Predicting HLA Class II Alloantigen Immunogenicity From the Number and Physiochemical Properties of Amino Acid Polymorphisms

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Background. We have shown previously that human leukocyte antigen (HLA) class I immunogenicity can be predicted by the number, position, and physiochemical differences of polymorphic amino acids (AAs). We have now modeled the structural and physiochemical polymorphisms of HLA class II alloantigens and correlated these with humoral alloimmunity in sensitized patients awaiting kidney transplantation.

Methods. Sera obtained from 30 patients with high levels of IgG HLA-specific antibodies were screened using single-antigen HLA antibody detection beads. A computer program was developed to determine the number of AA mismatches (after interlocus and intralocus subtraction) and their hydrophobicity and electrostatic mismatch score for each mismatched HLA-DR and -DQ specificity. Regression methods were used to compare these variables with the occurrence and magnitude of alloantibody responses.

Results. HLA-specific antibody was detected against 879 (55%) of 1604 mismatched HLA specificities evaluated. There was a strong correlation between increasing number of AA mismatches and the occurrence ($P<0.001$, odds ratio 3.85 per AA) and magnitude of alloantibody responses ($P<0.001$); only 6% of alloantigens with 0 to 2 mismatched AA-induced alloantibody (median fluorescence intensity 37) compared with 82% of alloantigens with more than or equal to 20 mismatched AAs (median fluorescence intensity 9969). Hydrophobicity and electrostatic mismatch scores also correlated closely with alloantibody response ($P<0.001$), but neither variable had independent predictive value over the number of AA mismatches alone.

Conclusion. Differences in the number of polymorphic AA mismatches and their physiochemical properties for a given recipient HLA type are strong predictors of class II alloantigen immunogenicity and alloantibody response before kidney transplantation.

Keywords: Alloantigen immunogenicity, Sensitization, HLA antibodies, Kidney transplantation.

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The level of human leukocyte antigen (HLA) matching influences transplant outcome after deceased donor kidney transplantation, and in many countries, HLA matching is an important component of organ allocation (*1–4*). In the UK allocation scheme for deceased donor kidneys, most importance is attached to matching for HLA-DR because it is the HLA locus that most strongly influences transplant outcome. Renal transplants that are poorly matched for HLA-DR have a higher incidence of rejection, increased requirement for immunosuppression, reduced early graft function, and commonly lead to the development of HLA class II-specific alloantibodies (*5*). In contrast, matching for HLA-A and -B has little effect within the first 6 months but is associated with improved long-term transplant outcome (*6*, *7*).

For the purposes of organ allocation, the level of HLA-DR mismatch is based on serologically defined HLA-DR specificities that are assigned equal weighting and used to designate HLA-DR mismatch grade as 0, 1, or 2 (*3*, *4*). This approach regards all HLA-DR mismatches as having equal relevance and takes no account of potential differences in immunogenicity according to recipient HLA type. For a given recipient

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HLA type, the immunogenicity of a donor HLA-DR mismatch can be determined by the nature and location of the mismatched amino acid (AA) polymorphisms (*8*).

We and others have shown that the immunogenicity of HLA-A and -B alloantigens can be determined by the difference in the number of AA mismatches (AAM) (*9–12*) and their relative hydrophobicity and electrostatic charge (*13*). The approach is based on a computer algorithm developed to allow intralocus and interlocus comparison of mismatched HLA-A and -B specificities with the corresponding HLA class I type and to determine the number, position, and physiochemical disparity of polymorphic AAs. In this study, we have applied our approach for defining HLA immunogenicity to HLA class II and report, for the first time, that the difference in the number and physiochemical properties of mismatched AAs predicts the alloantibody response to HLA class II alloantigens in sensitized patients awaiting renal transplantation.

