Multiple Loci in the HLA Complex Are Associated with Addison's Disease

Beate Skinningsrud,* Benedicte A. Lie,* Ewa Lavant, Joyce A. Carlson, Henry Erlich, Hanne E. Akselsen, Kristina Gervin, Anette B. Wolff, Martina M. Erichsen, Kristian Løvås, Eystein S. Husebye,** and Dag E. Undlien**

Department of Medical Genetics (B.S., H.E.A., K.G., D.E.U.), Oslo University Hospital, Ullevål, N-0407 Oslo, Norway; Institute of Medical Genetics (B.S., B.A.L., D.E.U.), University of Oslo, N-0315 Oslo, Norway; Institute of Immunology (B.A.L.), Oslo University Hospital, Rikshospitalet, N-0027 Oslo, Norway; Clinical Chemistry (E.L., J.A.C.), University Hospital, S-205 05 Malmö, Sweden; Roche Molecular Systems (H.E.), Alameda, California 94501; and Section of Endocrinology (A.B.W., K.L., E.S.H.), Institute of Medicine, University of Bergen, and Department of Medicine (M.M.E., K.L., E.S.H.), Haukeland University Hospital, N-5021 Bergen, Norway

Context: A strong association between autoimmune Addison's disease (AAD) and major histocompatibility complex class II-encoded *HLA-DRB1-DQA1-DQB1* haplotypes is well known. Recent evidence from other autoimmune diseases has suggested that class I-encoded *HLA-A* and *HLA-B* gene variants confer *HLA-DRB1-DQA1-DQB1*-independent effects on disease.

Objective: We aimed to explore AAD predisposing effects of *HLA-A* and *-B* and further investigate the role of *MICA* and *HLA-DRB1-DQA1-DQB1* in a much larger material than has previously been studied.

Design: *HLA-A*, *-B*, *-DRB1*, and *-DQB1* and a microsatellite in *MICA* were genotyped in 414 AAD patients and 684 controls of Norwegian origin.

Results: The strongest association was observed for the *DRB1* locus, in which the DRB1*03:01 and DRB1*04:04 conferred increased risk of AAD, particularly in a heterozygous combination [odds ratio 22.13; 95% confidence interval (11.39–43.98); $P = 6 \times 10^{-20}$]. After conditioning on *DRB1*, association with AAD was still present for *HLA-B* and *MICA*, suggesting the presence of additional risk factors.

Conclusions: The major histocompatibility complex harbors multiple risk loci for AAD, in which *DRB1* appears to represent the main risk factor. **(*J Clin Endocrinol Metab* 96: E1703–E1708, 2011)**

The major histocompatibility complex (MHC) in humans, the human leukocyte antigen (HLA) complex, spans approximately 3.6 Mb on chromosome 6p21.3 and harbors a large number of genes involved in immune function. Among them are the classical HLA class I (*HLA-A*, *HLA-B*, and *HLA-C*) and class II (*HLA-DRB1*, *HLA-DQB1*, *HLA-DQA1*, *HLA-DPB1*, and *HLA-DPA1*) genes, which encode antigen presenting molecules. Variations within HLA have been found associated with almost every autoimmune disease, with risk estimates exceeding those of other genetic susceptibility factors identified in these complex diseases. Strong linkage disequilibrium (LD) in the region has, however, complicated the identification of the causal variants. Moreover, because the HLA class I and II genes are the most polymorphic loci in the human genome, the majority of studies have been restricted to small cohorts because of the comprehensive genotyping involved.

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* B.S. and B.A.L. contributed equally to this study, and ** E.S.H. and D.E.U. contributed equally to this study as first and last authors, respectively.

Abbreviations: AAD, Autoimmune Addison's disease; APS I, autoimmune polyendocrine syndrome type I; CI, confidence interval; HLA, human leukocyte antigen; KIR, killer immunoglobulin receptor; LD, linkage disequilibrium; MHC, major histocompatibility complex; OR, odds ratio.

