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EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Human leucocyte antigen class II genotype in susceptibility and resistance to co-amoxiclav-induced liver injury

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Background & Aims: Co-amoxiclav is one of the most common causes of drug-induced liver injury (DILI). Although there are previous reports of genetic associations between HLA class II and co-amoxiclav-related DILI, studies to date have been based on very small numbers from single centres only. In order to address this problem we have investigated the role of HLA class II *DRB1* and *DQB1* in 61 cases of co-amoxiclav DILI as part of a UK-wide multicentre study.

Methods: HLA alleles and genotypes were compared with those of 40 individuals exposed to co-amoxiclav without toxicity (treated controls) and 191 population controls.

Results: There were two significant findings from the study. First, HLA-*DRB1∗15* was increased in patients (53%) versus both treated (33%: OR = 2.29: 95% CI: 1.00–5.26) and population controls (30%: OR = 2.59:95% CI: 1.44–4.68: $p = 0.002$). Second, *DRB1∗07* was found to be reduced in patients (9.8%) compared to both treated (35%: OR = 0.18: 95% CI: 0.06 – 0.52: p = 0.0011, pc = 0.0154) and population controls (29%: OR = 0.266: 95% CI: 0.11 – 0.65: p = 0.0019, pc = 0.0266).

Conclusions: These results confirm the previously reported significant genetic risk for HLA-*DRB1∗15* and also provide evidence of a protective effect of the HLA-*DRB1∗07* family of alleles. HLA alleles and haplotypes may be particularly important in susceptibility and resistance to co-amoxiclav-DILI, but it remains to be seen whether this effect is due to the identified alleles or others in close linkage disequilibrium elsewhere on the MHC.

Introduction

Most drug-induced liver injury (DILI) is due to idiosyncratic reactions that are not predictable from drug dosage or concentration [1] However, drug-induced toxicity remains a major cause of termination of clinical trials of new therapeutic agents and adverse hepatic reactions accounted for 24% of post-marketing withdrawals in the United Kingdom in the period from 1975 to 2005 [2]. Co-amoxiclav is a combination of the potent β-lactamase inhibitor clavulanic acid and amoxicillin and is now among the most commonly prescribed antimicrobials worldwide. Hepatotoxicity following co-amoxiclav administration was first reported in 1988 [3] and is now one of the most common causes of DILI in Europe and the US [4–6].Co-amoxiclav DILI is seen at a rate of approx. 1 in 10,000 prescriptions, but fatalities are rare with the majority of patients making a full recovery [4,7,8]. Though most cases in UK surveys were cholestatic or mixed [4,8] a recent survey based in Spain found a higher incidence of hepatocellular cases which may also be associated with a lower average age for these patients [9].

Two previous studies have reported significant associations between HLA class II and susceptibility to co-amoxiclav-induced DILI, but these were both based on relatively small numbers [10,11]. In the first of these studies Hautekeete et al. found that 57% of 35 Belgian patients with co-amoxiclav DILI carried the *DRB1∗1501* allele compared to 11% of population controls [10]. In a second study O'Donohue et al. reported that the same allele was present in 70% of 20 patients from the west of Scotland [11]. Further analysis of both series confirmed that the *DRB1∗1501* allele was almost exclusively found in combination with the *DQB1∗0602* allele [10,11]. This combination is expected in a Northern European population due to the very high level of linkage disequilibrium between these two alleles. Although these two studies concur, they are both relatively under-powered and may have overlooked other significant genetic associations particularly those with protective alleles. A more recent study from Spain of 27 co-amoxiclav DILI cases failed to find a significant increase in the *DRB1∗1501* allele in patients, but did report a significantly higher frequency of *DQB1∗06* [12]. This latter observation is particularly interesting. There are many different *DQB1∗06* alleles. One of the *DQB1∗06* alleles, *DQB1∗0602*, is found almost exclusively with *DRB1∗1501* in Europeans and therefore the absence of an association with *DRB1∗1501* in Spain suggests that the *DQB1∗06* allele found here must be a different allele (or group of alleles) and cannot be *DQB1∗0602*. Unfortunately it is not possible to explore these findings further because only low resolution genotyping

Keywords: Drug-induced liver injury (DILI); Human leucocyte antigen (HLA); Immunogenetics; Cholestasis; Co-amoxiclav.