RESULTS

Sera obtained from 30 patients with the highest number of HLA class II alloantibody specificities were screened by single antigen beads using Luminex. This gave a total of 1604 mismatched HLA-DR and -DQ specificities of which HLA-specific antibody was detected (adjusted median fluorescence intensity [MFI] ≥1500) against 879 (55%). Of the HLA class II-specific alloantibodies that were defined as antibody positive, MFI values varied, ranging from 1504 to 25,776 with a median of 9294. The number of mismatched AA for the 1604 mismatched HLA-DR and -DQ specificities represented on the single-antigen beads ranged from 0 (after intralocus or interlocus subtraction) to 51 (median 11, interquartile range [IQR] 7–17). The total hydrophobicity mismatch score (HMS) and electrostatic mismatch score (EMS) for each mismatched HLA class II specificity also varied; for HMS, the median value was 14.6 (IQR 7.4–25.2) and for EMS, the median value was 15.8 (IQR 8.7–26.0).

Relationship Among AAM, HMS, and EMS and Occurrence of an Antibody Response

We first explored the relationship between the number of mismatched AA, HMS, and EMS and the occurrence of an alloantibody response (defined as adjusted MFI≥1500). As the number of mismatched AA increased, so too did the likelihood of an alloantibody response to the mismatched HLA-DR and -DQ specificities (Table 1, $P<0.001$). Alloantibody was detected in only 1 (1.4%) of the 73 mismatched HLA-DR and -DQ combinations that contained zero amino acid mismatch (after intralocus or interlocus subtraction). For example, a mismatch for HLA-DR17 (DRB1*0301) has no AAM in a patient with HLA-DRB1*1301, 0801, and DRB3*0101. Similarly, DR17 (DRB1*0301) carries no AAM in a patient HLA with HLA-DRB1*1101, *1301, and DRB3*0101, and both examples may be considered nonimmunogenic. The frequency of alloantibody response increased to 83% when there were 23 or more mismatched AAs. Similarly, as the HMS and EMS of the mismatched HLA class II specificities increased, so too did the occurrence of an alloantibody response (Table 2, $P<0.001$).

The values for AAM, HMS, and EMS correlated closely (AAM vs. HMS, $r=0.97$; AAM vs. EMS, $r=0.96$; and HMS vs. EMS, $r=0.97$), and all three variables were equally effective for prediction of an alloantibody response (Figs. 1 and 2). The use of two or more of these three variables in combination did not confer additional predictive value, and we chose to focus mainly on the AAM in further models used to investigate the relationship with the alloantibody response. As shown in Figure 1, the association between the presence of alloantibody and number of AAM was highly significant ($P<0.001$) with an odds ratio of 3.85 (95% confidence interval 3.24–4.57) per 1 unit change in log(AAM+1).

TABLE 1. Relationship between amino acid mismatches and the occurrence of alloantibody response to mismatched HLA-DR and -DQ specificities represented on single-antigen beads

Total no. AAM	No. mismatched specificities	Frequency of alloantibody response, MFI≥1500 (%)
0	73	1 (1)
1	76	4 (5)
2	31	6 (19)
3	42	17 (40)
4	37	9 (24)
5	66	22 (33)
6	56	23 (41)
7	99	47 (47)
8	88	37 (42)
9	100	64 (64)
10	90	54 (60)
11	87	57 (66)
12	81	49 (60)
13	80	51 (64)
14	51	35 (69)
15	65	41 (63)
16	62	42 (68)
17	60	38 (63)
18	36	23 (64)
19	31	20 (65)
20	24	18 (75)
21	28	21 (75)
22	17	13 (76)
23–51	224	187 (83)

HLA, human leukocyte antigen; AAM, amino acid mismatch; MFI, median fluorescence intensity.

Relationship Between AAM and the Magnitude of an Antibody Response

We next considered the relationship between the number of mismatched AA and the magnitude of HLA-DR and -DQ alloantibody responses detected in the patient sera. The results are plotted as boxplots stratified according to the number of mismatched AA (Fig. 3). Median regression analysis showed that mismatched specificities with increasing number of AAMs were associated with progressively stronger alloantibody responses ($P<0.001$). The magnitude of alloantibody binding increased from a MFI of 37 (IQR 8–124) for alloantigens with 0 to 2 AAMs to a MFI of 9969 (IQR 2679–14,884) for alloantigens with more than or equal to 20 AAMs.

