Public Epitope Specificity of HLA Class I Antibodies Induced by a Failed Kidney Transplant: Alloantibody Characterization by Flow Cytometric Techniques

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Background. Patients whose kidney grafts fail develop alloantibodies that react with many HLA molecules. We analyzed the epitope specificity of HLA class I alloantibodies in the sera of 55 patients who had been sensitized by kidney grafts, and investigated the immunogenicity of various polymorphic epitopes.

Methods. HLA class I alloantibodies were detected and characterized by flow cytometry (FlowPRA beads). Potential "immunizing epitopes" were identified by comparing the amino acid sequences of HLA class I antigens/alleles of the donor, recipient and the antibody-reactivity pattern.

Results. In the 55 anti-HLA class I-positive patients, 82 different antibody reactivity patterns were identified; all but 5 (94%) were determined by a "public epitope" of donor HLA-A and/or -B molecules. Forty-five of 50 patients who showed HLA-A Res-MMs with their donors produced HLA-A antibodies, but only 31 of 51 subjects with HLA-B Res-MMs produced HLA-B antibodies ($P=0.001$; O.R.=5.81). The antibody patterns were specific for a "single" epitope of the mismatched donor molecules in 91% of patients. Forty-three of the 120 (36%) mismatched HLA-A and/or -B epitopes were positively correlated with antibody production. The polymorphic determinants of higher immunogenic capacity were b80N (Bw6-associated) and ab82–83LR (Bw4-associated) public epitopes.

Conclusions. The humoral immune response against a kidney graft mainly produces HLA class I antibodies specific for "public epitopes" of mismatched donor molecules. A "single" donor-epitope may determine the production of a spread antibody pattern. In renal transplantation, epitope matching is better than HLA antigen matching for avoiding or minimizing development of HLA antibodies.

Keywords: HLA epitopes, Residue matching, Kidney transplant, Alloantibody, Flow cytometry.

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Several reports have described the beneficial effect of HLA matching on long-term graft survival in kidney transplantation (*1–4*). Conventional donor/recipient HLA-A, -B and -DR compatibility takes into consideration six serologically defined HLA antigens, that is to say, six highly polymorphic "private epitopes" of specific HLA antigens. On the other hand, it has been shown that the humoral immune response against the mismatched HLA antigens of the graft determines the production of antibodies that react with multiple HLA specificity (*5–6*). The development of this wide variety of antibodies, especially HLA class I antibodies, can be understood by analysis of the molecular structure of class I antigens (*7*). The high polymorphism of the major histocompatibility complex is not reflected in the number of HLA antigens. Information on the amino acid sequences of HLA class I antigens has resulted in the identification of multiple polymorphic determinants that act as alloantigens. Some of these determinants, called "public epitopes", are shared by two or more HLA molecules, while others, defined as "private epitopes", represent epitope configurations unique to individual HLA class I antigens (*8*). As a result, the high percentage of panel-reactive antibodies (% PRA) in patients whose kidney grafts fail (*9*) may be caused by a single antibody directed against widely shared "public epitopes", rather than by many different antibodies specific for individual HLA molecules (*10–11*). It has been shown that matching for "public epitopes" shared by HLA antigens belonging to cross-reacting antigen groups is associated with an allograft survival rate similar to that of classical HLA private antigen matching (*12–13*). Thus, the immunogenicity of a kidney graft is related to differences in amino acid sequences of HLA molecules between donor and recipients.

The aim of this study was to use sensitive, allele-specific FlowPRA class I Single Antigen beads (*14*) to characterize HLA class I donor-specific antibodies (DSA) in the sera of patients who had previously received kidney grafts. By analyzing the amino acid sequences of serologically and/or genomically defined HLA class I antigens of the donor and recipient, and the alloantibody reactivity pattern, we were able to define the epitope specificities of the HLA class I DSA. For each patient, we also defined the number and type of potential "immunizing epitopes" and then investigated the immunogenicity of various mismatched HLA-A and -B epitopes.

MATERIALS AND METHODS

Patients

The study population consisted of 84 patients who had been alloimmunized by kidney transplants. Forty-six patients (55%) had re-entered the transplant waiting list after graft

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failure, 13 (15%) lost their graft during our follow-up and the remaining 25 (30%) suffered from chronic allograft dysfunction.

Presensitization status was investigated by using a flow cytometry based technique. Out of 84 positive subjects, only 9 were found to be PRA positive, although none showed HLA DSA. Pretransplant crossmatch was performed by using complement dependent lymphocytotoxicity in PRA negative subjects, while both lymphocytotoxicity and flow cytometry were used in the PRA positive ones.

