ELSEVIER

The Number of Amino Acid Residues Mismatches Correlates With Flow Cytometry Crossmatching Results in High PRA Renal Patients

A. L. Lobashevsky, R. W. Senkbeil, J. L. Shoaf, A. K. Stephenson, S. B. Skelton, R. M. Burke, M. H. Deierhoi, and J. M. Thomas

ABSTRACT: Highly sensitized renal transplant candidates present a group at high risk for acute and chronic rejection. The probability of finding compatible donors for these recipients is significantly lower in comparison to those who have low PRA values. As a consequence, these patients spend longer time on the waiting list and become tethered to dialysis. The results of final cross match (XM) are critical for making a decision about whether such a candidate receives an organ or not. The degree of donor and recipient HLA compatibility predicts the results of XM. The goal of this study was to expand a variety of acceptable HLA-AB mismatches (MM) for high PRA kidney recipients using the HLAMATCHMAKER algorithm. This strategy focuses on the fine structural features of HLA polymorphism comprising amino acid residues or triplets (AAT), which are located in α-helical coils of HLA molecules and are available to antibodies. We analyzed serum samples from thirty-nine highly alloimmunized recipients (PRA $\geqslant$ 85%). The level of sensitization was detected using FlowPRA Class I Screening Test. This group of transplant candidates included thirteen recipients who demonstrated negative results of final T/B FCXM and twenty-six, who were FCXM positive. The application of the HLAMATCHMAKER algorithm based on the HLA class I donor and recipient typing allowed us to detect the total number of AATMM as well as the number of immunogenic AAT in both FCXM negative and FCXM positive groups of recipients. Significantly greater numbers of both total and highly immunogenic AATMM have been emerged in the group of FCXM positive patients. Furthermore, the results of this analysis have shown a high degree of probability of positive FCXM if the number of highly immunogenic AATMM was $\geqslant 1$ ($\chi^2 = 22.9$ Yate's correction; $p = 0.000001$). We did not observe overlapping between antibody specificity and permissible HLA-AB MM detected using the HLAMATCHMAKER strategy. Thus, the number of highly immunogenic AATMM can serve as a reliable predictive value for final FCXM results in highly sensitized renal transplant candidates. The HLAMATCHMAKER algorithm appears to be the proper strategy to find donors for high PRA recipients. *Human Immunology 63, 364–374 (2002).* © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: high PRA renal patients; HLA matchmaker; flow cytometry crossmatch

ABBREVIATIONS

PRA	panel reactive antibody
FCXM	flow cytometry crossmatch
IVIG	intravenous immunoglobulin
CREG	cross-reactive group
MM	mismatches
HLA	human leukocyte antigens
NIMA	noninherited maternal antigens
AMM	acceptable mismatches
AAT	amino acid triplets

From the Tissue Typing Laboratory, University of Alabama Hospitals, Birmingham, AL, USA.

Address reprint requests to: Dr. Andrew Lobashevsky, Alabama Regional Histocompatibility Laboratory, University of Alabama Hospital, P290 West Pavillion 618 18th Street S., Birmingham AL 35294, USA; Tel: (205) 934-8948; Fax: (205) 934-9039; E-mail: andrew.lobashevsky@ccc.uab.edu

Received October 22, 2001; revised January 4, 2002; accepted January 14, 2002.

0198-8859/02/$–see front matter
PII S0198-8859(02)00371-3

INTRODUCTION

It is generally accepted that immunological factors play a critical role in success of kidney transplantation. Numerous reports indicate that the long-term prognosis of graft survival is associated with the degree of donor/recipient HLA matching and level of patient sensitization [1–5]. Hyperacute rejection, a major cause of graft loss in the first decade of clinical transplantation, has been virtually eliminated by ABO blood group matching and prospective crossmatch (XM) testing. The presence of preformed donor specific antibodies, which are associated with hyperacute rejection, is a contraindication for transplanation. However, the precise role of antibodies in the pathogenesis of acute and chronic rejection is complex and still controversial, as both cellular and humoral immune components can participate in each of these processes [6–12]. The involvement of alloantibody in non-hyperacute rejection is implicated through an association between poor graft outcome and anti-donor HLA antibodies, including low titer antibodies [13–15].

Patient's serum reactivity is routinely tested against a panel of allogeneic lymphocytes from healthy donors to monitor the level of allosensitization. Panel reactive antibody (PRA) greater than 10% is usually consistent with the presence of alloantibodies. A definition of the specificity of these antibodies is useful for organ allocation to avoid occurrence of positive XM tests. High PRA levels, typically in the range of 50%–80%, are indicative of broadly reactive antibodies. Most high PRA patients have antibodies that exhibit specificity to one or two common or public HLA epitopes, with only a small percentage showing private epitope specific alloantibodies [16–20]. These highly sensitized patients are disadvantaged for transplantation because few donors will be XM non-reactive with their sera. Thus, the higher the PRA value, the lower the probability of finding a XM nonreactive donor.

Several strategies have been proposed for increasing the probability of finding a suitable donor for transplantation and also improving the transplant outcome in highly sensitized patients. There are two fundamentally different methodological approaches in current practice. One of these is targeted on the immunogenetic issue of identifying a suitable donor. The second involves reducing alloantibodies in the serum of the highly sensitized recipient by plasmapheresis or immunoabsorbtion. Success in the latter strategy is still encumbered by a rapid rebound of antibody production [21, 22]. Another approach to reduce alloantibody involves the administration of intravenous immunoglobulin (IVIG). The mechanism by which IVIG treatment reduces antibody and also inhibits mixed lymphocyte responsiveness in vitro [12, 23] is uncertain, although one hypothesis suggests a role for anti-idiotypic antibodies [24].