TABLE 2. Relationship between HMS and EMS and the occurrence of alloantibody response to mismatched HLA-DR and -DQ specificities represented on single-antigen beads

HMS/EMS deciles	Frequency of alloantibody response using HMS (%)	Frequency of alloantibody response using EMS (%)
1	17/164 (10)[a]	10/164 (6)
2	47/159 (30)	57/157 (36)
3	56/158 (35)	74/160 (46)
4	88/163 (54)	78/161 (48)
5	102/160 (64)	85/160 (53)
6	96/164 (59)	102/161 (63)
7	102/155 (66)	107/162 (66)
8	114/163 (70)	105/159 (66)
9	115/158 (73)	117/160 (73)
10	142/160 (89)	144/160 (90)

[a] No. alloantibody positive (normalized MFI≥1,500) mismatched HLA combinations/total no. combinations (%=proportion positive).

HLA, human leukocyte antigen; EMS, electrostatic mismatch score; HMS, hydrophobicity mismatch score; MFI, median fluorescence intensity.

The strength of this relationship, however, was less pronounced for alloantigens with more than 25 AAMs because of the relatively small number of observations above this point in the dataset, and this was reflected on the fit of the relevant part of the regression curve (Fig. 3).

Analysis of AAM and Antibody to HLA-DR and -DQ

The differential immunogenicity of HLA-DR compared with HLA-DQ alloantigens was next examined. For those specificities associated with an antibody response, analysis of alloantibody levels using mean and median regression and after adjustment for the number of AAM showed that mismatched HLA-DR specificities were associated with stronger alloantibody responses (higher MFI levels) than HLA-DQ specificities ($P<0.0001$) and with an increase in the likelihood of a positive response ($P<0.0001$). These findings suggest that HLA-DR mismatches have higher immunogenic potential per AAM than that of HLA-DQ.

DISCUSSION

The ability of donor HLA class II alloantigens to provoke an alloantibody response varies and is likely to depend on the recipient HLA class II type. Until recently, it was not possible to predict whether a particular HLA class II alloantigen mismatch was likely to be immunogenic. However, the availability of AA sequence data for all HLA class II alleles now enables detailed comparisons of structural differences between mismatched HLA alloantigens. In addition, single-antigen bead Luminex technology now enables the detailed definition of HLA class II alloantibody responses in sensitized patients. By combining this information, we have, for the first time, examined the relationship between AA sequence differences of HLA-DR and -DQ mismatches and their ability to stimulate an alloantibody response. Our results show that the number of polymorphic AAM and their physiochemical properties for a given recipient HLA type are strong predictors of HLA class II alloantigen immunogenicity and alloantibody response. Comparison of the relative immunogenicity of HLA-DR and -DQ, after adjustment for the number of AAM, suggested that HLA-DR mismatches were more immunogenic than HLA-DQ. Polymorphisms in HLA-DR are confined to the β chain, whereas for HLA-DQ, both the α and β chains are polymorphic. Whether there is a difference in immunogenicity of AA substitutions between the α and β chains of HLA-DQ is not clear from our analysis because there were too few examples where AAMs were confined exclusively to the α or β chain to allow meaningful analysis. When there are AAMs in both the α and β chains of HLA-DQ, these may contribute in combination to a B-cell epitope, making comparison of their relative contribution particularly problematic and beyond the scope of this study.