All patients were followed up for at least six months after the start of DSA production (mean follow-up ± SD=44.7±31.7 months). Serological HLA-A, -B, -C and -DR typing information of donors and recipients were available. When it was necessary to confirm that the detected HLA class I DSA were specific for the mismatched HLA antigens of the graft, low and/or high resolution PCR-SSP was used to define patient and/or donor HLA class I alleles.

Anti-HLA Antibody Screening

In total, 768 serum samples were tested for IgG anti-HLA PRA using FlowPRA class I and class II screening beads (One Lambda, Canoga Park, CA), which consisted of a pool of 30 different bead populations each coated with purified HLA class I antigens derived from a single cell line (*15*). Briefly, class I and class II beads (5 μl of each type) were incubated with 20 μl of patient serum for 30 min at room temperature. After two washes in wash buffer, 100 μl of properly diluted FITC-conjugated goat anti-human IgG was added to each tube and samples were incubated for 30 min in the dark. Finally, the tubes were washed twice, after which 0.5 μl of fixing solution was added. Negative (FL-NC, One Lambda) and positive (FL1- and FL2-PC, One Lambda) control sera were included in each assay. Flow cytometric analysis was performed using FACSCalibur and Cell Quest software (Becton Dickinson, Mountain View, CA). At least 10,000 beads were collected from each tube. Two gates were set on the dot plot of SSC (side scatter) vs. FL2 (fluorescence 2) to analyze class I (FL2-negative particles) and class II (FL2 high-fluorescence particles) beads separately.

The percentage of beads that shifted to the right of the cut-off point set on the FL1 histogram (anti-human IgG) of the negative control serum represented the amount of class I and/or class II antibodies (=%PRA) of the test sample.

Flow Cytometric Identification of Anti-HLA Class I DSA

To determine if the detected HLA class I antibodies were specific for the mismatched donor antigens, at least two FlowPRA class I-positive sera for each patient were analyzed using HLA class I Single Antigen beads (One Lambda). This assay consisted of 64 different bead populations coated with purified (or recombinant) HLA-A or -B single antigens and divided into eight groups (sets 1–8). Each set of beads contained eight different bead populations that can be separated from each other by the FL2 channel of a flow cytometer because each bead population has a unique FL2 channel-shift (*14*). The testing procedure was the same as that used for the FlowPRA Screening test. Briefly, each set of beads was incubated separately with the test sample, negative and positive control sera, and then stained with FITC-conjugated goat anti-human IgG. Flow cytometric analysis was performed by gating each bead population on a FL1 vs. FL2 dot plot of the negative control.

A positive reaction of the test serum was indicated by a FL1 shift of beads to the right of the gate set using the negative control sample. It is noteworthy to underline that this technique allows to define the allele-specificity of DSA. Table 1 lists the HLA-A and -B alleles present on the Single Antigen beads used in this study and, when appropriate, the HLA equivalents.

Identification of Immunizing Epitopes

To identify mismatched donor epitopes that were recognized as "non-self" by the recipient immune system and which consequently determined DSA production, we first determined the amino acid sequences of the HLA-A, -B and -Cw antigens/alleles of donors and recipients using an online database (www.ebi.ac.uk/imgt/hla/index/html). In this way, we were able to define amino acid mismatches, that is, donor/recipient residue mismatches (Res-MMs).

To determine the number and type of "potential" immunizing epitopes, which might be composed of one or more amino acid residues located in close proximity to each other, we also identified the amino acid sequences of HLA-A and -B alleles corresponding to antibody reactivity patterns. In defining donor/recipient Res-MMs, we only considered amino acid substitutions on extracellular portion of HLA class I molecules. Table 2 shows an example of this methodological approach.

We also investigated the immunogenic capacity of the 120 mismatched HLA-A and/or -B epitopes by analysis of the ratio between the number of sensitizations due to a specific epitope and the number of MMs for the same residues.

Statistical Analysis

Data analysis was performed using Fischer's exact test and the Mann–Whitney test or the unpaired *t* test (Welch corrected) when appropriate (Instat Software; GraphPad Software Inc., San Diego, CA). A *P* value of 0.05 or less was considered significant.

