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Opposing Effects of HLA–DRB1*13 Alleles on the Risk of Developing Anti–Citrullinated Protein Antibody–Positive and Anti–Citrullinated Protein Antibody–Negative Rheumatoid Arthritis

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Objective. **The effect of non–shared epitope HLA–DRB1 alleles on rheumatoid arthritis (RA) is poorly understood. This study was undertaken to investigate the effects of several HLA–DRB1 alleles, independent of the shared epitope, on the risk of developing anti–citrullinated protein antibody (ACPA)–positive or ACPA-negative RA in a large case–control study.**

Methods. **HLA typing for the DRB1 gene was performed in 1,352 patients with RA and 922 controls from the Swedish Epidemiological Investigation of Rheumatoid Arthritis study. Relative risks (RRs) and 95% confidence intervals (95% CIs) were calculated.**

Results. **DRB1*13 was found to protect against ACPA-positive RA when stratifying for the shared epitope and using a dominant genetic model (RR 0.41 [95% CI 0.26–0.64]). Furthermore, DRB1*13 neutralized the effect of the shared epitope in ACPA-positive RA (RR 3.91 [95% CI 3.04–5.02] in patients who had the shared epitope but not DRB1*13, and RR 1.22 [95% CI 0.81–1.83] in patients with both the shared epitope and DRB1*13, as compared with patients negative for both the shared epitope and DRB1*13). However, we did not replicate the previous published risk of ACPA-negative RA conferred by DRB1*03 when a dominant genetic model was used (RR 1.29 [95% CI 0.91–1.82]). Similarly, no significant effect of DRB1*03 on RR for ACPA-negative RA was seen using the recessive genetic model (RR 1.18 [95% CI 0.6–2.4]). In contrast, the combination of DRB1*03 and DRB1*13 was significantly associated with increased risk of developing ACPA-negative RA (RR 2.07 [95% CI 1.17–3.67]).**

Conclusion. **Our findings indicate that the DRB1*13 allele plays a dual role in the development of RA, by protecting against ACPA-positive RA but, in combination with DRB1*03, increasing the risk of ACPA-negative RA.**

Rheumatoid arthritis (RA) is characterized by chronic inflammation of synovial joints, resulting in progressive destruction of cartilage and bone. Both genes and environment contribute to development of this chronic disease; therefore, RA is referred to as a complex disease (1). The major genetic risk factors for RA are associated with loci in the HLA class II region. Since the function of HLA class II molecules is presentation of antigenic peptides to T helper cells, allelic variations probably influence the antibody repertoire, including anti–citrullinated protein antibodies (ACPAs), in RA. The HLA–DR β-chain molecule seems to be the most important contributor to the development of the disease; in particular, certain subsets of DRB1*01, *04, and *10 represent the so-called "shared epitope" alleles (2). Specific alleles of this type encode a conserved amino acid sequence (QKRAA, QRRAA, or RRRAA) at positions 70–74 in the third hypervariable region (HVR3) of the β-chain (corresponding to exon 2 of the

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DRB1 gene) (2). Shared epitope alleles have dominated the research on HLA–DRB1 since the discovery of the importance of these alleles as risk factors for RA development, and relatively little is known about non–shared epitope HLA–DRB1 alleles. Previous studies have demonstrated that some non–shared epitope HLA–DRB1 alleles, such as *0103, *03, *0402, *07, *08, *11 (except *1107), *12, and *13, are associated with protective effects (3–8).

ACPAs are present in 50–70% of RA patients and are rare in healthy individuals (found in <2%) (9). Furthermore, these antibodies have been demonstrated to precede development of RA by several years (10), and anti-citrulline immunity has subsequently been hypothesized to be causatively involved in the development of RA (11). Based on this and the evidence that major genetic as well as environmental risk factors (11–13) differ between ACPA-positive RA and ACPA-negative RA, these 2 subsets of RA are increasingly viewed as 2 different conditions in an etiopathogenetic context (14). More specifically, the risks conferred by DRB1 shared epitope alleles and PTPN22 R620W are specific for ACPA-positive RA (15,16), while the risks conferred by DRB1*03 and by allelic forms of DCIR and IRF5 are specific for ACPA-negative RA (7,17–19). There is thus a need to reanalyze the effects of alleles of DRB1 in ACPA-positive RA and ACPA-negative RA as separate entities. However, even if it is evident that such discrimination by ACPA status is important, further subtyping of different ACPA specificities might be essential, and every new antibody specificity has to be tested in relation to shared epitope alleles.