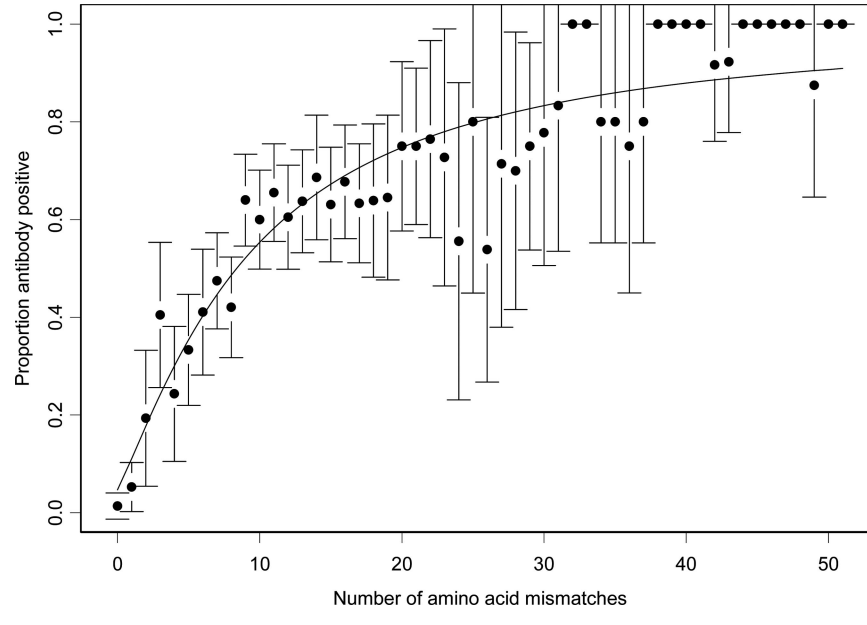

FIGURE 1. Relationship between the number of amino acid mismatches and the occurrence of alloantibody response to mismatched HLA-DR and -DQ specificities represented on single-antigen beads. The figure shows point estimates and 95% confidence intervals at each level of amino acid mismatch and the fitted logistic regression curve.

