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An increased risk of Crohn's disease in individuals who inherit the HLA class II *DRB3*0301* allele

(inflammatory bowel disease/major histocompatibility complex/disease susceptibility/HLA class II genotyping)

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ABSTRACT **The role of inflammatory T cells in Crohn's disease suggests that inherited variations in major histocompatibility complex (MHC) class II genes may be of pathogenetic importance in inflammatory bowel disease. The absence of consistent and strong associations with MHC class II genes in Caucasian patients with inflammatory bowel disease probably reflects the use of less precise typing approaches and the failure to type certain loci by any means. A PCR-sequence-specific oligonucleotide-based approach was used to type individual alleles of the HLA class II *DRB1, DRB3, DRB4*, and *DRB5* loci in 40 patients with ulcerative colitis, 42 Crohn's disease patients, and 93 ethnically matched healthy controls. Detailed molecular typing of the above alleles has previously not been reported in patients with inflammatory bowel disease. A highly significant positive association with the *HLA-DRB3*0301* allele was observed in patients with Crohn's disease ($P = 0.0004$) but not in patients with ulcerative colitis. The relative risk for this association was 7.04. Other less significant HLA class II associations were also noted in patients with Crohn's disease. One of these associations involved the *HLA-DRB1*1302* allele, which is known to be in linkage disequilibrium with *HLA-DRB3*0301*. These data suggest that a single allele of an infrequently typed *HLA* class II locus is strongly associated with Crohn's disease and that MHC class II molecules may be important in its pathogenesis.**

The pathogenesis of both forms of inflammatory bowel disease (IBD) is poorly understood. Ulcerative colitis (UC) is largely a mucosal disease, while in Crohn's disease (CD) the entire thickness of the bowel is affected, and granulomas are frequently a feature. IBD is frequently observed in families, and both UC and CD may be observed in different members of a given kindred. Immune mechanisms play an important role in IBD (1), and it is assumed that a combination of genetic, particularly immunogenetic, and environmental factors contributes to the development of these two disease states (2).

Many previous studies have explored the association between the class I and class II loci of the major histocompatibility complex (MHC) and IBD. Those dealing with MHC class I alleles have exclusively used serological methods for analysis, and while associations have on occasion been reported, no consistent pattern has emerged that could provide evidence for susceptibility (refs. 3–5 and selected references therein). While the rationale for postulating a direct role for antigen presentation by MHC class I molecules in IBD may be open to question, considerable evidence exists to suggest that the presentation of yet-to-be-defined peptides by MHC class II molecules plays a role in the pathogenesis of at least one form of IBD, namely Crohn's disease. In CD, activated helper T cells are a prominent feature, and the inflammatory lesions resemble those seen in other disorders in which inflammatory $CD4^+$ T cells play a prominent role (6–9). Genetic variation in MHC class II genes (10) would therefore be expected to represent an important component of genetic susceptibility to IBD. However, consistent and strong MHC class II associations in Caucasian IBD patients remain to be determined.

There are several reasons for the equivocal nature of the published information on MHC class II alleles in patients with IBD. Until quite recently, the limited resolving power of serotyping imposed significant constraints on the study of MHC class II associations. Indeed, as a recent study has emphasized, serological methods are particularly inappropriate for certain MHC class II alleles (11), including alleles linked to the DR2 serological specificity. The failure to identify linkage to the class II locus may be attributed to the virtual lack of systematic molecular genotyping studies in IBD using state-of-the-art approaches. In addition to the necessity for accurate and precise genotyping techniques, specific assignment of alleles also requires that all known alleles at a particular locus be typed. Unfortunately, few studies have been performed in IBD using systematic molecular typing approaches and, indeed, there has not been any report in which all of the known alleles of the *DRB1, DRB3, DRB4*, and *DRB5* loci were examined.

There are a few studies involving IBD patients in which a limited number of MHC class II alleles have been examined using molecular genotyping approaches. In Japanese UC patients, linkage to *DR2* has been shown to involve the *DRB1*1502* allele (12); this allele is relatively infrequent in Caucasians. In one of the earliest North American IBD studies in which limited molecular typing was used (in conjunction with serotyping), the approach involved largely a PCR and restriction-fragment-length-polymorphism-based typing method. This method is not as precise as the preferred sequence-specific oligonucleotide (SSO)-based approach. In that study (13), an association between the DR2 group and UC patients was reported but, when individual alleles were examined using SSO probes, there was no specific association with any particular *DRB1* gene linked to the *DR2* group. In the same study (13), using serological techniques, a relatively weak association of CD with the DR1/DQw5 haplotype was described.