Autoimmune destruction of the adrenal cortex is the main cause of Addison's disease in Western countries. More than half of all autoimmune Addison's disease (AAD) patients have coexisting nonadrenal organ-specific autoimmunity, most often with concomitant thyroid autoimmunity and/or type 1 diabetes, a syndrome referred to as autoimmune polyendocrine syndrome type II (1). Aside from the rare cases found in the monogenic autoimmune polyendocrine syndrome type I (APS I) caused by mutations in the autoimmune regulator (*AIRE*) gene, the etiology of AAD is assumed to be multifactorial. Although the identities of underlying genetic and environmental risk factors are still incompletely understood, HLA class II variations were found to be associated with AAD already in 1986 (2). The disease susceptibility has been mapped to two HLA haplotypes, *i.e.* DRB1*03:01-DQA1*05-DQB1*02 and DRB1*04:04-DQA1*03-DQB1*03:02, with a large risk increment when they occur in a heterozygous combination (2, 3).

Our aim was to explore the effects of HLA class I (*A* and *B*) and II (*DRB1* and *DQB1*) genes for AAD susceptibility by genotyping a large cohort of cases and controls because the role of HLA class I polymorphisms in AAD is still unexplored but were recently shown to have effects on type 1 diabetes susceptibility (4). The gene MHC class I polypeptide-related sequence A (*MICA*), located between the HLA class I and class II genes, was also included because of its earlier reported associations with AAD, although in modest sample sizes ($n_{cases} < 50$) (5, 6).

Patients and Methods

Patients and controls

This study included 414 unrelated Norwegian AAD patients and 684 Norwegian blood donor controls described earlier by Erichsen *et al.* (7). The patients were diagnosed by having either low basal serum cortisol together with high ACTH or a pathological ACTH stimulation test, excluding those with adrenoleukodystrophy, congenital adrenal hyperplasia, pharmacologically induced adrenal failure, bilateral adrenalectomy, or APS I. An autoimmune etiology was demonstrated in more than 95% of the patients, *i.e.* they had at least one concomitant organ-specific autoimmune disease or a family member with AAD (APS I excluded) or at least one positive organ-specific autoantibody. If we had samples from more than one family member with Addison's disease, only the patient with the lowest age of onset was included in this study (22 individuals).

The study was performed according to the Helsinki Declaration and was approved by the regional ethical committees of western and southeastern Norway.

HLA typing

HLA-A, *-B*, *-DRB1*, and *-DQB1* genotyping was performed at a two- to four-digit level. A PCR-based, sequence-specific oligonucleotide probe system for typing *A*, *B*, and *DRB1* was performed as described earlier (8). *DQB1* was analyzed by DELFIA hybridization assay (PerkinElmer Life Sciences, Turku, Finland). Samples with ambiguous HLA class II results were subjected to sequencing-based typing by AlleleSEQR kit (Abbott Molecular, Abbott Park, IL) and analyzed by Assign version 3.5 (Conexico Genomics; Abbott Molecular). *DQA1* was not genotyped because it easily can be deduced from the *DRB1-DQB1* haplotype due to virtually complete LD. Primers for amplifying the *MICA* microsatellite were identical to those described earlier (9). All sequencing and microsatellite analyses were performed on an ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA).

Grouping of HLA alleles

The degree of polymorphisms among *HLA-A*, *-B*, *-DRB1*, and *-DQB1* alleles result in a number of very rare alleles. To be able to use the information they provide but also minimize the statistical biases they may confer, we merged some subtypes differing only at the third and fourth digit level into a two-digit level allele group. When the alleles in this manuscript are given at a two-digit level, it means that groups of subtypes have been formed. Conversely, the alleles given at a four-digit level have been genotyped to this resolution, and no merging has been performed.