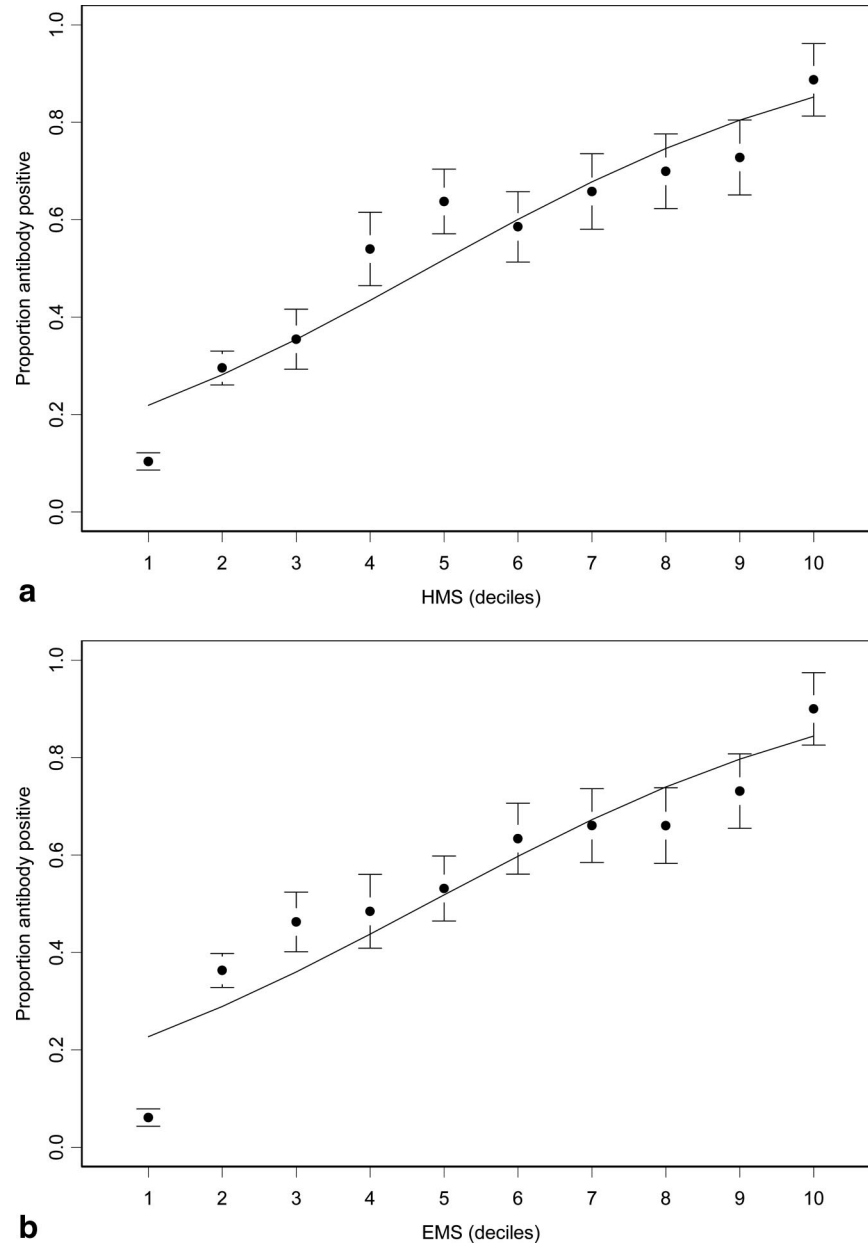

FIGURE 2. Relationship between hydrophobicity mismatch score (HMS) (a) and electrostatic mismatch score (EMS) (b) and the occurrence of alloantibody response to mismatched HLA-DR and -DQ specificities represented on single-antigen beads. Each figure shows point estimates and 95% confidence intervals at each deciles of HMS (a) and EMS (b) and the fitted logistic regression curve.

A key assumption made in our analysis is that an AA polymorphism at a given position in an HLA alloantigen that is shared by any one of the different recipient HLA class II alleles within the same locus will not be immunogenic. This concept, designated intralocus subtraction (for AA sequence comparisons within the same locus) or interlocus subtraction (for AA sequence comparisons across different HLA loci), was first proposed and validated by Duquesnoy (*14*, *15*) who showed using the HLAMatchmaker computer program that analysis of HLA class I and class II immunogenicity based on AA triplets and eplets after interlocus and intralocus AA sequence subtraction correlated with alloantibody production and graft survival after renal transplantation (*10*, *16*, *17*).

We and others showed previously that increasing number of triplet and eplet AA mismatches between HLA class I alloantigen and recipient HLA class I type correlated with the occurrence and magnitude of the alloantibody response (*10–12*). We also showed that simply enumerating the number of AA mismatches after interlocus AA subtraction provided a strong correlation with alloantibody formation to mismatched HLA-class I specificities (*13*). The results of this study demonstrate that this approach is also applicable to HLA class II and that enumerating the number of AA mismatches after intralocus and interlocus subtraction enables determination of alloantigen immunogenicity and allows prediction of the ability of HLA class II alloantigens to stimulate an alloantibody response.

The correlation between the number of mismatched AAs and alloantibody response to HLA class II observed in this study is better than that demonstrated in our previous study of HLA class I alloantibody responses (*13*). This may be explained by the different approach used for selection of sensitized patients in the two studies. In our analysis of HLA class

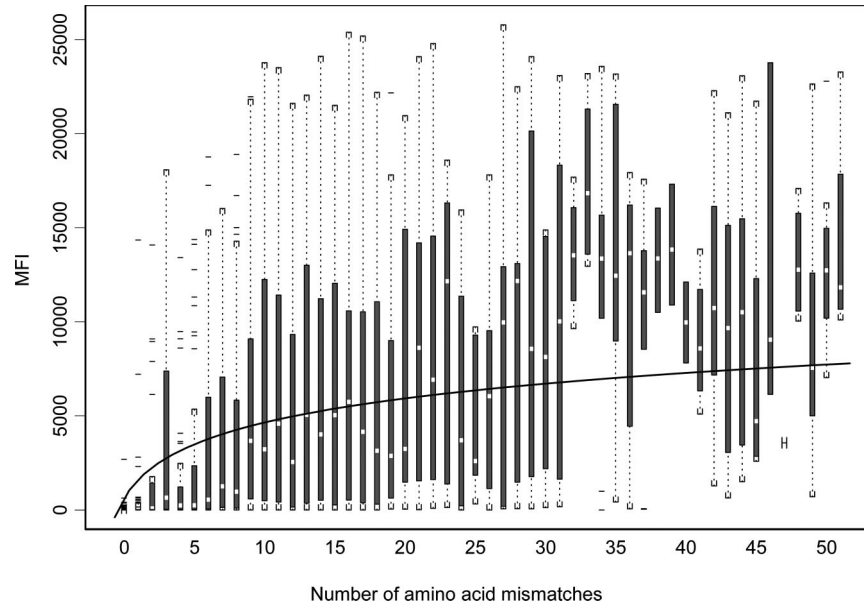

FIGURE 3. Relationship between number of amino acid mismatches and HLA-DR and -DQ specific antibody strength. The figure shows *box* and *whisker plots* at each level of amino acid mismatch and the fitted median regression curve. MFI, median fluorescence intensity.