Received 22 March 2010; received in revised form 29 April 2010; accepted 20 May 2010

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Please cite this article in press as: Donaldson PT et al. Human leucocyte antigen class II genotype in susceptibility and resistance to co-amoxiclav-induced liver injury. J Hepatol (2010), doi:10.1016/j.jhep.2010.05.033

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was performed in the Spanish study [12]. Altogether, the differences in findings between these three studies may be explained either by population variation, different patterns of linkage disequilibrium between northern and southern Europe, or the fact that hepatocellular as opposed to cholestatic liver injury was more common in the Spanish DILI patients in contrast to the other studies in northern Europeans.

Recent genetic studies indicate that the human MHC plays a major role in susceptibility to a wide range of adverse drug reactions including flucloxacillin DILI associated with the MHC 57.1 haplotype [13], transaminitis following treatment with the anticoagulant ximelagatran associated with the HLA class II *DRB1∗0701* allele [14], and hypersensitivity to the anti-retroviral agent abacavir which has also been found to be associated with the MHC 57.1 haplotype [15]. Consequently there is increasing interest in the role of the MHC in adverse drug reactions and the potential that these genetic associations may have in helping to explain the pathogenesis of these idiosyncratic disorders.

The aims of the present study were to investigate the role of the HLA class II in promoting susceptibility and resistance to co-amoxiclav-induced liver injury in a large group of patients recruited as part of a national study.

Materials and methods

Patients

We studied 61 cases of co-amoxiclav-induced DILI collected retrospectively and prospectively between October 2004 and December 2007 as part of the UK-wide DILIGEN study. Initial inclusion criteria for suspected DILI were either: (a) clinically apparent jaundice or bilirubin >40 μmol/L (after exclusion of cases due to haemolysis), or (b) an ALT >5× ULN (upper limit of normal) or (c) an ALP >2× ULN plus any raised bilirubin above ULN. The causal relationship of liver injury to co-amoxiclav was established using Council for International Organizations of Medical Sciences (CIOMS) scale [16] (details of the patient group are given in Table 1). Ethical approval for the UK-wide study was from Leeds East Research Ethics committee and the study protocol conforms to the ethical guidelines of the 1975 Helsinki Declaration. Informed consent was obtained from all patients and controls participating in the study. Cases were identified by searching the histological databases and discharge records at 10 UK Regional Liver Units for cases of DILI or cholestasis/hepatitis of unknown aetiology. In addition, direct contact with gastroenterologists throughout the UK was made by advertising the study through the British Society for Gastroenterology (BSG) website and by mailing to members of the British Association for the Study of the Liver (BASL). All cases were of self-reported white European ethnic origin.

Table 1. Clinical data on all DILI patients.

Sex (F/M)	26/35
Age at onset (years)	63 (31 – 84)
Time to onset (days)	17 ± 20
Total days on drug	7 ± 15
Histology	
Cholestatic	32 (53%)
Hepatocellular	16 (26%)
Mixed	13 (21%)
ICC score	
3 – 5 possible	7 (11%)
6 – 8 probable	26 (43%)
>8 highly probable	28 (46%)
Peak Bilirubin (μmol/L)	174 ± 169
Peak ALT (U/L)	293 ± 431
Peak ALP (U/L)	400 ± 322

Controls

Two distinct groups of controls were included. The first group (population controls) was composed of 191 unrelated individuals of white European ancestry [17]. The second group (treatment controls) were unrelated individuals, of self-reported white European ethnic origin, recruited from hospitals and general practices throughout the UK who had been prescribed a course of co-amoxiclav, but had not reported any symptoms consistent with DILI to the prescriber after completing the course.