We also grouped the *HLA-B* alleles into killer immunoglobulin receptor (KIR) ligands based on previous reports of association between this functional grouping and autoimmune diseases (10, 11). The *HLA-B* KIR ligands can be separated into two main groups (Bw4 and Bw6) determined by the motifs at amino acid positions 77–83 in *HLA-B*, in particular the amino acid at position 80. In general, the following *HLA-B* alleles belong to the Bw6 group: HLA-B*07, HLA-B*08, HLA-B*15, HLA-B*18, HLA-B*35, HLA-B*39, HLA-B*40, HLA-B*41, HLA-B*42, HLA-B*45, HLA-B*46, HLA-B*48, HLA-B*50, HLA-B*54, HLA-B*55, HLA-B*56, HLA-B*67, HLA-B*73, HLA-B*78, HLA-B*81, HLA-B*82, HLA-B*83, whereas Bw4 is comprised of HLA-B*13, HLA-B*27, HLA-B*37, HLA-B*38, HLA-B*44, HLA-B*47, HLA-B*49, HLA-B*51, HLA-B*52, HLA-B*53, HLA-B*57, HLA-B*58, and HLA-B*59.

Statistical analyses

The software UNPHASED version 2.403 with COCAPHASE was used for global association tests, including the conditional analyses for allele main effects, and to calculate the LD (12). Haplotype frequencies were inferred by PHASE version 2.1.1 (13). The haplotype method (14) was used to explore allele frequencies of *A*, *B*, *MICA*, and *DQB1* stratified on the different *DRB1* alleles. Testing of potentially independent effect was also investigated by the approach described by Svejgaard and Ryder (15).

Results

All loci tested showed association with AAD (Table 1), and the frequency distribution between cases and controls for all alleles at the tested loci are given in Supplemental Table 1–5, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. All geno-

TABLE 1. *P* values from global regression analyses of the HLA loci to reveal independent associations

		Conditional locus					
	Unconditional	**A**	**B**	**MICA**	**DRB1**	**DQB1**	**DRB1-DQB1**
Test locus							
A	2.6e-009		0.007	1.9e-5	0.23	0.0003	0.20
B	6.6e-022	1.3e-14		3.3e-12	0.005	9.9e-011	0.003
MICA	8.8e-014	5.0e-9	0.01		0.001	4.0e-10	0.007
DRB1	7.2e-052	2.3e-40	6.1e-28	3.4e-41		4.4e-020	
DQB1	1.4e-031	2.3e-25	8.0e-19	7.7e-28	0.09		

typing data for each individual can be downloaded from Supplemental Table 6 and 7. HLA class II genotype distributions in our AAD material and subphenotypes are detailed by Erichsen *et al.* (7). The focus of the present paper was to elucidate which of the genotyped HLA loci confine a main effect, in addition to explore whether the HLA class I genes and/or *MICA* contribute with additional effects.

Conditional analyses point to *DRB1* as the main effect gene

HLA-DRB1 displayed the most significant association ($P = 7 \times 10^{-52}$). Because strong LD exists across the MHC, conditional logistic regression analyses were performed to evaluate which locus showed the strongest association and which associations were independent of each other. *DRB1* was significantly associated with AAD when conditioning on each of the other loci. After conditioning on *DRB1*, two loci, *HLA-B* and *MICA*, still appeared to be associated ($P = 0.005$ and $P = 0.001$, respectively), whereas the *HLA-A* and *DQB1* associations were lost.

DRB1*03:01 and DRB1*04:04 were strongly predisposing to AAD [odds ratio (OR) 2.93; 95% confidence interval (CI) (2.12–4.04); $P = 5 \times 10^{-11}$ and OR 3.30; 95% CI (2.27–4.81); $P = 4 \times 10^{-10}$, respectively]. The *DQB1* alleles in strong LD with these *DRB1* alleles, *i.e.* DQB1*02 and DQB1*03:02, also showed strong association, although with somewhat lower OR values [OR 1.83; 95% CI (1.35–2.47); $P = 7 \times 10^{-5}$ and OR 1.53; 95% CI (1.11–2.09); $P = 0.008$, respectively]. For *HLA-B*, the strongest effect was seen for the B*08 allele [OR 2.56; 95% CI (1.94–3.39); $P = 4 \times 10^{-11}$]. For *MICA*, only MICA5.1 showed disease association [OR 1.78; 95% CI (1.30–2.42); $P = 0.0003$]. Several other alleles were less significantly ($0.0001 < P < 0.01$) associated with increased (HLA-A*01) and decreased (HLA-A*02, B*15, DRB1*01, DRB1*07:01, DRB1*13:01, DRB1*13:02, DQB1*05:01, DQB1*06:03, DQB1*06:04) disease risk.