TABLE 1. HLA-A and -B alleles that coated the single antigen beads used for the antibody identification

	Specificity of single antigen beads
HLA-A	*0101, *0201, *0301, *1101, *2301, *2402, *2501, *2601, *2902, *3001, *3101, *3201, *3301, *3401, *3601, *4301, *6601, *6801, *6901, *7401, *8001.
HLA-B	*0702, *0801, *1301, *1401 (B64), *1402 (B65), *1501 (B62), *1502 (B75), *1503 (B72), *1510 (B71), *1512 (B76), *1513 (B77), *1516 (B63), *1801, *2705, *3501, *3701, *3801, *3901, *4001 (B60), *4002 (B61), *4102, *4201, *4402, *4501, *4601, *4701, *4801, *4901, *5001, *5101, *5201, *5301, *5401, *5501, *5601, *5701, *5801, *5901, *6701, *7301, *7801, *8101, *8201.

The HLA equivalents are quoted in parentheses when appropriate.

TABLE 2. Example of the method used for the identification of the "immunizing epitope"

		Amino-acid residues						
	HLA antigens/alleles	**44**	**67**	**150**	**152**	**158**	**166**	**167**
Donor MM	A1	K	M	V	A	V	**D**	**G**
	A3	R	V	A	E	A	E	W
	A26	R	V	A	E	A	E	W
Recipient	B35	R	F	A	V	A	E	W
HLA class I typing	B55	R	Y	A	E	A	E	W
	Cw3	R	Y	A	E	A	E	W
	Cw4	R	Y	A	E	A	E	W
Antibody reactivity pattern	A1	K	M	V	A	V	**D**	**G**
	A23	R	V	A	V	A	**D**	**G**
	A24	R	V	A	V	A	**D**	**G**
	A80	R	V	A	R	A	**D**	**G**

The number and type of Res-MMs were determined from analysis of amino acid sequences of donor HLA-A or -B MMs and recipient HLA-A, -B and -Cw antigens/alleles Reg-MMs, that acted as "immunizing epitope," were identified from analysis of the amino acid sequence of antigens corresponding to the antibody pattern. In this example, the HLA-A1 MM with the donor determined the antibody pattern (A1, A23, A24 and A80) because of recognition of the "166-167DG" mismatched donor epitope.

RESULTS

Results of HLA antibody screening showed that 21 (25%) of the 84 patients screened for DSA production produced only HLA class I antibodies, whereas 34 (40%) produced both HLA class I and HLA class II antibodies. HLA class II antibodies were exclusively present in the sera from the remaining 29 (35%) subjects.

In the 55 anti-HLA class I-positive patients, antibody characterization by FlowPRA class I Single Antigen beads revealed a total of 82 antibody reactivity patterns; 50 of these were due to the recognition of HLA-A Res-MMs, 29 to HLA-B Res-MMs and the remaining three were specific for residues common to HLA-A and -B donor molecules. None of the patients developed antibodies against HLA epitopes of their own HLA class I antigens/alleles.

The correlation between HLA-A and/or -B Res-MMs and anti-HLA antibody production showed that all but five patients who had HLA-A Res-MMs with their donors produced HLA-A antibodies. In contrast, only 31 of the 51 subjects with HLA-B Res-MMs were positive for HLA-B antibodies ($P=0.001$; O.R.$=5.81$). Moreover, the incidence of HLA-A Res-MMs in anti-HLA-A positive patients was greater than the incidence of HLA-B Res-MMs in anti-HLA-B positive patients (mean value $\pm$ SD, min-max values: 10.7 ± 6.3, 1–33 vs. 7.8 ± 4.2, 1–20; $P=0.0003$). Conversely, there was no difference in the incidence of Res-MMs between the anti-HLA-positive and anti-HLA-negative patients.

Tables 3 and 4 summarize potential immunizing epitopes of the mismatched HLA-A and -B donor molecules. HLA antigens/alleles, MMs and antibody reactivity patterns are reported as HLA equivalents for all patients except patient 9, who was typed as B*4403 and produced B*4402-specific antibodies. In some instances, we also detected HLA antibodies that were related to the patients' presensitization status (data not shown).

Tables 3 and 4 show that 76 of the 82 antibody patterns (93%) were due to recognition of a "public epitope" of donor HLA class I molecules. Antibodies specific for the "private epitopes" of the mismatched donor antigens were detected in four patients (numbers 4, 19, 25 and 46) for HLA-A antibodies and in two patients (numbers 25 and 28) for HLA-B antibodies.

