type 1 diabetes but also the risk of having certain islet autoantibodies (Table 5). The data strongly suggest that *DRB3* on the *DRB1**03:01:01 haplotype affects the risk of having GADA or ZnT8RA (positive association) or IA-2A (negative association). Patients with *DRB1**07:01:01-*DRB4**01:01:01 have an increased risk for GADA at the time of clinical diagnosis. Therefore, determining to what extent the *DRB3*, *DRB4*, and *DRB5* β-chains are able to form heterodimers with the *DRA* α-chains is critical. It cannot be excluded that peptide presentation on *DRB3*, *DRB4*, or *DRB5* heterodimers may induce immune responses related to autoimmunity. It was found, for example, that peptides from group A streptococcal vaccine epitopes are effectively presented on *DRB3*, *DRB4*, and *DRB5* heterodimers (36).

In conclusion, *HLA-DRB3*, *-DRB4*, and *-DRB5* genetic factors should be taken into account when dissecting the role of *HLA-DR* in the risk for islet autoimmunity and progression to the clinical onset of type 1 diabetes. The contribution of these heterodimer proteins in the immune response to infectious agents may be of particular interest in studying the etiology of islet autoimmunity and the progression to the clinical onset of type 1 diabetes.

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Appendix

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