Next, we constructed haplotypes to explore how these separate allele associations were carried together. Several predisposing and protective haplotypes were seen (Tables 2 and 3). All haplotypes associated with significantly increased risk for AAD carried either DRB1*03:01 or DRB1*04:04 (together with DQB1*02 and DQB1*03:02), and compound heterozygote carriers of these two haplotypes showed a highly increased risk of developing AAD [OR 32.28; 95% CI (14.36–72.54); $P = 4 \times 10^{-17}$]. There was a significant overdominance of the high-risk compound heterozygotes compared with the homozygotes of either DRB1*03:01-DQB1*02 ($P = 0.0001$) or DRB1*04:04-DQB1*03:02 ($P = 0.015$) [Fisher's exact test

TABLE 2. Haplotypes with diverging frequencies between cases and controls: haplotypes that are significantly associated with increased disease risk and haplotypes not present in controls

HLA haplotype					**Addison's disease**		**Healthy controls**			
A	**B**	**MICA**	**DRB1**	**DQB1**	**Counts**	**%**	**Counts**	**%**	**OR (95% CI)**	***P***
31	40	5.1	04:04	03:02	21	2.5	6	0.4	5.91 (2.37–14.70)	2×10^{-5}
03	08	5.1	03:01	02	22	2.7	8	0.6	4.64 (2.06–10.47)	5×10^{-5}
03	07	5.1	03:01	02	8	1.0	3	0.2	4.44 (1.17–16.78)	0.03
02	40	5.1	04:04	03:02	14	1.7	6	0.4	3.90 (1.49–10.20)	0.003
01	08	5.1	03:01	02	172	20.8	98	7.2	3.40 (2.61–4.43)	5×10^{-21}
02	08	5.1	03:01	02	23	2.8	12	0.9	3.23 (1.60–6.52)	0.001
03	07	5.1	04:04	03:02	9	1.1	5	0.4	3.00 (1.00–8.97)	0.04
26	07	5.1	04:04	03:02	8	1.0			Not present in controls	
02	27	4	04:04	03:02	6	0.7			Not present in controls	
03	07	5.1	04:01	05:03	6	0.7			Not present in controls	
24	40	5.1	04:04	03:02	6	0.7			Not present in controls	

TABLE 3. Haplotypes with diverging frequencies between cases and controls: haplotypes that are significantly associated with decreased disease risk and haplotypes not present in cases

HLA haplotype					Addison's disease		Healthy controls			
A	B	MICA	DRB1	DQB1	Counts	%	Counts	%	OR (95% CI)	*P*
02	40	5.1	13:02	06:04	3	0.4	22	1.6	0.22 (0.07–0.75)	0.008
02	15	5	13:01	06:03	2	0.2	14	1.0	0.23 (0.05–1.03)	0.04
02	44	5.1	15	06:02			14	1.0	Not present in cases	
29	44	6	07:01	02			14	1.0	Not present in cases	
03	35	9	01	05:01			13	1.0	Not present in cases	
02	07	5.1	14:01	05:03			11	0.8	Not present in cases	
24	35	9	01	05:01			10	0.7	Not present in cases	
2501	1801	4	15	06:02			10	0.7	Not present in cases	

was performed as published by Svejgaard and Ryder (15), data not shown].

No haplotype including DRB1*03:01-DQB1*02 or DRB1*04:04-DQB1*03:02 was found protective. Similarly, HLA-B*08 always predisposes to AAD, whereas *HLA-A*, *MICA*, and other *HLA-B* alleles were present on both protective and predisposing haplotypes (*e.g.* A*02, MICA5.1, and B*40). Although MICA5.1 was the only common denominator on the predisposing HLA haplotypes, its presence even on the protective haplotypes suggests that it is not the primary causal risk factor. Furthermore, the risk conferred by homozygosity for MICA5.1 was significantly lower than that for the DRB1*03:01-DQB1*02/DRB1*04:04-DQB1*03:02 genotype [OR 2.66; 95% CI (2.06–3.42) *vs.* OR 32.28; 95% CI (14.36–72.54)]. Taken together, the association tests, including conditional logistic regression and haplotype analyses, indicate *DRB1* as the primary risk locus in AAD.