Analysis of the number of HLA-A and/or -B donor epitopes involved in the production of alloantibodies showed that in 50 of the 55 (91%) anti-HLA-positive patients, alloantibody development was caused by recognition of a single epitope (composed of one or a few amino acid residues located in close proximity to each other) of the mismatched donor HLA-A and/or -B molecules. In four cases (patients 32, 39, 54 and 55), antibody patterns were determined by recognition of two or three scattered epitopes of the donor HLA-A molecule. It was not possible to define the potential immunizing epitopes of patient 3, who produced an antibody pattern (anti-A1 and anti-A36 antibodies) specific for three different epitopes (44K, 150V and 158V) of the donor HLA-A1 molecules. In three patients, an epitope shared by donor HLA-A and -B molecules (the 82–83LR epitope in patients 14 and 53 and the 62G epitope in patient 17) might be related to both anti-HLA-A and -B antibody development.

Forty-three (36%) of the 120 mismatched epitopes were positively correlated with antibody production (Table 5); 18 (42%) were HLA-A specific residues, 12 (28%) were characteristic of HLA-B molecules and the remaining 13 (30%) were common to HLA-A and -B. Considering only epitopes that showed MMs and/or positive correlations >1, a high incidence of positive correlations was found for b80N (100%), ab82–83LR (75%), a151-H (67%), ab62-G (50%), a127K (38%), ab80I (37%), a62Q (33%) and ab81A (29%) "public" epitopes, and for a161-D (60%) private epitope. It is noteworthy that in three patients (21, 24 and 33), Res-MMs for the 82–83LR Bw4-associated epitope were due to differences in the amino acid sequences of HLA-A molecules only. Moreover, in patients 10 and 37, amino acidic residue variation within the HLA-Bw4 complex of HLA-B molecules determined a particular pattern of alloantibodies related to the recognition of the 80-I Res-MM.

Despite the high incidence of MMs for several of the remaining 77 (63%) epitopes (28 of HLA-A, 34 of HLA-B and 14 of both) (Table 6), they were not associated with antibody development.

TABLE 3. Detected HLA-A antibody patterns and potential immunizing epitope/s

Patient	Antigen MMs	Antibody reactivity patterns	Res-MMs (No.)	Immunizing epitopes
1	A**31**	A25, 26, 29, **31**, 32, 33, 34, 43, 66, 74	10	246S
2	A**24**	A23, **24**	17	65G
3	A**1**	A**1**, 36	17	44K, 150V, 158V
4	A**3**	A**3**	8	161D
5	A**68**	A1, 3, 11, 25, 26, 29, 30, 31, 32, 33, 34, 36, 43, 66, **68**, 69, 74, 80; B13, 35, 37, 44, 53, 57, 58, 75, 77	6	95I