I antibody responses, highly sensitized patients were selected on the basis of more than 85% IgG panel reactive antibodies. This approach is not optimal, particularly in the case of HLA class II, where the more limited number of different HLA-DR and -DQ specificities that are commonly expressed, means that HLA class II alloantibodies to a single mismatch would give high panel reactivity without necessarily reflecting sensitization to a broad range of HLA class II specificities. Patients in this study were selected not based on panel reactivity, but instead based on antibodies to a high number of different HLA class II specificities, which maximizes the opportunity for assessment of relative immunogenicity. In many cases, the development of antibodies to a high number of different HLA specificities reflects previous exposure to multiple HLA class II mismatches, but it is important to recognize that in some patients, broad antibody reactivity arises from exposure to only a limited number of HLA class II mismatches that have widely expressed epitopes. Therefore, it is possible that the strong correlation observed between AAM and HLA class II-specific alloantibody production may be biased by a small number of commonly expressed immunogenic epitopes. Analysis of HLA class II alloantibody responses in patients after a limited antigenic exposure, such as a single pregnancy, would help to address this point but would require a large recipient cohort.

Our findings are of potential clinical importance in the identification of HLA class II mismatches that are less likely to result in high levels of alloantibody after transplantation. There is now clear evidence that the emergence of donor-specific HLA class II antibodies after renal transplantation is associated with reduced long-term graft survival (*18–21*), and avoidance of kidneys bearing highly immunogenic HLA class II mismatches may improve transplant outcome (*16*, *22*, *23*). The ability to predict HLA class II mismatches most likely to provoke an alloantibody response is also of relevance in the context of retransplantation. Many patients listed for transplantation have had a previous failed transplant, and the majority are sensitized to a broad range of HLA class I or class II alloantigens, making retransplantation problematic (*24*). By avoiding donor HLA mismatches that are predicted to be most immunogenic when undertaking a first transplant, the risk of subsequent high levels of alloantibody sensitization may be reduced.

In contrast to our previous analysis of HLA class I immunogenicity, the physiochemical properties of mismatched AAs did not give any additional predictive value over that provided by enumerating the number of mismatched AAs after intralocus and interlocus subtraction. The number of mismatched AAs and their physiochemical scores were more closely correlated with the alloantibody response to HLA class II in this study than was observed for HLA class I in our previous study, which may explain why there was no added predictive value in considering AA mismatches and their physiochemical properties separately for HLA class II mismatches. The physiochemical properties of AAs are the major determinants of protein-protein interactions, such as alloantibody binding, and are therefore the likely underlying explanation for the strong correlation with HLA class II alloantibody responses. However, in the context of this study, simply enumerating AAM alone is sufficient to adequately reflect the physiochemical differences between mismatched HLA class II epitopes that determine alloantibody binding.

In conclusion, differences in the number of polymorphic AAM and their physiochemical properties on a mismatched HLA class II alloantigen for a given recipient HLA-DR and -DQ type are a strong predictor of class II alloantigen immunogenicity and alloantibody response before kidney transplantation. This information may be useful when considering kidney allocation policies to improve graft survival and minimize the risk of posttransplant allosensitization.

MATERIALS AND METHODS

Study Design

The approach taken to determine alloantigen immunogenicity was based on the assumption that if a patient produced an alloantibody to a given HLA

specificity, then the mismatched alloantigen is immunogenic in the context of that patient's HLA type. Selecting only those patients who have produced antibodies to a wide number of different HLA specificities for analysis increases the likelihood that lack of a specific antibody response is due to low immunogenicity rather than a lack of previous encounter with the relevant alloantigen. The validity of this approach is supported by the findings of our previous analysis of immunogenicity to HLA class I in highly sensitized patients selected on the basis of strong panel reactive antibodies (>85% IgG panel reactive antibodies). Certain serologically defined HLA class II specificities are present in a high proportion of the population (e.g., HLA-DR52 is present in approximately 80% of individuals), so that the presence of alloantibody to a single HLA class II mismatch may result in a high level of panel reactivity without reflecting encounter and response to a broad range of HLA class II specificities. Instead of selecting patients with high panel reactivity, therefore, we identified patients with antibodies to the greatest number of the 17 different HLA-DR and 7 different HLA-DQ serologic specificities.