Determination of the HLA-DRB1 and DQB1 allele and genotype distribution

All genotypes were performed using a standard polymerase chain reaction protocol using commercial kits (Invitrogen Ltd., Scotland). All samples were genotyped for *DRB1* using low resolution genotyping (*DRB1∗01–DRB1∗16*). High resolution *DRB1* and low resolution *DQB1* (*DQ1–DQ9*) genotyping were performed on selected samples, as appropriate.

Statistical analysis

Two tailed probabilities were calculated for allele and genotype distributions using Fisher's exact test on Prism software (Graphpad). No correction factor was necessary for the comparison of data for *DRB1∗1501* and *DQB1∗0602* because associations with these alleles have been identified in previous studies [10,11], however a correction factor of 14 was applied for all other *DRB1∗* allele families (i.e. the number of *DRB1∗* alleles or families tested at low resolution). Associations with alleles were tested by counting the individuals positive for each allele as suggested by Svejgaard and Ryder [18] and Odds Ratios (OR) presented in place of relative risk as the accepted standard.

Results

Patient cohort

Characteristics of the patient cohort are summarized in Table 1. Briefly, in line with previous reports [4,10,11], there were more males than females with a mean age of 63 years. Causality for co-amoxiclav DILI was scored as probably or highly probable in 89% of cases on the basis of ICC scoring. The average length of drug treatment was 7 days with DILI developing on average 17 days after the start of treatment though this period varied widely between patients. In line with previous studies on Northern Europeans, 77% of cases had either a cholestatic or mixed phenotype. The majority of the cases (77%) fulfilled Hy's law [19] showing ALT at least three times ULN and bilirubin two times ULN.

HLA class II genotyping

There were two significant differences in *HLA-DRB1* allele distribution (Table 2). First, DILI patients had a significantly higher frequency of *DRB1∗1501* than population controls: 53% of patients versus 30% (OR = 2.59; 95% CI = 1.44–4.68: $p = 0.002$). Second, there was a significantly lower frequency of *DRB1∗07* alleles in the DILI patients versus both population and treatment controls: 9.8% of patients versus 29% of population controls (OR = 0.26; 95% CI = 0.11–0.65, equivalent to a 3.8-fold reduced risk: $p = 0.0019$, p-corrected = 0.0266) and 35% of treatment controls (OR = 0.18; 95% CI = 0.06–0.52, equivalent to a 5.5-fold reduced risk: $p = 0.0011$, p-corrected = 0.0154). Testing for *DQB1* indicates that all patients with *DRB1∗1501* also carry *DQB1∗0602* as expected. Further DQ genotyping was not per-

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Table 2. HLA phenotype distribution in cases and controls.

DRB1 allele family	Cases	Treated Controls	Healthy Controls	Probability (p and pc as appropriate)
	N = 61	N = 40	N = 191	
01	11 (18)	6 (15)	35^{1} (18.3)	ns
0103	0	0	10^{1} (5.2)	ns
15	32^{5} (53)	13^{1} (33)	57^{3} (30)	^{1}p = 0.0655, ns ^{2}p = 0.0020
16	2 (3.2)	0	1 (0.5)	ns
03	15^{1} (25)	12^{2} (30)	59^{3} (31)	ns
04	16^{3} (26)	8 (20)	54^{14} (28)	ns
11	6 (9.8)	7 (18)	20 (11)	ns
12	2 (3.2)	1 (2.5)	7 (3.7)	ns
13	11 (18)	8^{1} (20)	34^{2} (18)	ns
14	3 (4.9)	2 (5)	8 (4.2)	ns
07	6^{1} (9.8)	15^{1} (35)	56^{6} (29)**	^{1}pc = 0.0154 ^{2}pc = 0.0266
08	5 (8.2)	1 (2.5)	7 (3.7)	ns
09	2 (3.2)	1 (2.5)	2 (1.1)	ns
10	1 (1.6)	0	2 (1.1)	ns

Superscript after frequency indicates number of homozygotes for each allele in each subgroup. Superscript 1 before *p* indicates comparison with treated controls, and superscript two indicates comparison with population controls. **17/56 (30%) *DRB1*07* haplotypes tested carried *DQB1*03* and 39/56 (70%) carried *DQB1*02*.