6	A**3**	A1, **3**, 11, 25, 26, 29, 30, 31, 32, 33, 36, 43, 66, 68, 69, 74, 80; B57, 58, 63	10	66N
8	A**26**	A25, **26**, 32, 33, 34, 43, 66, 74	6	114Q
9	A**23**	A1, **23**, 24, 80	10	166–167DG
11	A**1**	A**1**, 23, 24, 80	8	166–167DG
13	A**26** A**30**	A1, 3, 11, 25, **26**, 29, 31, 32, 33, 34, 36, 43, 66, 68, 74, 80; B27, 44, 47A1, 3, 11, **30**, 31, 32, 36, 74	19	116D 62Q
14	A**24**	A23, **24**, 25, 32; B38, **49**, 51, 52, 53, 57, 58, 59, 63, 77	15	80–83IALR
16	A**31**	A1, 3, 11, 30, **31**, 32, 36, 74	7	62Q
17	A1, **2**	A**2**; B**57**, B58	19	62G
18	A**68**	A2, 23, 24, **68**, 69	5	127K
19	A**3**	A**3**	1	161D
20	A**1**	A**1**, 26, 29, 36, 80	12	76A
21	**A24**, 34	A23, **24**, 25, 32; B38, 49, 51, 52, 53, 57, 58, 59	13	80–83IALR
22	A**29** A**2**	A2, 23, 24, **29**, 30, 31, 33, 68, 69, 80; A**2**; B57, B58	14	105S 62G
24	A**24**	A23, **24**, 25, 32; B38, 49, 51, 52, 53, 57, 58, 59	16	80–83IALR
25	A**3**	A**3**	2	161D
26	A**24**	A2, 23, **24**, 68, 69	12	127K
29	A**11**	A1, **11**, 25, 26, 43, 66, 80	10	163R
30	A**2**, 74	A**2**, 68, 69	12	142T, 145H
31	A**24, 68**	A2, 23, **24**, 68, 69	20	127K
32	A**31**	A1, 3, 11, 30, **31**, 32, 36		62Q
	A**2**	A**2**, 23, 24, 68, 69 A**2**; B57, B58	18	127K 62G
33	A**1** A**24**	A**1**, 2, 3, 11, 24, 25, 26, 34, 36, 43, 66, 68, 69 A23, **24**, 25, 32; B38, 49, 51, 52, 53, 57, 58, 59	33	151H 80–83IALR
34	A**30**	A2, 3, 11, **30**, 31, 33, 34, 66, 68, 69, 74; B37, 47	13	77D
35	A**24**	A2, 3, 11, 23, **24**, 25, 26, 29, 30, 31, 32, 33, 34, 66, 68, 69	12	67V
36	A**1**	A**1**, 3, 11, 31, 32, 36, 74	13	62Q
37	A**2**	A**2**, 23, 24, 68, 69	11	127K
38	A**11**	A1, **11**, 25, 26, 43, 66	2	163R
39	A**2**	A**2**, 68, 69 A**2**; B57, B58	7	142T, 145H 62G
40	A**26**	A25, **26**, 29, 31, 32, 33, 34, 43, 66	13	246S
43	A**24**	A1, 23, **24**, 80	17	166–167DG
44	A**26**	A25, **26**, 34, 66	9	149T
45	A**1**	A**1**, 2, 3, 11, 24, 25, 26, 34, 36, 43, 66, 68, 69	13	151H
46	A3, **29**	A**29**	13	102H
48	A**1**, 66	A1, 23, 24, 80	17	166–167DG
49	A**2**	A**2**, 3, 23, 24, 29, 30, 31, 32, 33, 34, 68, 69, 74; B8, 18, 38, 39, 41, 42, 54, 55, 59, 64, 65, 67	13	163T
50	A**3**	A1, **3**, 11, 25, 26, 29, 30, 31, 32, 33, 36, 43, 66, 68, 69, 74, 80; B57, 58, 63	8	66N
51	A**1**, 11	A**1**, 26, 29, 36, 43, 80	12	76A
52	A**1**, 2	A**1**, 26, 29, 36, 43	9	76A
53	A2, **32**	A23, 24, 25, **32;** B38, 49, 51, 52, 53, 57, 58, 59, **63**, 77	18	80–83IALR
54	A**1**	A**1**, 11, 25, 26, 43, 66 A**1**, 23, 24, 80	10	163R 166–167DG
55	A**2**, 30	A**2**, 23, 24, 68, 69 A**2**, B57, B58	11	127K 62G