The immunogenetic approach of finding a compatible donor for highly sensitized patients originated in the 1980s when Sanfilippo *et al.* [25] proposed kidney allocation based on serologic epitopes, well before the HLA amino acid sequences were established. In this scheme, HLA antigens with serum crossreactivity were sorted into cross-reactive groups (CREGs). This HLA categorization was further refined by Rodey and Fuller [26], who postulated that the majority of high PRA sera contained antibodies recognizing public HLA epitopes that are expressed on different HLA molecules. They demonstrated that most serum cross-reactivity could be explained by conserved or public epitopes on individual HLA molecules [17, 20]. Consequent analysis revealed that kidney graft survival in recipients who received kidneys from donors with multiple CREG mismatches (MM) was decreased compared to those without CREG MM [3, 16, 27]. This observation led to the concept of "permissible" or "acceptable" MM in transplant immunology. However, due in part to variable alloantibody affinities and sensitivities of screening procedures and the fact that some CREGs were not clearly defined, allocation of kidneys based on the number of CREG MM was not widely adopted [28].

Another effort to examine permissible HLA MM for sensitized patients was that reported by Maruya *et al.* [29]. These investigators retrospectively studied a large cohort of kidney transplants mismatched for single HLA-A, -B and -DR antigens. Reasoning from the outcome perspective, donor/recipient incompatibilities were classified as "permissible" or immunogenic depending on the fate of the transplant. The kidney recipients judged to have received a permissible mismatch had graft survival rates equivalent to zero-A, B, DR-mismatched grafts. The elegant study of Takemoto developed a molecular CREG concept based on public or shared amino acid residues among HLA antigens [28]. Based on the HLA-A, B loci amino acid sequences, he demonstrated that five-year survival was 66% for zero DR, zero-residue mismatched kidneys in comparison to 59% for kidneys with antigen MM.

Serologic non-reactivity against non-inherited maternal HLA antigens (NIMA) and associated CREGs is another way to identify suitable donors with acceptable MM (AMM) for high PRA patients. Recent clinical studies show that kidney transplants mismatched for NIMA have a better prognosis compared to grafts mismatched for non-inherited paternal antigens [30, 31]. The Eurotransplant program uses still another algorithm

to define an AMM for highly immunized patients. They construct special panels of cells, each having only one HLA-A or -B MM with the sensitized patient. The HLA antigens that produce negative reactions are considered AMMs, which have been demonstrated even for patients with a 100% PRA [32].

The amino acid sequence polymorphism of HLA reflects the immunogenicity of epitopes that can elicit alloantibody production and effector T cell activation. Amino acid residues at exposed sites on α-helical coils but not in β-pleated sheets of HLA class I molecules are accessible for alloantibody binding, as has been demonstrated by crystallography data [33]. Based on these observations, a new HLA matching algorithm was proposed by R. Duquesnoy as an alternative strategy for identifying potential donors for highly immunized patients [34]. HLAMATCHMAKER, a computer-based algorithm, uses a molecular approach to define the degree of HLA compatibility. This program converts each class I HLA allele into a linear structure of amino acid triplets (AAT) and then determines those donor AAT, which are shared or not shared with the recipient [35].

In this study we investigated the relationship between the number of immunogenic AAT MM and results of the final FCXM using the HLAMATCHMAKER algorithm and to examine if AMM could be identified for high PRA kidney recipients at our institution.

MATERIALS AND METHODS

Patients

Final FCXM serum samples were analyzed from 39 highly sensitized (PRA $\geqslant$ 85%) patients with various end-stage renal diseases. A total of seventy FCXM assays were performed. Twelve patients underwent FCXM with more than one potential donor. Table 1 reports the demographic characteristics of patients involved in the study.

TABLE 1 Demographic values of the patients used in the study

	FCXM positive	FCXM negative	Total
Age[a]	17–68	19–65	39
Ethnicity[a]			
African-Americans	12	5	17
Caucasians	14	8	22
Sex[a]			
Male	11	5	16
Female	17	6	23
Donor source[a]			
CD[b]	18	7	25
LD[c]	6	8	14
Total number of patients	26	13	39

[a] The numbers are not statistically different ($p > 0.05$).

[b] Cadaveric donor.

[c] Living donor.

Abbreviations: FCXM = flow cytometry crossmatching.

Histocompatibility Testing

All donors and recipients in this study were ABO identical. HLA-A, -B, -Cw, -DR, and -DQ tissue typing was performed by the standard microlymphocytoxicity method using commercially available tissue typing trays. In cases when serological typing was not possible, PCR-SSP low resolution methodology was performed.

FCXM

Final FCXM was performed prospectively using a current patient serum sample according to ASHI standards. Details of the procedure are described elsewhere [36]. Scoring was determined by relative binding of FITC goat antihuman IgG in recipient serum compared to background controls. Control values were obtained with donor cells incubated for the primary incubation with either 10% NHS-RPMI (negative media control), nontransfused male AB+ serum (negative serum control), or a pool comprised of at least five high PRA (95%–100%) positive sera (positive serum control). Patient serum samples were run in duplicate. A positive FCXM was defined as a reproducible shift in the median channel of fluorescence (MCS) of $\geqslant$ 40 channels to the right of the negative serum control T cell peak and $\geqslant$ 70 channels compared to the negative serum control B cell peak. Autologous FCXM with recipient serum was performed in parallel. When the FCXM result was positive only with donor B cells, we further examined specificity using the FlowPRA Specific class I and class II HLA antibody screening tests (One Lambda, Canoga Park, CA, USA). If negative reactions were observed in the latter tests, a positive B-cell FCXM was assigned as having a non-HLA specificity.