Table 3. Clinical Data on DILI patients with and without *DRB1*1501*.

	DRB1*15 positive N = 32	DRB1*15 negative N = 29
Sex (F/M)	14/18	12/17
Age at onset (years)	65 ± 11	62 ± 13
Time to onset (days)	14 ± 14	18 ± 25
Total days on drug	7 ± 6	7 ± 21
Histology		
Cholestatic	19 (59%)	13 (45%)
Hepatocellular	8 (25%)	8 (28%)
Mixed	5 (16%)	8 (28%)
ICC score		
3 – 5 possible	4 (13%)	3 (10%)
6 – 8 probable	14 (44%)	12 (41%)
>8 highly probable	14 (44%)	14 (48%)
Peak Bilirubin (μmol/L)	175 ± 154	173 ± 184
Peak ALT (U/L)	247 ± 296	296 ± 552
Peak ALP (U/L)	399 ± 214	387 ± 409

formed in these patients. *DQB1** genotyping of the six patients who were *DRB1*07* positive was complicated by the fact that one of these six was homozygous for *DRB1*07*. Therefore, of the seven *DRB1*07* haplotypes carried by these six patients, five were found to have *DQB1*02* and two were found to have *DQB1*03* based on the most probable *DRB1–DQB1* combination. Though these are very small numbers this distribution of *DRB1*07–DQB1*02* and *DRB1*07–DQB1*03* haplotypes is in keeping with the expectation for this population, whereby approximately 30% of *DRB1*07* haplotypes are expected to carry *DQB1*03* with the majority of the remainder carrying *DQB1*02* [13,20]. These data do not indicate a specific reduction in either haplotype and may indicate the absence of any effect of *HLA-B*5701* which is found on the *DRB1*0701–DQB1*0303* haplotype.

When the current data were pooled with data for *DRB1*1501* from the previous studies of Hautekeete [10] and O'Donohue [11] there was a very significant association with *DRB1*1501*. Sixty-six (57%) out of a total of 116 co-amoxiclav DILI patients were *DRB1*15/DRB1*1501* positive compared to 30% of our population controls (OR = 3.1; 95% CI = 1.92–5.02: $p = 4.3 \times 10^{-6}$). Data for *DRB1*07* were not available for the O'Donohue study [11], making further validation of the *DRB1*07* association difficult at this stage.

Clinical characteristics were also compared on the basis of *DRB1*1501* genotype (Table 3). There were no significant differences for any of the factors examined between the two genotype groups.

Discussion

There are two major findings in the present study. Firstly we have confirmed the previously reported association with the *DRB1*1501–DQB1*0602* haplotype in co-amoxiclav DILI in a comparatively large national study. Second, we report a novel protective association with the *DRB1*07* family in co-amoxiclav DILI. With respect to *DRB1*1501* our data show similarity with both the Belgian [10] and the west of Scotland studies [11]. Our *DRB1*1501* data are only statistically significant at the 5% level when comparing the DILI patients and population controls. The lack of statistical significance with the treatment control group is due to the relatively small size of that group ($N = 40$). Thus, even though there is a 20% difference (53% versus 33%) in the frequency of *DRB1*15* between the two groups, this is not statistically significant ($p = 0.0655$). However, the inclusion of this control group is important as it establishes the fact that the association with *DRB1*15* is not due to a genetic association with infectious illness requiring co-amoxiclav treatment but directly linked with co-amoxiclav DILI *per se*. In contrast to our study, neither of the two previous studies included a treatment control group in their analysis.