DISCUSSION

Almost all patients who reject renal transplants develop alloantibodies against the HLA incompatibilities of graft cells (*9*, *17*). In this study, we used HLA-allele-specific FlowPRA Single Antigen beads to accurately characterize HLA class I antibodies in the sera of patients who had rejected previous kidney grafts. This analysis allowed us to identify all the anti-HLA-A and/or -B antibodies that developed as a result of graft

TABLE 4. Detected HLA-B antibody patterns and potential immunizing epitopes

Patient	Antigen MMs	Antibody reactivity patterns	Res-MMs (No.)	Immunizing epitopes
1	B**60**	B7, 8, 13, 18, 35, 38, 39, 41, 42, 46, 48, 51, 52, 53, 54, 55, 56, 57, 58, 59, **60**, 61, 62, 63, 67, 71, 72, 76, 78, 81, 82	7	113-H
4	B**50**, **61** (**Bw6**)	B7, 8, 18, 35, 39, 41, 45, 46, 48, **50**, 54, 55, 56, 60, **61**, 62, 64, 65, 67, 71, 72, 73, 75, 76, 78, 81, 82	9	80-N
5	B**51** (Bw4)	35, 44, 45, 46, 49, 50, **51**, 52, 56, 57, 58, 62, 63, 71, 72, 75, 77, 78, 82	6	163-L
7	B**18** (**Bw6**)	B7, 8, **18**, 35, 39, 41, 45, 46, 48, 50, 54, 55, 56, 60, 61, 62, 64, 65, 67, 71, 72, 73 (only 80-N), 75, 76, 78, 81, 82; A25 (only 77-S), 32 (only 77-S)	4	77-S, 80-N
8	B38, **51**	B18, 35, 37, **51**, 52, 58, 78	7	45-T
9	B**45**	B8, 37, 41, 42, 44, **45**, 82	5	156-D
10	B**51**	B38, 49, **51**, 52, 53, 57, 58, 59, 77; A23, 24, 25, 32	5	80-I
12	B13, **39** (**Bw6**)	B7, 8, 18, 35, **39**, 41, 45, 46, 48, 50, 54, 55, 56, 60, 61, 62, 64, 65, 67, 71, 72, 73, 75, 76, 78, 81, 82	1	80-N
13	B**44**	B13, 27, 41, **44**, 45, 47, 49, 50, 60, 61, 73	11	24-T
14	B**49** (**Bw4**)	B13, 27, 37, 38, 44, 47, **49**, 51, 52, 53, 57, 58, 59, 63, 77; A23, **24**, 25, 32	12	82–83LR
	B51 (Bw4)	B7, 8, 18, 35, 38, 39, 42, **51**, 53, 54, 55, 56, 59, 64, 65, 67, 71, 73, 75, 78, 81, 82; A25, 26, 33, 34, 66, 68, 69		63-N
15	B**14**, **18** (**Bw6**)	B7, 8, **18**, 35, 39, 41, 45, 46, 48, 50, 54, 55, 56, 60, 61, 62, **64**, **65**, 67, 71, 72, 73, 75, 76, 78, 81, 82	12	80-N
17	B**57**	B**57**, 58; A**2**	5	62-G
22	B14, **53** (**Bw4**)	B13, 27, 37, 38, 44, 47, 49, 51, 52, **53**, 57, 58, 59, 63, 77; A23, 24, 25, 32	4	82–83LR
23	B**44**, **51** (**Bw4**)	B13, 27, 37, 38, **44**, 47, 49, **51**, 52, 53, 57, 58, 59, 63, 77; A23, 24, 25, 32	20	82–83LR
24	B**35**	8, 13, **35**, 41, 44, 45, 47, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 62, 63, 71, 75, 76, 77, 78, 82	4	12-M
25	B**8**	**8**	7	9-D
26	B**60**	7, 13, 27, 47, 48, **60**, 61, 73, 81; A80	3	163-E
27	B**8**	7, **8**, 13, 18, 27, 35, 37, 38, 39, 41, 42, 44, 45, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 59, 60, 61, 62, 64, 65, 67, 73, 75, 76, 77, 78, 81, 82	14	66-I
28	B**18**	**18**	4	30-G
35	B**27**	7, 13, **27**, 48, 60, 61, 73, 81; A80	6	163-E
37	B**51**	B38, 49, **51**, 52, 53, 57, 58, 59, 63, 77; A23, 24, 25, 32	6	80-I
38	B**7**	B**7**; A1, 36	7	156-R
41	B**38** (Bw4)	B**38**, 39	4	158-T
42	B**51** (**Bw4**)	B13, 27, 37, 38, 44, 47, 49, **51**, 52, 53, 57, 58, 59, 63, 77; A23, 24, 25, 32	7	82–83LR
43	B**38**, **53** (**Bw4**)	B13, 27, 37, **38**, 44, 47, 49, 51, 52, **53**, 57, 58, 59, 63, 77; A23, 24, 25, 32	10	82–83LR
44	B**51** (**Bw4**)	B13, 27, 37, 38, 44, 47, 49, **51**, 52, 53, 57, 58, 59, 63, 77; A23, 24, 25, 32	9	82–83LR
45	B**8**	B**8**, 37, 41, 42, 4402, 45	6	156-D
47	B**8**	B7, **8**, 42, 61, 81	10	97-S
49	B**18**, **45**	B8, 13, **18**, 27, 35, 37, 38, 39, 41, 42, 44, **45**, 47, 48, 53, 54, 56, 57, 58, 59, 60, 61, 67, 81, 82; A2, 23, 24, 29, 31, 32, 33, 68, 69, 74	14	152-V
50	B**7**, 21	B**7**, 13, 27, 47, 48, 60, 61, 73, 81; A80	13	163-E
53	B**63** (**Bw4**)	B13, 27, 37, 38, 44, 47, 49, 51, 52, 53, 57, 58, 59, **63**, 77; A23, 24, 25, **32**	9	82–83LR

sensitization. It is important to emphasize that the FlowPRA class I Single Antigen technique allowed us to identify most of the antibodies specific for antigens sharing the same immunizing epitopes in each serum sample.

Contrary to a recent report (*18*), our study showed that only five of the 55 (9%) anti-HLA class I-positive patients developed alloantibodies with specificity restricted to the mismatched HLA-A or -B antigen/s of the graft, that is to say alloantibodies specific for the "private" epitopes of the mismatched donor HLA class I molecules. A broad HLA-A and/or -B sensitization was found in more than 90% of the study population. These findings strongly confirm previous observations (*19–20*) that most anti HLA class I antibodies detected in serum samples from sensitized patients are specific for epitopes shared by different HLA antigens, the so called "public" epitopes.