PRA

Class I PRA was analyzed in serum samples of both FCXM positive and negative patients using the FlowPRA Class I Screening Test (One Lambda) as described earlier [36]. The test results were scored on the basis of peak fluorescence shift to the right of the negative control. To confirm the results two additional tests were performed. First, the patient's serum was tested by flow cytometry against a cell pool that represented the major CREGs as described previously [37]. Secondly, the patient's serum was tested for HLA antigen specificity using the FlowPRA Specific class I HLA Antibody Detection Test (One Lambda), according to the manufacturer recommendations and as described [36]. The latter

TABLE 2 Results of final FCXM analysis

	Positive FCXM	Negative FCXM			
	T+/B+	T+/B−	T−/B+	T−/B−	Total
Tests	54[a]	1[b]	4[c]	11	70
Patients	26		13		39

[a] Autologous and allogenic anti-class I antibodies were found in four patients.

[b] Non-HLA antibodies caused this reactivity.

[c] Two patients had autologous antibodies only, and two patients had antibody to donor HLA class II only.

Abbreviations: FCXM = flow cytometry crossmatch; HLA = human leukocyte antigen.

technology enabled us to identify the non-reactive antigens in the bead panel. If the serum sample demonstrated positive reactivity with all members of the specificity bead panel (*i.e.*, 100% PRA), serum dilutions were made in attempt to define negative reactions.

HLA Matchmaker Algorithm

The principle of the AAT triplet MM algorithm was developed by Duquesnoy [34, 35]. A detailed description of the program and instruction for use can be downloaded from the Transplant Pathology Internet Service (http://tpis.upmc.edu). Briefly, HLA matchmaker applies two principles: (1) each HLA antigen represents a distinct array of polymorphic triplets as potential immunogenic epitopes; (2) sensitized patients cannot produce alloantibodies against antigenic epitopes expressed on their own HLA molecules. In this model, donor/recipient HLA compatibility is assessed through intra- and interlocus triplet comparisons. The results of the analysis provide the user with a list of HLA-A, -B AMM and the relative degree of mismatching (*i.e.*, the number of mismatched donor immunogenic AAT) for that particular allogeneic combination. Notably, the HLA matchmaker algorithm can define mismatched HLA antigens with zero AATMM.

Data Analysis

Statistical analysis was performed using the χ^2 test, Fisher exact test and Student's *t*-test using Stagraphics Plus 3.0 software (Manugistic, Inc., Rockville, MD).

RESULTS

FCXM Analysis in Highly Sensitized Patients

Table 2 illustrates the final FCXM results of 39 patients involved in the study. Among 70 final FCXM assays performed, twelve recipients were crossmatched with multiple donors. Sixty-seven percent of the recipients (26/39) demonstrated a positive T and B-cell FCXM, and an overall seventy seven percent (54/70) of FCXM tests were scored positive. Among these, auto-reactive antibodies were detected in four serum samples. However, consequent platelet absorption and specificity analysis confirmed the concomitant presence of allogeneic anti-class I antibodies in all four of these samples.

Sixteen FCXM were scored negative for anti-HLA antibodies. The majority of these were T- and B-cell negative. Those samples that demonstrated T pos./B neg. or T neg./B pos. final FCXM results were subjected to further analysis including autologous FCXM, platelet absorption and class I/II PRA beads analysis, which collectively ruled out the presence of anti-HLA class I antibodies. These confirmatory analyses enabled us to judge these pretransplant serum samples as true negative for anti-HLA class I antibodies.

TABLE 3 Class I PRA analysis

Test	Positive FCXM $n = 54$	Negative FCXM $n = 16$	*p* Value
Flow beads	94.8 ± 4.0	91.0 ± 3.0	>0.05
Flow specificity beads[a]	92.0 ± 8.0	88.0 ± 9.0	>0.05
Flow pool[b]	81.0 ± 14.2	84.0 ± 11.8	>0.05

[a] Pool of beads included 32 members.

[b] T-lymphocyte cell pool comprised 6 members.

Abbreviations: FCXM = flow cytometry crossmatch; PRA = panel reactive antibody.

PRA Analysis in Sensitized Patients

PRA was determined in the same serum sample that was used for the final FCXM. The results are shown in the Table 3. There was no statistically significant difference in PRA values between FCXM positive and negative groups. All three flow cytometry methods used for PRA detection (see Materials and Methods) demonstrated comparable levels of alloreactivity between patients with positive or negative final FCMX.

Determination of HLA Compatibility at the AAT Level

The HLAMATCHMAKER computer algorithm was used in order to define the degree of MM at AAT level in seventy highly sensitized recipient/donor combinations as described by Duquesnoy [35]. The number of immunogenic AAT MM was calculated for HLA-A and -B loci. These results are presented as mean values in Table 4 and as the distribution of AAT MM in Figure 1, respectively. Significantly greater numbers of total, highly immunogenic and medium immunogenic AAT MM were observed in the group of FCXM positive donor/recipient combinations. However, no significant difference was found in the numbers of low immunogenic AAT between FCXM positive and FCXM negative groups (data not shown). We did not observe any pref-

TABLE 4 The number of immunogenic AAT MM in FCXM positive and FCXM negative patients

Mismatched immunogenic triplets	FCXM pos.	FCXM neg.	*p* Value
Total[a]	7.6 ± 4.6	2.9 ± 2.5	0.000083
Highly immunogenic	2.1 ± 2.4	0.6 ± 1.4	0.00095
Medium immunogenic	4.2 ± 3.5	1.7 ± 2.1	0.00088

[a] Total number of mismatched triplets includes those of high, medium, and low immunogenicity.

Abbreviations: AAT = amino acid triplets; FCXM = flow cytometry crossmatch; MM = mismatches.

erential distribution of the number or degree of AAT MM and the HLA loci from which were encoded. We also did not find any significant differences between the number of AAT MM when recipients were analyzed on the basis of race, sex or age.

FIGURE 1 The number of highly immunogenic triplet mismatches in FCXM positive and negative high PRA patients. Mean and standard deviation values are presented in Table 4. Abbreviations: FCXM = flow cytometry crossmatch; PRA = panel reactive antibody.