In this study, we used DNA acquired from a large case–control population-based study in which cases were dichotomized according to ACPA status, and care was taken to obtain reasonable statistical power to analyze both of these RA subsets. Our analysis focused on the impact of HLA–DRB1 genetic variations in development of the 2 subsets of RA, with major emphasis on non–shared epitope HLA–DRB1 alleles.

PATIENTS AND METHODS

Patients. The source of data for our investigation was a population-based case–control study called the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) (11,20), from which 1,352 cases and 922 controls were included in the present study. Of the 1,352 cases, 820 (60.7%) were ACPA positive and 532 (39.3%) were ACPA negative. The details of the EIRA study have been described previously (20). Briefly, a case was defined as a person in the study base who received a new diagnosis of RA from a rheumatologist (within 1 year after the onset of symptoms in 85% of the cases) and fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 criteria for the classification of RA (21). Cases were recruited from all public and a majority of private rheumatology units in the study area. For each potential case, a control subject was randomly selected from the Swedish national population registry, taking into consideration the subject's age, sex, and residential area. The ethics committee of Karolinska Institutet approved the study. The age, sex, and ACPA status of the subjects are presented in Table 1.

Table 1. Baseline characteristics of the controls and RA cases

	Controls (n = 922)	Cases (n = 1,352)
Age, mean ± SD years*	52.1 ± 11.8	50.9 ± 12.5
Sex, no. (%) female	670 (72.7)	968 (71.6)
No. (%) ACPA-positive	NA†	820 (60.7)

* Age at the beginning of the study for controls; age at onset of rheumatoid arthritis (RA) for cases.
† Anti–citrullinated protein antibody (ACPA)–positive controls (n = 18) were excluded from the study. NA = not applicable.

HLA typing was performed using sequence-specific primer–polymerase chain reaction (PCR) (DR low-resolution kit; Olerup SSP, Saltsjöbaden, Sweden), and the PCR products were loaded onto 2% agarose gels for electrophoresis. An interpretation table was used to determine the specific genotype according to the recommendations of the manufacturer (22). The HLA–DRB1 allelic groups studied were DRB1*01, DRB1*03, DRB1*04, DRB1*07, DRB1*08, DRB1*09, DRB1*10, DRB1*11, DRB1*12, DRB1*13, DRB1*14, and DRB1*15.

Detection of antibodies to citrulline-containing peptides was performed using the Immunoscan RA (Mark 2) enzyme-linked immunosorbent assay (Euro-Diagnostica, Malmö, Sweden). A level of >25 units/ml was regarded as being positive according to instructions in the kit and as confirmed by the Clinical Immunology Laboratory at Uppsala University Hospital (Uppsala, Sweden).

Statistical analysis. The association between distinct DRB1 alleles and the risk of developing ACPA-positive or ACPA-negative RA was estimated by calculating the odds ratio (OR) and the 95% confidence interval (95% CI) by means of logistic regression. ORs were interpreted as relative risks (RRs) due to the population-based design of our study. A separate analysis was conducted for each of 12 different allelic groups, in which each individual was classified as having or not having the specific allele, regardless of the second allele (dominant model). Potential confounding from the HLA–DRB1 shared epitope was accounted for by adjusting the calculated ORs for shared epitope (no or any). All 12 allelic groups were analyzed, but for some groups the number of observations was too small to allow a meaningful analysis (DRB1*09, DRB1*11, DRB1*12, DRB1*14, and DRB1*16).

When number permitted (DRB1*03,*13, and *15), we also computed ORs for homozygosity for each allelic group for the risk of developing ACPA-positive RA or ACPA-negative RA in comparison with subjects without the alleles in question.