Sequence-specific oligonucleotide probes have been used in two studies to type a limited number of alleles in Caucasian IBD patients. In one study specifically examining the association of *DR2* with UC, a relatively large number of Caucasian patients were molecularly typed for individual alleles of this group, but there were no positive associations in UC patients (14). In fact, there was a trend toward a negative association

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Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel disease; MHC, major histocompatibility complex; UC, ulcerative colitis; SSO, sequence-specific oligonucleotide

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with one *DR2* allele, *DRB1*1601*. The authors of this report assumed that the use of less accurate methods in previous studies (13) might have led to the apparent association of *DR2* with UC. Differences in the ethnic compositions of the two study groups might also have contributed to this discrepancy. Another genotyping study of class II MHC alleles in IBD (actually a study examining disease progresssion in primary sclerosing cholangitis in which UC patients formed a control group) was performed on Caucasian subjects in England (15). A small number of MHC class II alleles, including *DR2, DR3, DR4, DR12, DRB3*0101*, and *DRB5*0101*, were examined using SSO-based typing, and no associations were observed with IBD. In a recent and detailed study on MHC class II alleles in Japanese patients with CD (16), a positive association with *DQB1*0401* and *DQB1*0402* was observed. A negative association of *DQA1*0102* with CD was observed in this Japanese study.

In the present study, we have used PCR–SSO techniques to molecularly type individual alleles of all *DRB1, DRB3, DRB4*, and *DRB5* loci and to compare the frequency of the known alleles among ethnically matched populations consisting of 40 UC patients, 42 CD patients, and 93 healthy controls. A very strong association between HLA *DRB3*0301* and Crohn's's disease is reported.

METHODS

Patients. The diagnosis of UC or CD was documented in 82 patients seen at the Center for the Study of Inflammatory Bowel Disease at the Massachusetts General Hospital by conventional endoscopic, histological, and clinical criteria. All patients were Caucasian. Information regarding the ethnicity of each patient was obtained.

Ethnically Matched Controls. Of the 93 healthy controls studied, 41 were Caucasian volunteers, and 52 were randomly selected spouses of patients with Huntington disease. These latter controls were all of European extraction. Information regarding ethnicity was available for all controls.

MHC Class II Typing. Genomic DNA was isolated from all subjects. These DNA samples were from either peripheral blood leukocytes or B lymphoblastoid cell lines derived by Epstein–Barr virus transformation. High-molecular-weight genomic DNA was prepared using standard procedures. Genotyping of all DRB alleles was performed using a PCR–SSO approach as described in the protocols of the 11th International HLA Workshop (17). Briefly, PCR amplification of genomic DNAs using generic primers yielded a 274-bp product containing all *DRB1, DRB3, DRB4*, and *DRB5* alleles, depending on a patient's particular haplotype. After dot blotting of 100 ng of these PCR products on Hybond N+ membranes (Amersham), prehybridization was carried out overnight at 54°C in a buffer containing 3 M tetramethylammonium chloride (to facilitate hybridization and washing independent of G+C content of the SSO probe), 50 mM Tris·HCl, pH 8.0/2 mM EDTA, pH 8/5× Denhardt's reagent/0.1% SDS/salmon sperm DNA at 100 μg/ml. Hybridization was done by using [γ-^{32}P]ATP 5′-labeled 18-mer SSOs at 54°C for 2 hr. Washing was done twice in 20 mM sodium phosphate, pH 7.0/0.3 M NaCl/10 mM EDTA/0.1% SDS at room temperature, once at 58.5°C, and then twice at 58.5°C in a buffer containing 3 M tetramethylammonium chloride, 50 mM Tris·HCl, 2 mM EDTA, and 0.1% SDS. The generic PCR products were screened with group-identifying probes to characterize samples as DRB1-DR1, -DR2, -DR3, -DR4, -DR7, -DR8, -DR9, -DR10, -DR11, -DR12, and/or -DR13/DR14. Probes were also used to identify *DRB3, DRB4*, and *DRB5* locus genes.

Separate PCR amplification reactions were performed to type individual alleles of the *DRB1* loci. Four different 5′ primers and the same 3′ primers used in generic DRB typing were used to characterize alleles of the *DRB1-DR1* group (*DRB1*0101–*0103*), the *DRB1-DR2* group (*DRB1*1501–*1503, DRB1*1601-*1602*), the *DRB1-DR4* group (*DRB1*0401–*0412*), and the DRw52-associated group (DR3: DRB1*0301–*0302; DR8: DRB1*0801–*0805; DR11: DRB1*1101–*1104; DR13: DRB1*1301–*1305; DR14: DRB1*1401–*1406).