Identification of HLA AMM in Highly Sensitized Patients Using HLA Matchmaker

The HLA matchmaker algorithm also enabled us to estimate the AMM HLA antigens for the highly sensitized patients. The results of such analysis are shown in Table 5 and 6. A highly sensitized patient MH typed as HLA-A11, A29; B44 (w4), B57 (w4) and presented with a PRA of 88%. Analysis by HLA matchmaker revealed that antigens with zero AATMM were A31, A74, B49, B52, B58, B63 presenting permissible or AMM. Further analysis of his serum revealed the following non-reactive antigens: A1, A80, A2, A24, A3, B73, B62. These negative HLA specificities carry unshared AAT, but none of them were recognized by the patient's antibodies. HLA matchmaker incorporates this information to identify additional AMM HLA for this patient. While the negative HLA specificities have unshared AAT MM with the recipient, they are negatively reactive (*i.e.*, inaccessible) AAT and thus, are nonreactive with the recipient's serum. The group of AMM HLA, therefore, includes antigens having zero AATMM with recipient (defined by HLA matchmaker), HLA specificities producing negative reactions with the high PRA patient's serum (defined by cell/

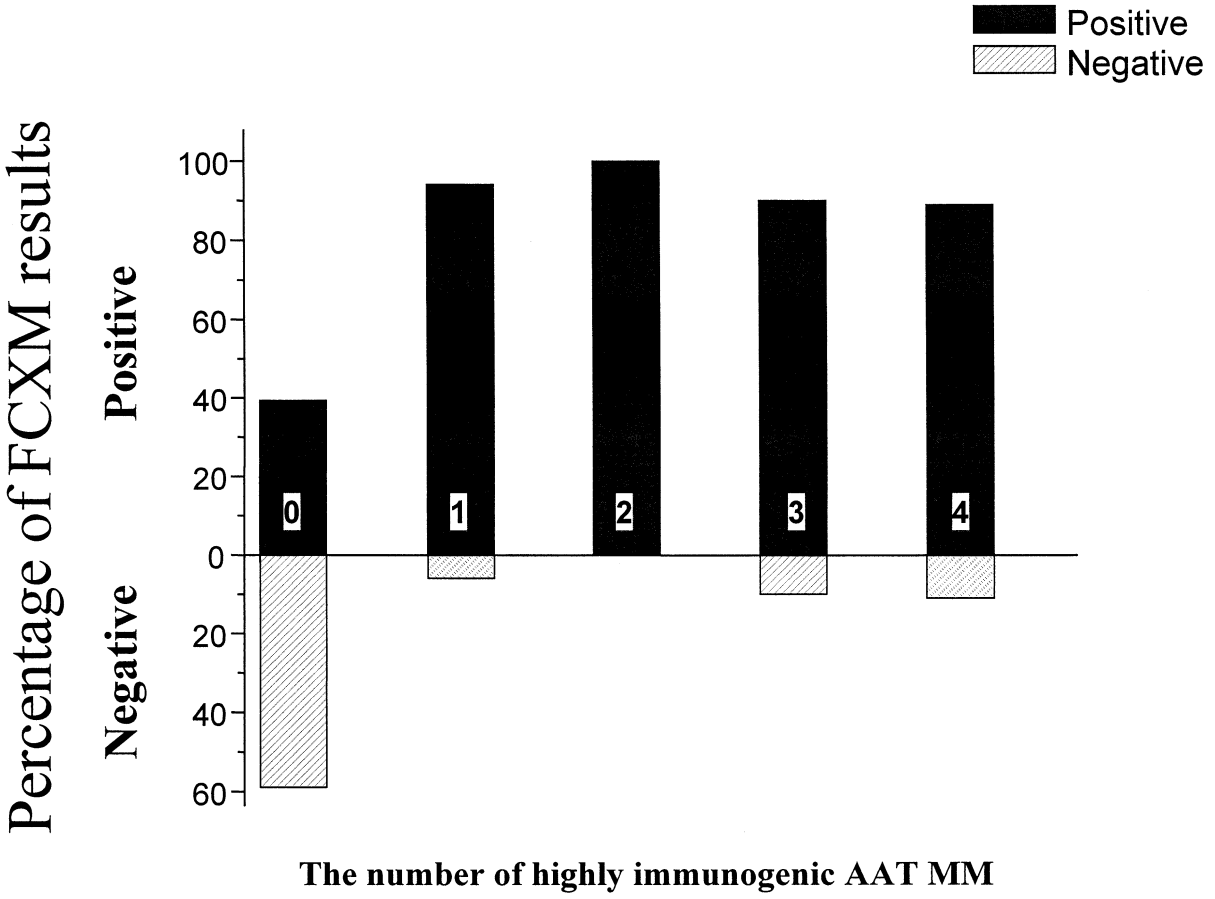

TABLE 5 The number of AMM HLA antigens with zero immunogenic AAT MM for highly sensitized patient MH

Patient HLA typing	AMM HLA	Final composition of AMM HLA
A11, 29; B44, 57	A31, 74;	A1, 2, 3, 24, 30, 31, 68, 74, 80;
	B49, 52, 58, 63[a]	B18, 35, 38, 39, 49, 51, 52, 58, 59, 62, 63, 73, 65, 78[d]
Antigens reacted negatively		
A1, 2, 3, 24, 80; B62, 73[b]	A30, 68;	
	B18, 35, 38, 39, 51, 59, 65, 78[c]	

[a] HLA having zero AATMM with the patient.

[b] HLA reacted negatively (not recognized by patient's antibodies) on cell/bead panel.

[c] This HLA cluster has unshared triplets with patient and were defined after nonreactive panel antigens had been processed by HLAMATCHMAKER.

[d] This final set of AMM HLA comprises bead or cell panel nonreactive antigens (have unshared triplets), those defined by HLAMATCHMAKER based on recipient typing (0 AATMM) and those after negative reactions have been incorporated (have unshared triplets).