Careful scrutiny of the previous studies also illustrates two other important points that are worthy of note. Both studies reported lower frequencies of *DRB1*15* in their population controls; Hautekeete et al. [10] acknowledge the abnormally low frequency of *DRB1*15* (11.7%) and in the Scottish study [11] *DRB1*1501* was found in 20% of controls, whereas we have found *DRB1*15* in 30% of our controls, a figure very similar to that reported across northern England (i.e. 27.8% in 15,000 pooled controls [21]). This type of variation between studies is common, especially in studies of HLA and can lead to false negative/false positive results, especially where the size of the genetic effect or sample is small.

The second important point relates to the two other alleles carried on the *DRB1*1501* haplotype: the second expressed *DRB* allele (*DRB5*0101*) and the *DQB1* allele (*DQB1*0602*). These alleles make up one of the most common haplotypes in the northern European population; the HLA-B7.1 haplotype, which also carries *DQA1*0102*, *HLA-B*07*, *HLA-Cw*0701*, and *HLA-A*03*. In the two earlier studies [10,11] and in the present study patients with *DRB1*1501* also have *DRB5*01* and *DQB1*0602*, reflecting the expected pattern of linkage disequilibrium. However, where there is such absolute linkage disequilibrium the "extra" knowledge provided by genotyping these other alleles is

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not informative, because any effect attributed to these alleles may be explained by linkage disequilibrium. In contrast, much more information may be gained by studying populations where this linkage disequilibrium is less pronounced. As such the Spanish data referred to above [12] are of particular interest.

DRB1∗1501 (the most common *DRB1∗15* allele) is strongly associated with primary sclerosing cholangitis (PSC) [22], may have a weak protective effect in type 1 autoimmune hepatitis (AIH), and may be associated (albeit weakly) with primary biliary cirrhosis (PBC) [23,24]. Interestingly, the study of O'Donohue et al. [11] included two patients with PBC which may be a confounding factor in their analysis. The DR2 antigen, which includes all members of the *DRB1∗15* family of alleles, has also been associated with hepatitis following nitrofurantoin [25], halothane-induced hepatitis in Japan [26], and with allergies to ragweed pollen [27]. All of the above associations may point to a strong role for the *DRB1∗15* allele family in immune and immune-allergic reactions following exposure to a variety of potential toxins and xenobiotic agents.

Our second observation is novel. The *DRB1∗07* family includes more than 16 major alleles with *DRB1∗0701* being the most common. This is an interesting association, because *DRB1∗07* haplotypes may be important in other causes of DILI associated with other commonly prescribed antibiotics, most notably flucloxacillin. However, in contrast to co-amoxiclav DILI, in flucloxacillin DILI, *DRB1∗07* is associated with an increased risk of disease and *DRB1∗15* is associated with a reduced risk [13]. Subsequent studies of the MHC in flucloxacillin DILI have mapped susceptibility closer to *HLA-B* (in particular *HLA-B∗5701*) on the *DRB1∗07–DQB1∗0303* haplotype. However, as stated above similar mapping studies of the *DRB1∗15* haplotype may be hampered by the exceedingly tight linkage disequilibrium found there.