Statistical Analysis. Two-tailed *P* values were calculated by using two-by-two contingency tables and Fisher's exact test. The relative risk was calculated as an odds ratio using the approximation of Woolf.

RESULTS

Initial group-specific typing of *DRB1* allelic groups failed to reveal any significant differences between patients with either form of IBD and controls (Table 1). At this level of analysis, no significant difference in allele group frequencies was observed between CD patients and controls.

We then examined individual *DRB1* alleles (Table 2) in patients and controls. No positive or negative association with the *DR2* group or with any *DR2*-linked alleles (*DRB1*1501, *1502, *1503, *1601*, and **1602*) was noted in UC patients. A negative association of a single *DRB1-DR4* allele was noted in CD patients (*DRB1*0401*; $P = 0.0179$; relative risk = 0.0767). A positive association of a single *DRB1-DR13*-linked allele

Table 1. MHC class II *DRB1* group alleles in IBD patients and healthy controls

	Allele presence, % (*n*)				
	CD (*n* = 42)	UC (*n* = 40)	Healthy controls (C) (*n* = 93)	*P* values (vs. C) CD	*P* values (vs. C) UC
DRB1 group alleles					
DR1	11.9% (5)	25.0% (10)	19.4% (18)	0.3330	0.4916
DR2	28.6% (12)	30.0% (12)	31.2% (29)	0.8413	1.0000
DR3	14.3% (6)	10.0% (4)	17.2% (16)	0.8036	0.4280
DR4	23.9% (10)	17.5% (7)	33.3% (31)	0.3154	0.0931
DR7	23.9% (10)	20.0% (8)	16.1% (15)	0.3401	0.6212
DR8	7.1% (3)	0.0% (0)	4.3% (4)	0.6768	0.3135
DR9	2.4% (1)	2.5% (1)	1.1% (1)	0.5270	0.5126
DR10	0.0% (0)	0.0% (0)	2.2% (2)	1.0000	1.0000
DR11	31.0% (13)	35.0% (14)	26.9% (25)	0.6812	0.4073
DR12	4.8% (2)	2.5% (1)	6.5% (6)	1.0000	0.6741
DR13	31.0% (13)	27.5% (11)	17.2% (16)	0.1115	0.2391
DR14	4.8% (2)	10.0% (4)	11.8% (11)	0.3439	1.0000

The number of individuals in each group inheriting a given allele is presented as a percentage. Actual number of individuals with a given allele is shown in parentheses.

Table 2. Molecular analysis of *HLA-DRB1* alleles in IBD patients and healthy controls

		Allele presence, % (*n*)				
	DRB1 allele	CD (*n* = 42)	UC (*n* = 40)	Healthy controls (C) (*n* = 93)	*P* CD vs. C	*P* UC vs. C
DR1	**0101*	11.9% (5)	7.5% (3)	9.7% (9)	0.7628	1.0000
	**0102*	0.0% (0)	7.5% (3)	5.4% (5)	0.3240	0.6965
	**0103*	0.0% (0)	10.0% (4)	4.3% (4)	0.3098	0.2411
DR2	**1501*	21.4% (9)	27.5% (11)	25.8% (24)	0.6687	0.8331
	**1502*	4.8% (2)	0.0% (0)	1.1% (1)	0.2283	1.0000
	**1503*	0.0% (0)	0.0% (0)	0.0% (0)		
	**1601*	2.4% (1)	2.5% (1)	4.3% (4)	1.0000	1.0000
	**1602*	0.0% (0)	0.0% (0)	0.0% (0)		
DR4	**0401*	0.0% (0)	7.5% (3)	12.9% (12)	**0.0179**†	0.5516
	**0402*	14.3% (6)	5.0% (2)	6.5% (6)	0.1900	1.0000
	**0403*	0.0% (0)	5.0% (2)	2.2% (2)	1.0000	0.5833
	**0404*	4.8% (2)	0.0% (0)	5.4% (5)	1.0000	0.3217
	**0405*	2.4% (1)	0.0% (0)	0.0% (0)	0.3111	
	**0406*	0.0% (0)	0.0% (0)	0.0% (0)		
	**0407*	0.0% (0)	0.0% (0)	5.4% (5)	0.3240	0.3217
	**0408*	2.4% (1)	0.0% (0)	1.1% (1)	0.5270	1.0000
DR11‡	**1101*	14.3% (6)	20.0% (8)	14.0% (13)	1.0000	0.4391
	**1102*	0.0% (0)	0.0% (0)	1.1% (1)	1.0000	1.0000
	**1103*	9.5% (4)	0.0% (0)	2.2% (2)	0.0750	1.0000
	**1104*	7.1% (3)	17.5% (7)	8.6% (8)	1.0000	0.1466
DR13‡	**1301*	7.1% (3)	15.0% (6)	10.8% (10)	0.7539	0.5633
	**1302*	19.0% (8)	5.0% (2)	5.4% (5)	**0.0234**†	1.0000
	**1303*	2.4% (1)	5.0% (2)	1.1% (1)	0.5270	0.2150
	**1304*	0.0% (0)	0.0% (0)	0.0% (0)		
	**1305*	2.4% (1)	2.5% (1)	0.0% (0)	0.3111	0.3008