Table 2. Relative risk of developing ACPA-positive or ACPA-negative RA conferred by different DRB1 alleles

DRB1 allele	No. of cases (n = 1,352)	No. of controls (n = 922)*	Frequency of genotype in ACPA subgroup, %†	RR (95% CI)‡
DRB1*01				
ACPA+	217	–	26	1.49 (1.19–1.88)
ACPA−	124	185	23	1.21 (0.93–1.58)
DRB1*03				
ACPA+	123	–	15	0.56 (0.44–0.72)
ACPA−	154	221	29	1.25 (0.98–1.60)
DRB1*04				
ACPA+	566	–	69	4.26 (3.47–5.23)
ACPA−	194	324	36	1.06 (0.84–1.33)
DRB1*07				
ACPA+	73	–	9	0.51 (0.38–0.70)
ACPA−	57	149	11	0.62 (0.44–0.86)
DRB1*08				
ACPA+	44	–	5	0.55 (0.38–0.81)
ACPA−	63	83	12	1.41 (0.99–2.00)
DRB1*09				
ACPA+	23	–	3	0.85 (0.48–1.48)
ACPA−	16	30	3	0.96 (0.52–1.81)
DRB1*10				
ACPA+	28	–	3	1.47 (0.81–2.64)
ACPA−	11	20	2	0.93 (0.43–1.99)
DRB1*11				
ACPA+	69	–	8	0.78 (0.56–1.10)
ACPA−	54	96	10	1.02 (0.71–1.46)
DRB1*12				
ACPA+	23	–	3	0.64 (0.38–1.10)
ACPA−	24	40	5	0.99 (0.58–1.68)
DRB1*13				
ACPA+	79	–	10	0.28 (0.21–0.37)
ACPA−	139	254	26	0.94 (0.74–1.21)
DRB1*14				
ACPA+	17	–	2	0.47 (0.26–0.85)
ACPA−	27	38	5	1.32 (0.79–2.20)
DRB1*15				
ACPA+	182	–	22	0.70 (0.56–0.87)
ACPA−	123	269	23	0.73 (0.56–0.93)

* Anti–citrullinated protein antibody (ACPA)–positive controls were excluded from the study.
† The frequency of genotypes in ACPA subgroups was calculated as the number of ACPA-positive or ACPA-negative rheumatoid arthritis (RA) cases for a specific DRB1 allele divided by the total number of cases within that ACPA subgroup (820 ACPA-positive cases; 532 ACPA-negative cases).
‡ The relative risk (RR) (95% confidence interval [95% CI]) was adjusted for age, sex, and residential area. Individuals without the allele being investigated were used as the reference group.

Finally, we tested for confounding specifically for DRB1*03 influence on ACPA-negative RA, from a second non–shared epitope allele. This analysis was performed only for DRB1*07, DRB1*08, DRB1*13, and DRB1*15, due to small numbers. All analyses were performed using the software packages StatView, version 5.0.1.0 and SAS, version 9.1.3.

RESULTS

Associations between specific HLA–DRB1 alleles and risk of developing ACPA-positive RA or ACPA-negative RA. All 3 shared epitope alleles (i.e., DRB1*01, *04, and *10) appeared to contribute to an elevated risk of ACPA-positive RA, although this increase was statistically significant only for DRB1*01 and *04 (Table 2). Conversely, DRB1*03, *07, *08, *13, *14, and *15 were significantly associated with protection against ACPA-positive RA. No allelic group was significantly associated with an increased risk of developing ACPA-negative disease. Instead, it was evident that DRB1*07 and *15 were associated with a significant protective effect. Notably, all of these findings reflected the effects of various DRB1 alleles in a heterozygous state, without correction for the second allele. Since this second allele (in particular if it is a shared epitope allele) might influence the effects, we expanded our analyses to investigate the combined effects of DRB1 alleles in heterozygous individuals. In this further analysis, we

Table 3. Relative risk of developing ACPA-negative RA conferred by DRB1*03 and of developing ACPA-positive RA conferred by DRB1*13, both with and without the shared epitope*