Patient Selection and Characterization of HLA-Specific Antibody Profiles

Serum samples obtained from all adult patients (approximately 600 patients) awaiting deceased donor kidney transplantation at the Cambridge Transplant Centre are routinely screened at 3 monthly intervals using Luminex-based HLA class I and II antibody detection beads and subjected, as appropriate, to antibody characterization using single-antigen HLA-specific antibody detection beads as described previously (*11–13*). In October 2009, the antibody profiles of all listed patients were examined, and the 30 patients with the highest number of HLA-DR and -DQ antibody specificities were selected for inclusion in this study. They comprised 28 (93%) white Europeans, 13 men and 17 women with a median age of 44 years (range 18–70 years). One patient was sensitized through blood transfusion alone, 2 through pregnancy, 3 through previous transplantation, 8 through pregnancy and transfusion, 12 through transfusion and previous transplantation, and 4 through a combination of transfusion, pregnancy, and previous transplantation. For each patient, the serum sample with the highest IgG HLA class II-specific antibody reactivity, representing the peak period of sensitization, was screened (at neat, 1 in 50 and 1 in 100 dilution) using LABScreen HLA class II single antigen antibody detection beads (LS2A01 lot 006, One Lambda Inc., Canoga Park, CA) as described previously (*11–13*). Antibody binding was assessed on a Luminex LABScan 100 (One Lambda Inc.), and the MFI value obtained was adjusted for background signal using the formula ([sample number of bead−sample negative control bead]−[negative control number of bead−negative control negative control bead]). The highest adjusted MFI value for IgG binding to individual HLA class II specificities was used for analysis of immunogenicity. As in our previous

TABLE 3. Haplotype associations between HLA-DR and -DQ specificities defined by PCR-SSP and the most common HLA-DRB1/3/4/5, -DQA1, and -DQB1 allele used for analysis

HLA-DR	HLA-DR	HLA-DQ	HLA-DRB1*	HLA-DRB*3/4/5	HLA-DQA1*	HLA-DQB1*
DR1	—	DQ5(1)	0101	—	0101	0501
DR15(2)	DR51	DQ6(1)	1501	B5*0101	0102	0602
DR15(2)	DR51	DQ5(1)	1502	B5*0101	0103	0501
DR15(2)	DR51	DQ6(1)	1503	B5*0101	0102	0602
DR16(2)	DR51	DQ5(1)	1601	B5*0202	0102	0502
DR16(2)	DR51	DQ5(1)	1602	B5*0101	0102	0502
DR17(3)	DR52	DQ2	0301	B3*0101	0501	0201
DR18(3)	DR52	DQ4	0302	B3*0101	0401	0402
DR4	DR53	DQ7(3)	0401	B4*0101	0302	0301
DR4	DR53	DQ8(3)	0401	B4*0101	0301	0302
DR4	DR53	DQ4	0405	B4*0101	0302	0401
DR11(5)	DR52	DQ7(3)	1101	B3*0202	0501	0301
DR11(5)	DR52	DQ6(1)	1101	B3*0202	0102	0602
DR12(5)	DR52	DQ7(3)	1201	B3*0202	0501	0301
DR12(5)	DR52	DQ5(1)	1201	B3*0202	0104	0501
DR12(5)	DR52	DQ7(3)	1202	B3*0301	0601	0301
DR13(6)	DR52	DQ6(1)	1301	B3*0101	0103	0603
DR13(6)	DR52	DQ6(1)	1302	B3*0301	0102	0604
DR13(6)	DR52	DQ7(3)	1303	B3*0101	0501	0301
DR13(6)	DR52	DQ2	1303	B3*0101	0201	0201
DR14(6)	DR52	DQ5(1)	1401	B3*0202	0104	0503
DR14(6)	DR52	DQ7(3)	1402	B3*0101	0501	0301
DR7	DR53	DQ2	0701	B4*0101	0201	0201
DR7	DR53	DQ9(3)	0701	B4*0101	0201	0303
DR8	—	DQ4	0801	—	0401	0402
DR8	—	DQ7(3)	0804	—	0401	0301
DR8	—	DQ6(1)	0803	—	0103	0601
DR9	DR53	DQ9(3)	0901	B4*0101	0302	0303
DR9	DR53	DQ2	0901	B4*0101	0302	0202
DR10	—	DQ5(1)	1001	—	0101	0501