Abbreviations: AAT = amino acid triplets; AMM = acceptable mismatches; HLA = human leukocyte antigens; MM = mismatches.

bead panel), and mismatched, but negatively reactive determinants (as defined by HLA matchmaker). Thus, the list of AMM HLA antigens is substantially expanded beyond those assigned as having a serologically negative status. Another example of HLA AMM identification including particular AAT for high PRA patient RA is presented in Table 6. The following zero AATMM HLA antigens were determined with the help of HLA matchmaker: A74, A69; B57, B59, B38, B51, B58. The pattern of alloantigens that reacted negatively with patient RA serum yielded an expanded set of acceptable HLA specificities (A1, A11, A23, A29, A68, A80; B35, B52, B72). These antigens have different numbers of immunogenic AATMM with the recipient (indicated in bold). Furthermore, the HLA matchmaker computer algorithm enabled us to define the array of HLA specificities having zero AATMM with negative reactions and various numbers of AATMM with the patient (in bold). The further analysis of the donor with A11, A23; B78 HLA phenotype revealed six immunogenic (Table 6. in bold) AATMM at the A locus. Furthermore, two of them were defined as highly immunogenic (in boxes). Two AATMM with a medium level of immunogenicity were detected at B locus (in bold). As indicated they were also mismatched with the recipient, but completely matched with the non-reactive HLA specificities.

Relationship Between the Number of Immunogenic AAT and Final FCXM Results

In this part of the study we attempted to answer the question as to whether there is a correlation between the number of immunogenic AATMM in highly sensitized recipient/donor combinations and the results of final FCXM. As shown in the Table 7, a significant correlation was detected between the number of highly immunogenic AATMM and results of final FCXM. A similar analysis was performed with the number of total and medium immunogenic AAT. These findings are presented in the Table 8. It is evident that a statistically significant relationship exists between the number of total and highly immunogenic AATMM and results of final FCXM. This observation is also confirmed by high predictive value for negative FCXM. In the case of highly immunogenic AATMM the relationship was stronger in comparison to the total number of immunogenic AATMM ($\chi^2 = 22.9$ vs. $\chi^2 = 5.1$).

Collectively, these data suggest that FCXM compatibility of particular highly immunized recipient/donor combination depends on the degree of immunogenic AATMM, particularly the highly immunogenic AATMM. Regression analysis was performed between the number of highly immunogenic AATMM (0 to 4) and the probability of a positive or negative final FCXM in highly sensitized recipients (Figure 2). In the case of zero highly immunogenic triplet MMs, a positive FCXM was observed in 41% of cases, whereas in the case of $\geq$ 1 MM these results were found in 90%–100% of assays. Thus, a single highly immunogenic AATMM was sufficient to more than double the probability of obtaining a positive crossmatch in highly sensitized patients.

DISCUSSION

Current histocompatibility standards recommend that methodology for pretransplant screening of allosensitization by PRA analysis and final crossmatching for kidney transplant candidates should be sufficiently sensitive to detect low concentrations of alloantibody. Accordingly, flow cytometry is widely used for this purpose [2, 13–15]. The emphasis on sensitivity emanates from observations that even low concentrations of anti-donor alloantibodies can exert a deleterious effect on graft outcome [7, 11, 38–40]. Usually, low levels of PRA are associated with readily definable alloantibodies to HLA private epitopes, and donors expressing the corresponding antigenic specificities are avoided [4, 16, 17, 20, 26]. However, when the PRA reaches 80% or higher, it is impossible to define HLA antigen specificities in the overwhelming majority of cases, because most of such antibodies are directed against public epitopes of HLA molecules [4, 19, 26, 34, 41]. Accordingly, the chances

TABLE 6 Identification of AMM for highly sensitized patient RA

Patient's RA typing	9[d]	45	62	66	70	74	76	80	82	90	149	151	156	163	166	AATMM with patient	AATMM with neg. reactions
A02			GE	RKV		H				A	AAH	AHV				N/A	N/A
A36		KME	QE	RNM			AN			D	AVH	VHA	R			N/A	N/A
B53			RN				EN	RIA	ALR	A						N/A	N/A
B63				RNM	ASA		EN	RIA	ALR	A						N/A	N/A
AMM[a]																	
A74				rnv[f]												0	N/A
A69				rnv	aqs											0	N/A
B57																0	N/A
B59		ee														0	N/A
B38			RN				EN	RIA	ALR	A						0	N/A
B51			RN				EN	RIA	ALR	A						0	N/A
B58			GE	RNM	ASA		EN	RIA	ALR	A						0	N/A
Negative reactions[b]																	
A01		KME	QE	RNM			AN			D	AVH	VHA	R	**R**	**DG**	2	N/A
A11			QE							D	AVH		**Q**	**R**		2	N/A
A23	**S**		**EE**	**GKV**			EN	RIA	ALR	A					**DG**	4	N/A
A29	**T**		**LQ**				AN			A						2	N/A
A68			RN							A	AAH	AHV	**W**			1	N/A
A80			**EE**			N	AN			D		**ARR**		**E**	**DG**	5	N/A
B35			RN				**ES**	**RNL**		A						2	N/A
B52				**QIS**			EN	RIA	ALR	A						1	N/A
B72				**QIS**			**ES**	**RNL**		A						3	N/A
0 AATMM with neg. reactions[c]																	
A30	**S**		QE							A						1	0
A24	**S**		**EE**	**GKV**			EN	RIA	ALR	A	AAH	VHA	**Q**		**DG**	5	0
A32			QE				**ES**	RIA	ALR	A						1	0
A31	**T**		QE							A						1	0
B39			RN				**ES**	**RNL**		A						2	0
B65			RN				**ES**	**RNL**		A						2	0
B71			RN				**ES**	**RNL**		A						2	0
B18			RN	**QIS**			**ES**	**RNL**		A						3	0
B62				**QIS**			**ES**	**RNL**		A			**W**			4	0
B64			RN				**ES**	**RNL**		A						2	0
B70			RN				**ES**	**RNL**		A						2	0
B75			RN	**QIS**			**ES**	**RNL**		A						3	0
B77			RN	**QIS**			EN	RIA	ALR	A						1	0
B78			RN				**ES**	**RNL**		A						2	0
Donor typing																	
A11			QE							D	AAH		**Q**	**R**		2	0
A23	**S**		**EE**	**GKV**[e]			EN	RIA	ALR	A					**DG**	4	0
B78			RN				**ES**	**RNL**		A						2	0

[a] Acceptable HLA MM defined by HLAMatchmaker.