*Part of nomenclature for MHC class II genes.
†Denotes statistical significance according to two-tailed Fisher's exact test.
‡In one DR11/DR13 UC patient and in one DR11/DR13 control, *DRB1*1101*, *-*1104*, *-*1301*, and *-*1302* alleles could not be precisely distinguished because of probe cross-reactivities. DQB typing will permit accurate assignment of the above alleles to be made in these two individuals.

(*DRB1*1302*; $P = 0.0234$; relative risk = 4.1412) was noted in CD patients (but not in UC patients) as compared with controls.

Examination of the alleles that make up the *DRB3, DRB4*, and *DRB5* loci (Table 3) revealed a very strong association of the *DRB3*0301* allele with CD ($P = 0.0004$). The relative risk for this association was 7.04. Interestingly, all the CD patients and controls that were *DRB1*1302* positive were also *DRB3*0301* positive. Population studies in Caucasians have shown that *DRB1*1302* is always linked to *DRB3*0301* (18–20). It is clear that the association seen in CD with *DRB1*1302* actually reflects linkage disequilibrium of *DRB1*1302* with *DRB3*0301*.

We reanalyzed all the data in Tables 1–3, segregating patients into non-Jewish and Jewish groups (data not shown). The numbers of Jewish patients and controls are at present too small for meaningful statistical interpretations to be made that are relevant to this group. Most of the results obtained from non-Jewish patients and controls generally mirrored the results described for the total patient and control population.

When *P* values were corrected, taking into account the number of observations made (21), the association of the *DRB3*0301* allele with CD remained very significant. We also reanalyzed our data against 456 North American Caucasian International Workshop Controls (22). The association of *DRB3*0301* with CD remained extremely significant in the context of this control group ($P < 0.0001$; relative risk = 9.20).

In Tables 1–3, the figures in each column represent the percentage of individuals in each group in whom a given allele was present. The actual number of individuals presenting with a specific allele is shown in parentheses. For individuals who were homozygous at a given locus, the allele in question was tabulated only once. We have also separately calculated allele frequencies including both alleles for homozygous individuals ($n = 2 \times$ number of individuals in the group). With this

Table 3. Molecular analysis of *HLA-DRB3, -DRB4*, and *-DRB5* alleles among IBD patients and healthy controls

	Allele presence, % (*n*)					
	HLA-DRB3			*HLA-DRB4*	*HLA-DRB5*	
	*-DRB3*0101*	*-DRB3*0201/02*	*DRB3*0301*	*DRB4*0101*	*DRB5*0101*	*DRB5 0102/02*
CD (*n* = 42)	11.9% (5)	45.2% (19)	28.6% (12)	40.5% (17)	21.4% (9)	2.4% (1)
UC (*n* = 40)	22.5% (9)	52.8% (21)	7.5% (3)	37.5% (15)	22.8% (9)	2.5% (1)
Healthy controls (*n* = 93) (C)	21.5% (20)	45.2% (42)	5.4% (5)	48.3% (45)	25.8% (24)	5.4% (5)
P values						
CD vs. C	0.2349	1.0000	**0.0004**†	0.4574	0.6687	0.6653
UC vs. C	1.0000	0.4553	0.6965	0.2616	0.8275	0.6679

*Part of nomenclature for MHC class II genes.
†Denotes statistical significance according to two-tailed Fisher's exact test.

approach, the association of *HLA-DRB3*0301* with CD (as compared to the healthy control group included in all the tables) also remained extremely significant ($P = 0.0002$).

Attempts were made to correlate inheritance of the *DRB3*0301* allele with age of onset, a family history of IBD, small bowel versus large bowel CD, stricturing versus fistulating disease, and the presence or absence of granulomas. None of these features of CD segregated in a significant manner with the inheritance of the *DRB3*0301* allele.