DRB1 allele	No. of cases (n = 1,352)	No. of controls (n = 922)	RR (95% CI)†
ACPA−			
No SE, DRB1*03	92	154	1.29 (0.91–1.82)
SE, no DRB1*03	234	412	1.26 (0.96–1.65)
SE, DRB1*03	55	66	1.48 (0.98–2.22)
ACPA+			
No SE, DRB1*13	27	155	0.41 (0.26–0.64)
SE, no DRB1*13	619	380	3.91 (3.04–5.02)
SE, DRB1*13	50	98	1.22 (0.81–1.83)

* ACPA = anti–citrullinated protein antibody; RA = rheumatoid arthritis; SE = shared epitope.
† Relative risk (RR) adjusted for age, sex, and residential area, and 95% confidence interval (95% CI) compared with subjects who had neither the shared epitope nor DRB1*03 or with subjects who had neither the shared epitope nor DRB1*13.

decided to exclude *09, *11, and *12 as categories for stratification due to the low numbers of observations of these alleles in our study population.

Risk conferred by different HLA–DRB1 alleles in relation to the presence or absence of shared epitope alleles. To assess the influence of shared epitope alleles on the associations found for non–shared epitope alleles, we used individuals who had neither the shared epitope nor the studied allele as a reference group. With regard to ACPA-positive disease, we observed that only DRB1*13 (of *03, *07, *08, *13, *14, and *15) was independent of the shared epitope in protecting against disease development (RR 0.41 [95% CI 0.26–0.64]) (Table 3). Notably, the protective effects of DRB1*07 and *15 for ACPA-negative disease were no longer observed after correction for shared epitope alleles (data not shown). Furthermore, we assessed the influence of DRB1*13 in combination with the shared epitope and, interestingly, observed that DRB1*13 completely "neutralized" the increased risk of developing ACPA-positive RA conferred by the shared epitope (RR 3.91 [95% CI 3.04–5.02] in subjects who had the shared epitope but not DRB1*13; RR 1.22 [95% CI 0.81–1.83] in subjects who had both the shared epitope and DRB1*13) (Table 3). For ACPA-negative RA we found that DRB1*03, in the absence of shared epitope alleles, did not contribute significantly to the risk of developing ACPA-negative disease (RR 1.29 [95% CI 0.91–1.82]) (Table 3).

Table 4. Relative risk of developing ACPA-negative RA conferred by different combinations of DRB1*03 and DRB1*13*

DRB1 allele	No. of cases (n = 1,352)	No. of controls (n = 922)	RR (95% CI)†
DRB1*03, No DRB1*13	77	127	1.18 (0.80–1.73)
DRB1*13, No DRB1*03	67	131	0.99 (0.67–1.47)
DRB1*03, DRB1*13	31	29	2.07 (1.17–3.67)

* Subjects with the shared epitope were excluded from the analysis. ACPA = anti–citrullinated protein antibody; RA = rheumatoid arthritis.
† Relative risk (RR) and 95% confidence interval (95% CI) compared with subjects who had neither DRB1*03 nor DRB1*13. The RR was not adjusted for age, sex, or residential area.

Risk of ACPA-negative RA conferred by the combination of DRB1*03 and DRB1*13. In addition to analyzing individuals who were heterozygous for various DRB1 alleles, we investigated the effects of DRB1 alleles in homozygous individuals. We observed that *03 homozygosity did not contribute significantly to ACPA-negative disease (RR 1.18 [95% CI 0.6–2.4] in 14 cases and 21 controls, using subjects without DRB1*03 as the reference group). To test whether the observed effect was due to confounding, the shared epitope was excluded from the reference group. Still, no significant risk from DRB1*03 was observed (RR 1.51 [95% CI 0.72–3.15] in 14 cases and 21 controls, using subjects without the shared epitope or DRB1*03 as the reference group). Furthermore, analysis of the interactions between *03 and other DRB1 alleles in ACPA-negative disease demonstrated that only the combination of *03 and *13 was significantly associated with risk of developing this subset of RA (RR 2.07 [95% CI 1.17–3.67]) (Table 4). The combination of DRB1*03 and *13 did not confer any risk of ACPA-positive RA among shared epitope–negative subjects (data not shown).