[b] HLA specificities producing negative reactions on bead/cell panel.

[c] HLA specificities, which are expected to produce negative reactions on bead/cell panel (defined by HLAMATCHMAKER).

[d] Only polymorphic amino acid residues positions are indicated.

[e] AATMM are indicated in bold; boxed triplets are highly immunogenic.

[f] Low case characters indicate monomorphic amino acid residues, upper case characters indicate polymorphic amino acid residues.

of finding a suitable donor for these patients are low, which in turn, results in an extended time on the transplant waiting list and poor quality of life related to dialysis.

Histocompatibility based strategies have been proposed in order to increase the chances of finding a cross-match negative donor for such highly sensitized patients. One approach is to define permissible or acceptable AMM (cross match negative) donor HLA antigens or CREGS [27, 28]. However, in situations where no clear allospecificity is defined, the assignment of "permissible" MM is precarious.

TABLE 7 Correlation analysis between the number of highly immunogenic AAT MM and results of final FCXM

Number of highly immunogenic AAT MM[a]	FCXM		Total
	Positive	Negative	
⩾1	46	3	49
0	8	13	21
	54	16	70

$\chi^2 = 22.9$ (Yate's correction); $p = 0.000001$.

Abbreviations: AAT = amino acid triplets; FCXM = flow cytometry crossmatching; MM = mismatches.

In this study we applied the Duquesnoy's HLA Matchmaker computer algorithm [34, 35], which is based on the identification of non-self (immunogenic) and self (acceptable) AAT to examine whether an acceptable donor could be identified in a population of highly synthesized renal transplant candidates. The basis of the HLA matchmaker strategy is the fact that inter- and intra-allelic differences of HLA antigens are related to the polymorphic amino acid residues. Polymorphic residues are located on the α-helical coils and β-pleated sheet of the peptide binding groove, respectively, with those exposed on the α-helical coils being critical (*i.e.*, immunogenic) for inducing antibody production [33–35] and generation of cytotoxic clones [42, 43]. Notably, comparative analysis of linear sequences of AAT indicates that some of them are shared by different HLA-A, -B, -Cw antigens. In other words, some mismatched HLA gene products can be nonimmunogenic because of AAT concordance between the donor and recipient HLA gene products. For example, if a particular donor and recipient have shared immunogenic AAT, additional donor amino acid residue MMs, which are not exposed on the α-helical coils, are unable to elicit alloantibody. Hence, despite the presence of mismatched residues on donor HLA gene products, as long as the AATs are shared with the recipient, the mismatch by definition is defined as potentially acceptable.

TABLE 8 Summary of 2×2 analysis performed with AAT MM of various immunogenicity

Immunogenicity of AAT	χ^{2a}	PV_N[b]	p Value[a]
Total[c]	5.1	62.5%	0.024500
Highly	22.9	85.0%	0.000001
Medium	3.2	70.5%	0.072000

[a] χ^2 test and p value were computed with Yates' correction.

[b] Predictive value of negative FCXM.

[c] Total number of triplets includes those of high, medium, and low immunogenicity.

Abbreviations: AAT = amino acid triplets; FCXM = flow cytometry crossmatching; MM = mismatches.

In this study we randomly selected 39 highly sensitized renal transplant candidates and analyzed the results of their final FCXM. Some of the transplant candidates were cross matched with more than one donor. A total of 70 FCXM assays were performed. Our data showed that in the case of positive FCXMs (54 assays), the number of immunogenic (high, medium, and low) AATMM significantly exceeded those in the group with negative FCXMs (16 assays, Table 4). Nonparametric 2×2 table analysis revealed a strong relationship ($\chi^2 = 22.9$; $p = 0.000001$) between the number of highly immunogenic AATMM and the FCXM result. However, no significant relationship was observed in the case of medium and low immunogenic AATMM. Thus, the χ^2 association was confined to the category of highly immunogenic AATMM.

The incidence of false positive FCXM was relatively low, *i.e.*, 15% (8/54) in those lacking any highly immunogenic AATMM compared with 85% of those having ⩾ 1 highly immunogenic AATMM. False positive results are possibly related to limitations in the HLA matchmaker algorithm. For example, the immunogenicity of a particularly mismatched AAT was assigned on the basis of that AAT representation in the most frequent allele in that antigen group. Thus, the AAT for all A2 antigens was assigned that associated with A*0201. Donor cells producing a positive FCXM in such a case might have another A*02 cluster allele, which could be recognized by the recipient's alloantibodies. Secondly, in PRA screening, negative reactions seen with a bead or cell panel can be false negative assignments if the panel is not fully representative. Thirdly, some AAT can establish hydrophobic and hydrogen bonds with other residues, forming three-dimensional structures, which might have different immunogenicity in eliciting antibody production [33].

Our results are in agreement with the observations of Oldfather et al., using AHG-CDC crossmatching in highly sensitized kidney recipients. These authors reported a high predictive value for negative crossmatch based on shared CREGs [18]. Similar findings have been observed by Takemoto [28], who reported that 30% of zero-residue MM patients had a positive crossmatch compared to 70% of cases with ⩾1 MM.