Multiple risk variants within the MHC

After having established *HLA-DRB1* as the main risk locus in this data set, we wanted to explore whether particular *HLA-B* and/or *MICA* alleles confer additional risk based on the residual associations seen after conditioning on *DRB1* (and *DRB1-DQB1*; Table 1). The associations seen for these two loci appear to be dependent on each other because conditioning on either of these loci together with *DRB1* eliminates the association seen at the other locus ($P = 0.17$ for *HLA-B* and $P = 0.74$ for *MICA*). This analysis will suffer from low power to differentiate these two loci in LD. Notably, if not including *DRB1* in the logistic regression analyses but only testing *MICA* and *HLA-B* against each other, *MICA* showed a weak association after conditioning on *HLA-B* ($P = 0.01$), whereas *HLA-B* was strongly associated after conditioning on *MICA* ($P = 3.3 \times 10^{-12}$). Importantly, the confounding effects from *DRB1* alleles, potentially influencing the two loci differentially, cannot be excluded in such analyses.

Therefore, to look more closely into these associations and their relationship, we conducted haplotype analyses. The most associated alleles at these loci are B*08 and MICA5.1. B*08 is usually carried on DRB1*03:01 haplotypes, and when comparing the distribution of *HLA-B* alleles on DRB1*03:01 haplotypes among cases and controls, an increased B*08 frequency was seen among AAD patients (85.6 *vs.* 79.4%), albeit not reaching statistical significance ($P = 0.07$). Overall, B*08 occurred at a greater frequency in patients than controls after stratification on different *DRB1* alleles (data not shown), and B*08 was found to be associated with AAD in subjects not carrying DRB1*03:01 [OR 1.84; 95% CI (1.22–3.13); $P = 0.007$].

The AAD-associated MICA5.1 is the most common *MICA* allele and is carried on several *DRB1* haplotypes. When comparing the distribution of MICA5.1 on DRB1*03:01 haplotypes, a significantly ($P = 0.01$) increased frequency was seen in patients compared with controls (93.3 *vs.* 86.2%). Likewise, MICA5.1 was associated among AAD patients not carrying DRB1*03:01 [OR 1.56; 95% CI (1.27–1.92); $P = 0.0003$]. Also, MICA5.1 occurred at an increased frequency on most other *DRB1* haplotypes, with one important exception, namely the DRB1*04:04, in which it was seen at equal frequency on DRB1*04:04 haplotypes among patients compared with DRB1*04:04 haplotypes among controls (63.2 *vs.* 63.5%).

Because both HLA-B*08 and MICA5.1 are in strong LD and both showed association after controlling for *DRB1*, we further wanted to explore whether we could tease apart the independent effects of these associations. On DRB1*03:01 haplotypes, HLA-B*08 was always carried together with the MICA5.1 allele, whereas MICA5.1 was carried together with several *HLA-B* alleles [although most commonly (>90%) with HLA-B*08]. MICA5.1 appears to influence the risk of AAD on the DRB1*03:01 haplotypes also in the absence of HLA-B*08 [OR 2.36; 95% CI (1.01–5.53); $P = 0.047$], whereas the opposite test is impossible to perform due to complete LD.

Classification of HLA-B alleles as ligands for KIR

Molecules encoded by the *HLA-B* alleles also serve as ligands for natural killer cell receptors and not only as

antigen presenting molecules for T cells. Hence, one could envisage that the *HLA-B* association was functionally connected to whether the allele encoded ligands for KIR or not (*i.e.* Bw4 *vs.* Bw6 classification of *HLA-B* alleles). To test this, the *HLA-B* alleles were grouped into Bw4 and Bw6 (including among others B*08) according to KIR binding motifs, defined by amino acids 77–83 (16). The Bw6 group was seen at a higher frequency in cases compared with controls (79.9 *vs.* 68.0%, OR 1.56, $P = 7 \times 10^{-6}$); however, this association vanished after conditioning on *DRB1* ($P = 0.35$). Hence, classification of the *HLA-B* alleles into KIR ligands revealed an association inferior to the associations seen for the classical *HLA-B* alleles (Table 1 and Supplemental Table 1).