DISCUSSION

These studies were undertaken with the expectation that susceptibility to IBD, particularly to CD, would be linked to specific MHC class II genes. The rationale for assuming an association of CD with the class II locus derives from studies showing that activated $CD4^+$ helper T cells play a role in the disease process (6–9). It remains unclear whether the T cells of interest are directed against a self-peptide or peptides or whether some unidentified pathogen plays a role in the process.

Association with MHC class II genes may reflect linkage disequilibrium to other MHC or non-MHC genes or may reflect a role for MHC class II molecules in disease pathogenesis. Positive MHC class II associations that are directly relevant to inflammatory diseases can generally be explained in one of two ways. The class II molecule of interest may present a specific peptide to T cells that play a role in the process of inflammation seen in the disease. Inheriting this specific MHC gene may therefore be an important component of the disease process. Alternatively, the associated MHC class II molecule bearing a self-peptide or peptides may be responsible for deleting specific T cells in the thymus that have the potential to protect an individual from the inflammatory disease. The identification of disease-specific MHC class II-restricted T-cell clones, if they exist, would represent an important step in establishing whether a particular MHC class II molecule plays a direct role in the inflammatory process. Similar explanations involving either a putative protective role of a peptide–MHC combination, or the generation of a "hole in the T-cell repertoire," may be relevant to understanding negative MHC class II associations with disease.

We have systematically analyzed individual alleles at all *DRB* loci in patients with CD, patients with UC, and ethnically matched controls. Our results suggest that in a subset of CD patients disease susceptibility is linked to inheritance of the *DRB3*0301* allele. This strong association suggests that the *DRB3*0301* allele may well be directly involved in disease pathogenesis in approximately one-third of Caucasian CD patients. The weaker association seen in CD with *DRB1*1302* reflects the known linkage disequilibrium between this *DRB1* allele and *DRB3*0301*. Although the association with *DRB3*0301* may well reflect a direct role for this *DRB3* allele in CD, the possibility that this association actually marks the involvement of a linked *DQ* gene remains to be excluded. We expect to establish whether the *DRB3*0301* association represents linkage disequilibrium and also to establish whether specific DPB1/DPA1 heterodimers or DQB1/DQA1 heterodimers are likely to be relevant, particularly in DRB3*0301-negative individuals.

A weaker negative association was observed with *DRB1*0401* in CD. Further studies are required with a larger group of patients to ascertain whether this negative association will achieve statistical significance. Clearly no single *DRB1, DRB3, DRB4*, or *DRB5* locus allele appears to be positively associated with UC in Caucasian patients. It is unclear whether this truly reflects the absence of a role for MHC class II-mediated events in the pathogenesis of UC. Because studies in Japanese patients have described the association of a specific *DRB1* allele with UC, extended studies on *DP* and *DQ* alleles in ethnically matched Caucasian patients might reveal a role for MHC class II genes in the pathogenesis of UC as well.

In their recent studies on Japanese CD patients, Nakajima *et al.* (16) conclude that *DQB1*04* (**0401/*0402*) confers susceptibility. A positive association is seen in Japanese CD patients with certain *DRB1* alleles, particularly *DRB1*0405* and *DRB1*0410*, both of which are in linkage disequilibrium with *DQB1*04*. Both *DQB1*0401* and *-*0402* are extremely rare alleles in Caucasians (22). Indeed, while in Caucasian patients with CD we have noted a positive association with *DRB1*1302*, this particular *DRB1* allele is negatively associated with CD in the Japanese population (16). This difference may be explained by the fact that the *DRB1*1302* allele is known to be in linkage disequilibrium with other specific alleles that confer either susceptibility or resistance to CD. The positive association seen with *DRB1*1302* in Caucasians with CD reflects linkage to *DRB3*0301*. Similarly in Japanese patients with CD, the negative association with *DRB1*1302* reflects the fact that this allele is in linkage disequilibrium with the "resistance allele" that is *DQA1*0102*. Given the very different frequencies of specific MHC class II alleles in Japanese and Caucasian populations, it is not surprising that very different alleles may account for susceptibility and resistance to CD in different ethnic groups. Ethnic differences in allele frequencies, rather than distinct pathogenetic mechanisms, are likely to account for different alleles being linked to susceptibility in Japanese and Caucasian CD patients.

The present studies have provided, to our knowledge, the first detailed and complete analysis of all *DRB* locus genes in patients with IBD. There was a highly significant association of CD with a single *DRB3* gene, *HLA-DRB3*0301*. These data suggest that MHC class II molecules may be important in the pathogenesis of IBD.

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