DISCUSSION

The major finding of this study is that the DRB1*13 allele plays a dual role in RA development. DRB1*13 appears to protect against ACPA-positive RA, whereas the same allele in combination with DRB1*03 is associated with an increased risk of ACPA-negative RA. These findings add to previous data on the genetic divergence between ACPA-positive and ACPA-negative RA, in this case in relation to risk from the DRB1*13 allele. Related to this basic finding, our data indicate that the previously demonstrated risk of ACPA-negative RA conferred by DRB1*03 is dependent on the simultaneous presence of the DRB1*13 allele in DRB1-heterozygous individuals.

The main goal of this study was to investigate the impact of different DRB1 alleles on the risk of 2

different variants of RA. The relatively large size of our study population (820 ACPA-positive RA cases, 532 ACPA-negative RA cases, and 922 controls) enabled us to both perform some new analysis on interactions between DRB1 alleles and reinvestigate associations that have previously been described in smaller patient populations (in which in most cases no division was made between ACPA-positive RA and ACPA-negative RA). In the initial analysis of ACPA-positive RA, we confirmed that the shared epitope alleles DRB1*01 and *04 are risk factors and that *13 is a major protector against disease development, as was previously demonstrated (11,23). However, our findings did not confirm the results of some previous studies of the effects of other non–shared epitope alleles, such as DRB1*03 (7,18). Besides differences in sample size between previous studies and the present study, the different results may be explained by the fact that the EIRA study is population based and may have fewer selection biases compared with previous studies.

Two previous reports have described an association between the DRB1*03 allele and development of ACPA-negative RA (7,17). In the first of those studies, findings in 171 ACPA-negative RA patients and 423 healthy controls were analyzed (7); the power of that study to identify an existing risk was ~85% (RR 1.92 [95% CI 1.25–2.92]). The present study had 99.9% power to identify an effect of DRB1*03 of this size on the risk of ACPA-negative RA, and 85% power to detect a significant effect with an OR of 1.5. Since the data in the study by Verpoort et al (7) were not corrected for possible shared epitope and DRB1*13 confounders, they may reflect a combined effect of DRB1*03 and DRB1*13. In the second of the published studies (17), 532 ACPA-negative patients were compared with 1,191 ACPA-positive patients (but not with healthy controls), yielding a difference between these 2 groups that corresponded to an RR of 1.6 (95% CI 1.2–2.1). We carried out the same analysis in the present study and confirmed a difference between ACPA-positive and ACPA-negative patients corresponding to an RR of 1.93 (95% CI 1.49–2.51), but, as described in Results, we did not observe any significant influence of DRB1*03 alone on the risk of ACPA-negative RA when we compared this patient group with healthy controls and corrected for the presence of shared epitope alleles. Instead, we found that a combination of DRB1*13 and DRB1*03 conferred an increased risk of ACPA-negative disease. In summary, our data indicated that DRB1*03 alone is unlikely to be a risk factor for ACPA-negative RA, at least in a Swedish population.

Since there are so many combinations of DRB1 allelic groups, in general, it is a difficult task to totally exclude the chance of associations with low risk. Our study provides relatively high statistical power for common, but not rare, DRB1 allelic groups in Caucasian populations. For example, after correction for shared epitope alleles, we observed 78% power to find an independent risk of RA for DRB1*15 with an OR of >1.5 in our study population.

With regard to the protective effect of DRB1*13 against ACPA-positive RA, we so far know very little about the possible mechanisms of such protection. Interestingly, DRB1*13 is also associated with protection against several viral diseases (24–28), suggesting that this allele may have a special capacity to affect the activity of some immune responses. Discovering the mechanisms of protection in RA and possibly also in viral infection is thus of considerable general interest.