TABLE 5. HLA-A (a) and/or -B (b) mismatched donor epitopes (n=43) that had positive correlations with antibody production

Immunogenic residues	MMs (No.)	Positive correlations (No.)	Positive correlations (%)
b9D	2	1	50
b12M	3	1	33
b24T	11	1	9
b30G	5	1	2
a44K	12	1	8
b45T	6	1	17
ab62G	12	6	50
a 62Q	12	4	33
ab63N	4	1	25
a 65G	9	1	11
b66I	1	1	100
ab66N	6	2	33
a 67V	1	1	100
a 76A	13	2	15
ab77D	1	1	100
ab77S	4	1	25
ab80I	19	7	37
b80N	4	4	100
ab81A	17	5	29
ab82–83LR	16	12	75
b97S	8	1	13
a 102H	1	1	100
a 105S	5	1	20
b113H	2	1	50
a 114Q	6	1	17
ab116D	19	1	5
a 127K	16	6	38
a 142T, 145H	11	1	9
a 149T	5	1	20
a 150V	13	1	8
a 151H	3	2	67
ab152V	3	1	33
b156D	11	2	18
ab156R	5	1	20
b158T	8	1	13
a 158V	13	1	8
a 161D	5	3	60
ab163E	10	3	30
b163L	6	1	17
a 163R	13	3	23
ab163T	2	1	50
a166–167DG	16	3	19
a 246S	7	2	29

TABLE 6. The 77 mismatched donor epitopes that only had negative correlations with antibody production

Nonimmunogenic residues	MMs (No.)
a9F/S/T, b9H/Y	34
a17S	2
b24S	4
b32L	10
b41T	11
b45E/K	20
b46A	4
a56R	5
a62E/L	11
a63Q	1
ab65R	2
ab66K	15
b67C/F/S, ab67M	43
b69T	2
a70H, b70K/N/S	10
b71T	2
a73I	2
a74H, b74Y	11
ab76V	1
ab77N	10
a79G	3
ab80T	2
a90D	4
b94I	5
a95V, b95W, ab95L	21
a97I/M, b97N/T	34
ab99F	8
b103L	8
a107W	9
a114E/R, b114N, ab114H	39
a116H, b116F/L/S, ab116Y	24
b131S	1
b143S	4
a144K	6
b145L	3
b147L	3
a152A/W, ab152E	15
a156Q, ab156W	18
b167S	5
ab171H	7
b177D	9
b178K	6
b180E	9
a184A	5
a193A	3
ab194I	6
b199V	4
a207S	4
a245V	3

This epitope specificity of anti-HLA class I antibodies was well represented in patient 9. To elucidate the antibody pattern of this patient, we performed high-resolution genomic typing of the B44 and B45 antigens of the patient and donor, respectively. HLA-B alleles corresponding to the antibody pattern (B*0801, B*3701, B*4102, B*4402, B*4501, B*8201) shared the 156D epitope with the donor's B*4501, but this epitope was mismatched with the patient's B*4403.

In our study, there were more HLA-A sensitized patients than HLA-B sensitized patients. This might have been due to the higher incidence of HLA-A Res-MMs in our patient population, which might have resulted from the HLA-DR, -B, -A matching priority used for organ assignment. Our data did not confirm previous evidence (*21*) of a strong correlation between the number of Res-MMs and antibody production. Although ours was a relatively small patient population, we did not detect any difference in the number of Res-MMs between the DSA-positive and DSA-negative groups of patients. This support the aforementioned hypothesis concerning the lack of correlation between the number of Res-MMs and DSA development. In fact, we detected production of HLA class I DSA not only in patients with high

numbers of HLA-A and/or -B Res-MMs with donors, but also in patients who exhibited one or few Res-MMs.

As HLA epitopes are considered the basic units of immunogenicity (*19*), we performed an epitope analysis of HLA class I antibodies which had been defined using the sensitive FlowPRA bead assay. We demonstrated that, in 91% of our patients, antibody patterns were due to recognition of a "single" epitope of the mismatched HLA-A and/or -B molecules of the donor, even when many donor/recipient Res-MMs were present.

Moreover, alloantibodies mainly identified a "single amino acid" epitope. The recognition by the recipient immune system of a unique Res-MM with donor HLA-A and-B molecules, as was the case for patient 12 (Table 4), was sufficient to give rise to the production of a spread antibody pattern. These findings are consistent with data of Park (*22*), who concluded that a "single amino acid" epitope could account for the development of multispecific sera.