The identification of non-reactive HLA specificities on a panel of typed cells or HLA antigen coated beads represents another, complementary approach to identifying AMM. These antigens may have immunogenic AATMM with recipient HLA, but they are not recognized by patient's alloantibodies. Moreover, the HLA-Matchmaker algorithm identifies additional HLA specificities, which are mismatched with the recipient at the

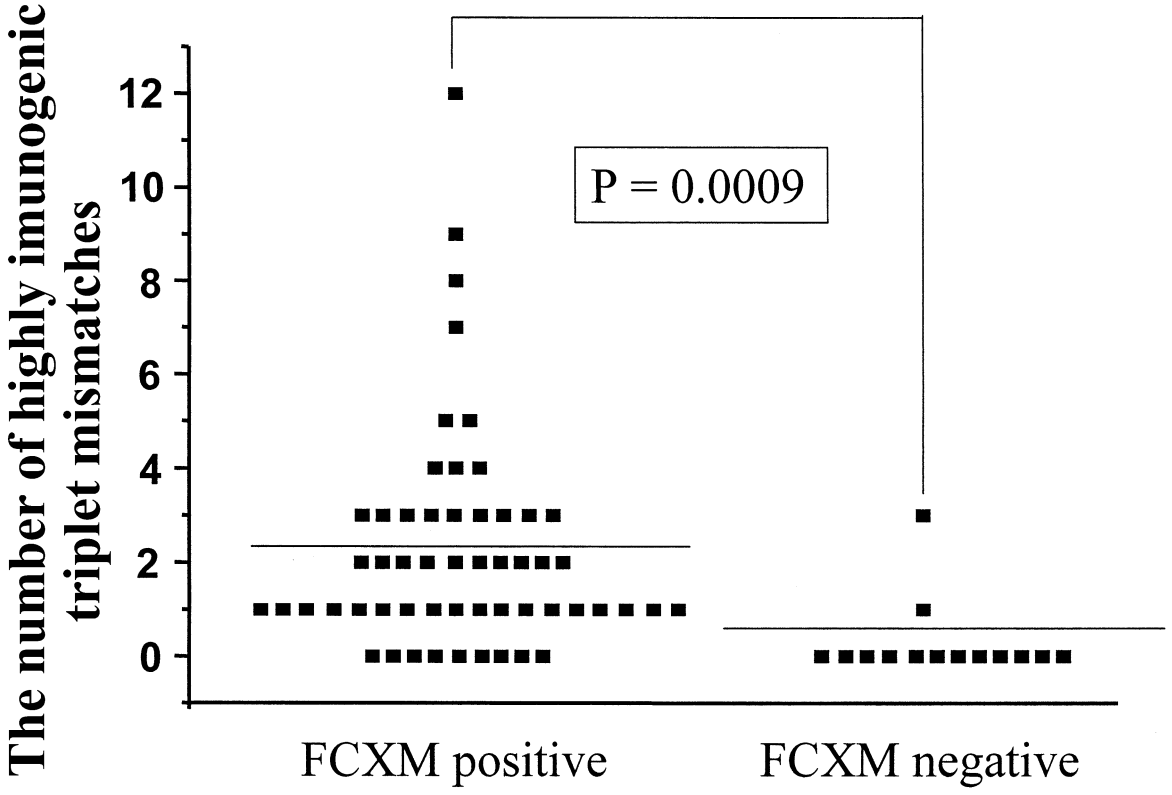

FIGURE 2 Nonparametric 2×2 table analysis of final FCXM results and degree of highly sensitized patient/donor compatibility on the level of AAT MM. Strong correlation was observed between the number of highly immunogenic AAT MM and positive FCXM. Abbreviations: AAT = amino acid triplets; FCXM = flow cytometry crossmatch; MM = mismatches.

amino acid triplet level but have no AATMM with the antigen specificities to which the recipient's serum reacts negatively.

A question that remains to be answered is whether the HLAMatchmaker algorithm can predict XM results obtained with HLA typed lymphocytes from unrelated healthy volunteers based on the number of highly immunogenic AATMM. Such studies are in progress in our laboratory and show encouraging preliminary results based on tests in seven patients.

Based on our results to date, application of the HLA Matchmaker computer algorithm to identify suitable donors for high PRA renal transplant candidates allows us to draw the following conclusions. First, on the basis of shared (*i.e.*, self) AAT between recipient and donor, there are additional non-shared HLA specificities which can be scored as AMM. Second, the array of non-reactive antigens (negative reactions on cell or bead panel) can substantially expand the scope of AMM HLA antigens. Third, the number of highly immunogenic donor AAT for which the high PRA recipient is mismatched correlates with results of the final FCXM.

In summary, the HLAMatchmaker computer algorithm, a user-friendly computer program, identifies inter- and intra-allelic structural differences of HLA antigens on the level of AAT. The composition of AMM for highly sensitized patient comprises the HLA specificities that have 0 AATMM with the recipient (defined by HLA Matchmaker) and the non-reactive antigens (defined by typed cell panel or beads and HLAMatchmaker). Our data suggest that definition of AMM may reveal the donors who are suitable for highly sensitized kidney recipients.

REFERENCES

1. Opelz G, Wujciak T, Dohler B, Scherer S, Mytilineos J: HLA compatibility and organ transplant survival. Collaborative Transplant Study. Rev Immunogenet 1:334, 1999.
2. Gebel HM, Bray RA: Sensitization and sensitivity: defining the unsensitized patient. Transplantation 69:1370, 2000.
3. Papassavas AC, Iniotaki-Theodoraki A, Boletis J, Kostakis A, Stavropoulos-Giokas C: Assignment of HLA-antigens in CREGs facilitates the detection of acceptable mismatches in highly sensitized patients. Hum Immunol 59:587, 1998.