Some previous studies have demonstrated a protective effect of the DRB1*13 allele on RA, but without dividing RA into ACPA-positive and ACPA-negative subsets (6,8). Clinical cohorts of RA patients are usually made up primarily of ACPA-positive patients, and this may be a reason the protective effect was originally described. The present study significantly refines this finding by demonstrating that DRB1*13 has a protective effect in patients with ACPA-positive RA only, while in combination with DRB1*03 it is a susceptibility allele for ACPA-negative RA.

By using step-by-step statistical analysis of HLA–DRB1 genotypes, we confirmed the importance of the DRB1*13 allelic group as a protective factor against ACPA-positive RA. This finding could be interpreted as evidence supporting the hypothesis of a second shared epitope allelic group, suggested previously as the "DERAA hypothesis" (5). Indeed, DRB1*13 (more specifically, *1301, *1302, and *1304), along with *0103, *0402, *1102, and *1103, carries the amino acids DE at positions 70–71, and it has previously been shown that this group is associated with protection against RA (23). However, due to a lack of data regarding 4-digit DRB1 genotypes in this study, we could not accurately test this hypothesis. Nevertheless, the frequency of non-DRB1*13 alleles (DRB1*0103, DRB1*0402, and DRB1*11) in the DERAA group in our study population was only 30%, and it is likely that DRB1*13 plays a major role in protection. A biologic explanation for the protective effect of DRB1*13 and DERAA in RA is needed but, so far, we have very little knowledge of what such a mechanism might be. Furthermore, we cannot rule out the possibility that HLA–DRB1 gene involve-

ment in gene–environment interaction is not indigenous but rather depends on another genetic factor in linkage disequilibrium with this locus, such as variations in HLA–DQ (29,30).

Due to the complexity of the allelic repertoire of the DRB1 gene, it is preferable for statistical evaluation to use groups of alleles instead of individual alleles. We chose to use DRB1*04 genotyping in this study without discrimination of alleles to facilitate such analysis. According to our estimate, the number of individuals carrying the non–shared epitope alleles of DRB1*04 was relatively low and did not affect the outcome considerably. Of 922 controls, 21 individuals had non–shared epitope alleles, and of 1,352 cases, 10 individuals had non–shared epitope alleles. We are also aware that discrimination by ACPA status in relation to HLA might be an oversimplification. In the near future, subtyping according to different autoantibody specificities may be an even more powerful criterion for dividing RA into clinically and physiologically meaningful subgroups. There is already a list of autoantigens awaiting such an approach, including filaggrin, fibrin, vimentin, and type II collagen.

HLA–DRB1 is a well-known polymorphic locus in the human genome, with >250 known alleles. It is a challenge to perform statistical evaluation with numerous genotypes, and the shared epitope hypothesis is a good background for grouping these alleles and/or genotypes into a reasonable number of categories for analysis. In the current study we analyzed the importance of all different allelic groups within HLA–DRB1, and, albeit with some loss of power due to stratifications, it was possible to identify specific effects for each group. However, due to multiple comparisons, these results must be interpreted with caution, and future replication in independent cohorts is needed.

In conclusion, our findings indicate that DRB1 alleles distinct from shared epitope–related alleles may have important roles in influencing susceptibility to and protection against RA. In light of the multitude of new data from major histocompatibility complex (MHC)–wide dense single-nucleotide polymorphism analysis that are now emerging for the MHC locus in relation to RA and other complex diseases, our findings emphasize the complexity of the relationships between the MHC and susceptibility to inflammatory diseases, and indicate the need for studies of distinct subsets of these diseases, as well as of interactions between different alleles of the same MHC genes.

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AUTHOR CONTRIBUTIONS

Dr. Padyukov had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Alfredsson, Klareskog, Padyukov.

Acquisition of data. Lundström, Källberg, Smolnikova, Rönnelid, Alfredsson, Padyukov.

Analysis and interpretation of data. Lundström, Källberg, Smolnikova, Ding, Alfredsson, Klareskog, Padyukov.

Manuscript preparation. Lundström, Källberg, Ding, Rönnelid, Alfredsson, Klareskog, Padyukov.

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