Unexpectedly, the same antigen mismatch resulted in the production of different antibody patterns because of the recognition of different epitopes of the mismatched HLA molecule. This might be partially explained by differences in specific amino acid sequences of HLA molecules between the donor and recipient. In fact, analysis of Res-MMs and immunizing epitopes of the 8 patients who developed anti-HLA antibodies as consequence of HLA-A2 mismatched kidneys, showed that all these patients had MMs for the 142T 145H epitope, but only two of them developed antibody patterns specific for this epitope (data not shown).

Our results also show that patients who rejected renal transplants did not always develop HLA antibodies against all the HLA class I mismatches of the graft. This is in agreement with the study of van Kampen (*22*), which showed that it is not always necessary to exclude repeated HLA class I mismatches for a subsequent transplantation because of the lack of primed cytotoxic T cells against all individual HLA class I donor–recipient mismatches.

Taken together, these findings suggest that the recipient humoral response to alloantigens is specific for the epitopes presented to alloreactive T cells. In fact, the development of a humoral response against the graft is determined by many factors, such as differences in specific amino acid sequences between HLA alleles of the donor and recipient, affinity of peptide binding to HLA molecules, competition between various peptides for binding to HLA class II molecules (*24*) and the capacity of the individual to develop specific alloreactive T cells (*25*).

Recent studies also showed that the immunogenicity of an HLA mismatch is affected by the HLA phenotype of the recipient (*26–27*) and that the development of antibodies against a particular HLA class I mismatch depends on the HLA-DR allele of the patient (*28–30*).

Analysis of this HLA-DR "restriction" of the humoral immune response to HLA class I mismatches in our patients (data not shown) showed a high incidence of DR1 and DR4 in patients who produced anti-Bw4 alloantibodies, which is in agreement with data of Fuller and Fuller (*28*). We also found a high incidence of DR6 in patients sensitized by a previous graft; this sensitization was mainly due to A1, A2 and A19 mismatches.

Finally, when we investigated the immunogenicity of the mismatched HLA-A and/or -B epitopes, we only detected antibodies to 36% of mismatched residues. This finding supports the theory that a small number of mismatched donor epitopes are recognized by the recipient immune system and that these determine alloantibody development.

The majority of immunizing residues was located inside alpha helices or loops of the HLA class I molecules but some were also found inside beta pleated sheets. Several considerations can be made regarding this last finding. In some cases, the produced antibody patterns could only be due to the recognition of one of those normally unexposed epitopes. During the indirect mechanism of allorecognition, recipient's APCs process and present graft derived HLA peptides which may contain unexposed epitopes. This could therefore lead to the production of specific antibodies which could nonetheless be clinically irrelevant since incapable of binding to their target epitope. On the other hand, conformational changes in the HLA molecule due to its binding to the peptide, may expose normally inaccessible residues.

High frequencies of positive correlations were observed for Bw4- and Bw6-associated epitopes (75% and 100%, respectively). These polymorphic determinants are located between amino acid positions 77 and 83 on the α_1 domains of HLA class I molecules and are alternately expressed on all HLA-B molecules. The Bw4-associated epitope is also expressed on a few HLA-A molecules (A23, A24, A25 and A32). Our data demonstrated the development of an antibody pattern specific for the Bw4-associated epitope in patients that exhibited Res-MMs either for the 82–83LR epitope of donor HLA-B molecules or for the 80–83IALR epitope of donor HLA-A molecules. The production of a particular antibody pattern specific for a unique residue (80-I) characteristic of a subgroup of HLA-B molecules bearing the Bw4 motif emphasizes the need to determine the epitope specificity of a detected alloantibody rather than its antigen specificity.

Lastly, we showed that in some cases, the type of amino acid at a particular position determined the immunogenicity of the corresponding epitope: in the case of position 62, the presence of glycine (G) or glutamine (Q) determined high immunogenicity (Table 5) while the presence of glutamic acid (E) or leucine (L) was not associated with antibody development (Table 6).

In conclusion, this study demonstrated that patients who were alloimmunized by a previous kidney graft produced HLA class I alloantibodies that were mainly specific for "public epitopes" of mismatched donor molecules. Independently of the number of donor–recipient Res-MMs, the recognition of a "single" donor epitope determines the formation of spread antibody patterns.

Identification of sensitizing donor epitopes and application of an amino acid-based matching could enhance organ allocation in re-transplant patients and significantly prolong survival of the second graft by preventing organ damage due to circulating alloantibodies, even if present at low levels or produced de novo by memory B cells. Moreover, the use of an amino acid-based matching for the first kidney transplant could minimize HLA "diversity" in the evaluation of donor-recipient HLA matching and consequently reduce the incidence of acute or chronic allograft rejection.

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