Discussion

This is the most comprehensive study of HLA complex variations, including both HLA class I and class II genes, conducted in AAD to date. Our results imply that multiple risk factors for AAD are located in the MHC and particularly point toward *DRB1* being a main locus directly involved in disease susceptibility. Additional risk is provided by the MHC class I region, notably by HLA-B*08 and MICA5.1. Strong LD exists between MICA5.1 and B*08 on the DRB1*03 haplotype, which makes it difficult to decipher these associations. The observation that MICA5.1 was not AAD associated on the DRB1*04:04 haplotype suggested that it is not a causal variant. In contrast, MICA5.1, unlike B*08, was associated also in the absence of the other allele. However, B*08 is rarely present without MICA5.1, making it impossible to test the association of B*08 in the absence of MICA5.1. Taken together, it is impossible to evaluate the relative contributions of these alleles and also to distinguish them from other potential risk factors within the same haplotype.

A limitation with our study design is that we have not performed a single-nucleotide polymorphism (SNP) screen across the entire MHC. Interestingly, dense SNP mapping studies of the MHC in type 1 diabetes have concluded that when the *HLA-DRB1-DQB1* effects were accounted for, additional associations was mainly attributed to *HLA-B*, and possibly *HLA-A*, rather than to SNP in other genes in the complex (4, 17, 18). However, it should be noted that alleles of both the class I and class II genes actually consist of multiple polymorphisms, thereby constituting a haplotype, which may detect a risk variant with more statistical power than a single SNP.

Alternatively, the scenario could be that none of the classical HLA loci genotyped in this study are causal but just markers for one single, yet-unidentified risk locus. Again, this interpretation appears unlikely, given findings in other autoimmune diseases, in which in general at least one classical HLA antigen-presenting locus has been found to be primarily involved (19). A recent study of the influence of the autoimmune risk haplotype A1-B8-DR3 on AAD concluded that AAD is not only simply associated with DRB1*03 but also with a highly conserved haplotype stretching from *DRB1* to *B* but not beyond into *HLA-A* (20). This observation is in line with our findings of multiple risk loci marked by DRB1*03:01 and HLA-B*08/MICA5.1. However, we could not confirm their finding of haplotype B8-DR3 to be more frequent in patients with a family history compared with the nonfamilial patient cohort. Although our sample set included few patients with a family history of Addison's disease, we found 11 B8-DRB1*03:01-DQB1*02 haplotypes of 44 possible (24%) in the patients with familial Addison's, compared with a frequency of 29% in the rest of the patient cohort.

The association with *DRB1* was particularly strong when DRB1*03:01 and DRB1*04:04 occurred in a heterozygous combination. Associations between these alleles and AAD has also previously been found (3, 7, 21), except for one study of Italian AAD patients (n = 166) in which no association was observed for DRB1*04:04 (22). On the other hand, these authors found a protective effect of DRB1*04:03 (22), but this allele is rare in our population (<2%). Furthermore, Triolo *et al.* (23) have reported that homozygosity for MICA5.1 (an allele carried together with both DRB1*03:01 and DRB1*04:04) defines 21-hydroxylase antibody-positive individuals at the highest risk of developing AAD, higher than the DRB1*03:01/*04:04 genotype. In our data set, DRB1*03:01/*04:04 is a far stronger predisposing genotype than MICA5.1 homozygosity.

Ultimately, the genetic architecture of HLA association in AAD may turn out similar to type 1 diabetes, in which both *HLA-DRB1* and *HLA-B*, and other genetic variants, possibly not at the classical HLA loci, all participate in the disease predisposition. To further differentiate the effect of loci in the MHC and pinpoint variants directly involved in AAD, dense variation mapping in larger cohorts, preferentially from different populations with potentially diverging recombination pattern, need to be investigated.

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Address all correspondence and requests for reprints to: Beate Skinningsrud, Department of Medical Genetics, Oslo University

Hospital, Ullevål Hospital, Pb 4956 Nydalen, N-0424 Oslo, Norway. E-mail: beate.skinningsrud@medisin.uio.no.

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