HLA, human leukocyte antigen; PCR-SSP, polymerase chain reaction using sequence specific primer.

studies, normalized IgG MFI values more than or equal to 1500 were considered positive (*11–13*).

Determination of Amino Acid Mismatches After Intralocus Subtraction

The HLA-DR and -DQ type of the 30 patients studied was determined by polymerase chain reaction using sequence specific primers DNA typing and then assigned the most common four digit DRB1, DQA1, and DQB1 allele for each HLA-DR and -DQ haplotype (Table 3) (*25*, *26*). All HLA-DQ α and β alleles may, in principle, also be present in cis and trans configuration, thereby forming two additional HLA-DQ specificities for each individual. However, alloantibodies to such specificities have, not to our knowledge, been described, suggesting that they are of limited clinical significance, and only cis-encoded molecules were considered for this analysis. For each patient, the AA sequence of their HLA-DR and -DQ type was compared with the AA sequence of each mismatched HLA-DR and -DQ specificity represented on the single-antigen antibody detection beads (31 HLA-DR alleles and 27 HLA-DQ alleles representing 17 and 7 specificities, respectively). Null alleles are not expressed at the protein level and were, therefore, not included in our analysis. A computer algorithm that allows interlocus AA sequence comparison for the β chains of HLA-DRB1/3/4/5 and intralocus AA subtraction for the α and β chains of HLA-DQA1 and -DQB1 alleles was then used to determine the number, position, and nature of polymorphic AA for each mismatched HLA-DR and -DQ specificity.

Determination of Hydrophobicity and Electrostatic Mismatch Scores

For each AA disparity identified earlier, mismatched AAs were each assigned a hydrophobicity value using the Hopp-Woods scale (*27*), and hydrophobicity mismatch value was determined as the difference in AA hydrophobicity values. Similarly, the difference between the isoelectric points (pI) of each mismatched AA was used to determine electrostatic mismatch values. In cases where the patient HLA class II type carried several AA polymorphisms at the same position, the lowest AA hydrophobicity mismatch value and lowest pI mismatch value were used in the analysis of immunogenicity. For each mismatched HLA-DR and -DQ specificity, the AA hydrophobicity and pI mismatch values were summed to give a total HMS and a total EMS, as described previously (*13*).

Statistical Analysis

The outcomes of interest were the occurrence (normalized MFI$\geq$1500) and magnitude (maximum MFI) of an antibody response to each mismatched HLA-DR and -DQ specificity represented on the single-antigen beads and their relationship to the total number of AA mismatches (AAMs), HMS, and EMS. Because of the high correlation among AAM, HMS, and EMS, we concentrated our analyses mainly on AAM. Logistic regression models were used to investigate the relationship between the occurrence of an alloantibody response (normalized MFI$\geq$1500) and the number of AAM. Previous studies (*12*, *13*) and exploratory analysis in this study showed that the optimal relationship between these variables was obtained using the log of the total number of AAM, that is, fitted the model

$$\text{logit}(p) = a + b \times \log(\text{number of mismatches} + 1),$$

where p is the proportion of positive responses and the coefficients a and b are estimated from the data (we add 1 to the total number of mismatches as log of zero is not defined). Results are presented as odds ratios and 95% confidence intervals, and significance levels relate to likelihood ratio tests comparing models with and without each variable.

The distribution of MFI values obtained against mismatched HLA-DR and -DQ specificities was skewed because of the high number of values at zero, and some very high values in patients with an antibody response. Therefore, we performed median regression of the MFI values to give more robust estimates of the relationship with AAM. Values are summarized as median and IQR, and significance levels are taken from Wald tests.

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