The *HLA-DRB1∗1501–DQB1∗0602* haplotype is important in a variety of different diseases, in particular the DQ molecule encoded on this haplotype seems to be uniquely capable of conferring protection against type 1 diabetes and susceptibility to severe narcolepsy [28–30]. Comparing alleles that confer susceptibility and resistance has permitted mapping to the crystal structure of the expressed molecule and to specific peptide binding pockets [31]. There are clear structural differences between the DR15 and DR7 antigens encoded by the alleles named above. Most *DRB1∗15* alleles encode the amino acids proline, arginine, glutamic acid, arginine, tyrosine, aspartic acid, tyrosine, alanine, alanine, and tyrosine at positions 11, 13, 14, 25, 30, 57, 60, 73, 74, and 78 of the DRβ polypeptide, whilst most *DRB1∗07* alleles encode glycine, tyrosine, lysine, glutamine, leucine, valine, serine, glycine, glutamic acid, and valine at these positions [32] (see Table 4). These differences are concentrated in the peptide-binding groove of the MHC molecule and as such may determine the functional significance of these genetic associations. Several of these amino acid differences are unique (or almost unique) to one or other of the two families of *DRB1* alleles including proline-11 and arginine-13 in the *DRB1∗15* family and glycine-11, lysine-14, glutamine-25, and leucine-30 in the *DRB1∗07* family. The amino acids at positions 11 and 13 interact with the 6th and 4th (respectively) pockets for binding of antigenic side chains and thus, these amino acids may be particularly important in determining differences in genetic susceptibility and resistance to co-amoxiclav-induced DILI. In addition when groups of amino acids are considered, even though individually they are not unique, they often form unique sequences which can contribute significantly to potential variations in the antigen binding properties of the expressed molecule. A good example of this can be seen comparing the most common amino acid sequences at positions 67–78 of the *DRB1∗15* and *DRB1∗07* allele families. *DRB1∗15* has the sequence ILEQARAAVDTY at positions 67–78 based on the single letter code and *DRB1∗07* has ILEDRRGQVDTV. None of these individual amino acids at these positions are unique to either allele family, but taken together these sequences create a unique sequence and as they reside over major sites for peptide antigen interaction on the expressed DR molecule, including the 4th, 6th, and 7th peptide-binding pockets [33,34], it is likely that these differences may be important in determining an individual's susceptibility or resistance to co-amoxiclav DILI.

Table 4. HLA allele families and major amino acid variations.

Position	11	13	14	25	30	57	60	73	74	78
Hypervariable Region	HVR1			HVR2				HVR3		
Binding pocket	P6	P4				P9			P4	
DR15										
Amino acid	Pro	Arg	Glu	Arg	Tyr	Asp	Tyr	Ala	Ala	Tyr
Polar	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes
Charge	-	Pos	Neg	Pos	-	Neg	-	-	-	-
DR7										
Amino acid	Gly	Tyr	Lys	Gln	Leu	Val	Ser	Gly	Glu	Val
Polar	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No
Polarity/Charge	-	-	Pos	-	-	-	-	-	Neg	-

Strong genetic associations have been found between specific HLA-DR molecular structures and autoimmune liver disease [22–24]. Generating molecular model based on comparison of amino acid sequences, as here, allows us to consider the potential functional impact of such simple genetic associations and brings attention to the underlying immunology.

These two genetic associations also need to be considered in the context of current studies of complex "diseases". Overall both *DRB1∗15* and *DRB1∗07* have a considerable effect in terms of disease risk. There is a 2.59-fold increased risk of co-amoxiclav DILI with *DRB1∗15* and a 3.8-fold reduced risk with *DRB1∗*07. These are quite large effects when measured against current observations for non-MHC genes in complex disease, but may still be considered small when measured against some studies of HLA and liver disease [22]. Nevertheless, these findings are in accord with earlier studies by our group and others [10,11] and confirm the importance of genetic factors in DILI following treatment with co-amoxiclav. However, the HLA genotypes and haplotypes discussed herein are common in the general population and therefore possession of these alleles is neither necessary nor sufficient for DILI to occur following treatment. DILI following co-amoxiclav treatment is almost certainly a complex trait and there may be more than one risk allele. Not only is there a need for further investigation of the MHC but also of non-MHC alleles in co-amoxiclav-induced DILI.

Conflict of interests

The authors who have taken part in this study do not have a relationship with the manufacturers of the drugs involved either in

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the past or present and did not receive funding from the manufacturers to carry out their research.

Acknowledgements

The grant support for this study was supplied by the UK Department of Health (Pharmacogenetics Initiative ref PHGX10A). Special thanks go to the research committees of the BASL and BSG for help with publicising the study, to the patients for their participation and to all those who informed us of eligible cases.

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Please cite this article in press as: Donaldson PT et al. Human leucocyte antigen class II genotype in susceptibility and resistance to co-amoxiclav-induced liver injury. J Hepatol (2010), doi:10.1016/j.jhep.2010.05.033