4. Scornik JC, LeFor WM: Antibodies to crossreactive HLA antigens. Evaluation by cytotoxicity, flow cytometry, and inhibition of monoclonal antibody binding. Transplantation 43:235, 1987.
5. Opelz G: Five-year results of renal transplantation in highly sensitized recipients. Collaborative Transplant Study. Transpl Int 9 (Suppl. 1):S16-9, 1996.
6. Shirwan H: Chronic allograft rejection. Do the Th2 cells preferentially induced by indirect alloantigen recognition play a dominant role? Transplantation 68:715, 1999.
7. Khauli RB, Stoff JS, Fan PY, Ayvazian P, Medawar WA, Habbal A, Lovewell T, Bigwood P, Valliere SS, Pullman JM, Blute RD, Harland R: Chronic rejection of renal grafts: the role of acute rejection (1). Transplant Proc 33:2693, 2001.
8. Theruvath TP, Saidman SL, Mauiyyedi S, Delmonico FL, Williams WW, Tolkoff-Rubin N, Collins AB, Colvin RB, Cosimi AB, Pascuai M: Control of antidonor antibody production with tacrolimus and mycophenolate mofetil in renal allograft recipients with chronic rejection. Transplantation 72:77, 2001.
9. Grimm PC, Nickerson P, Jeffery J, Savani RC, Gough J, McKenna RM, Stern E, Rush DN: Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal-allograft rejection. N Engl J Med 345:93, 2001.
10. Marcen R, Pascual J, Teruel JL, Villafruela JJ, Rivera ME, Mampaso F, Burgos FJ, Ortuno J: Outcome of cadaveric renal transplant patients treated for 10 years with cyclosporine: is chronic allograft nephropathy the major cause of late graft loss? Transplantation 72:57, 2001.
11. Ponticelli C, Tarantino A, Vegeto A: Renal transplantation, past, present and future. J Nephrol 12 (Suppl 2): S105-10, 1999.
12. Jordan SC, Quartel AW, Czer LS, Admon D, Chen G, Fishbein MC, Schwieger J, Steiner RW, Davis C, Tyan DB: Posttransplant therapy using high-dose human immunoglobulin (intravenous gammaglobulin) to control acute humoral rejection in renal and cardiac allograft recipients and potential mechanism of action. Transplantation 66:800, 1998.
13. Bray RA: Flow cytometry in human leukocyte antigen testing. Semin Hematol 38:194, 2001.
14. Talbot D: Flow cytometric crossmatching in human organ transplantation. Transpl Immunol 2:138, 1994.
15. Scornik JC, Bray RA, Pollack MS, Cook DJ, Marrari M, Duquesnoy R, Langley JW: Multicenter evaluation of the flow cytometry T-cell crossmatch: results from the American Society of Histocompatibility and Immunogenetics–College of American Pathologists proficiency testing program. Transplantation 63:1440, 1997.
16. Laundy GJ, Bradley BA: The predictive value of epitope analysis in highly sensitized patients awaiting renal transplantation. Transplantation 59:1207, 1995.
17. Delmonico FL, Fuller A, Cosimi AB, Tolkoff-Rubin N, Russell PS, Rodey GE, Fuller TC: New approaches to donor crossmatching and successful transplantation of highly sensitized patients. Transplantation 36:629, 1983.
18. Oldfather JW, Anderson CB, Phelan DL, Cross DE, Luger AM, Rodey GE: Prediction of crossmatch outcome in highly sensitized dialysis patients based on the identification of serum HLA antibodies. Transplantation 42:267, 1986.
19. Duquesnoy RJ, White LT, Fierst JW, Vanek M, Banner BF, Iwaki Y, Starzl TE: Multiscreen serum analysis of highly sensitized renal dialysis patients for antibodies toward public and private class I HLA determinants. Implications for computer-predicted acceptable and unacceptable donor mismatches in kidney transplantation. Transplantation 50:427, 1990.
20. Rodey GE, Neylan JF, Whelchel JD, Revels KW, Bray RA: Epitope specificity of HLA class I alloantibodies. I. Frequency analysis of antibodies to private versus public specificities in potential transplant recipients. Hum Immunol 39:272, 1994.
21. Kupin WL, Venkat KK, Hayashi H, Mozes MF, Oh HK, Watt R: Removal of lymphocytotoxic antibodies by pretransplant immunoadsorption therapy in highly sensitized renal transplant recipients. Transplantation 51:324, 1991.
22. Hiesse C, Kriaa F, Rousseau P, Farahmand H, Bismuth A, Fries D, Charpentier B: Immunoadsorption of anti-HLA antibodies for highly sensitized patients awaiting renal transplantation. Nephrol Dial Transplant 7:944, 1992.
23. Jordan SC, Tyan D, Czer L, Toyoda M: Immunomodulatory actions of intravenous immunoglobulin (IVIG): potential applications in solid organ transplant recipients. Pediatr Transplant 2:92, 1998.
24. Tyan DB, Li VA, Czer L, Trento A, Jordan SC: Intravenous immunoglobulin suppression of HLA alloantibody in highly sensitized transplant candidates and transplantation with a histoincompatible organ. Transplantation 57:553, 1994.
25. Sanfilippo F, Vaughn WK, Spees EK, Heise ER, LeFor WM: The effect of HLA-A, -B matching on cadaver renal allograft rejection comparing public and private specificities. Transplantation 38:483, 1984.
26. Rodey GE, Fuller TC: Public epitopes and the antigenic structure of the HLA molecules. Crit Rev Immunol 7:229, 1987.
27. Thompson JS, Thacker LR: CREG matching for first cadaveric kidney transplants (TNX) performed by SEOPF centers between October 1987 and September 1995. Southeastern Organ Procurement Foundation. Clin Transplant 10:586, 1996.
28. Takemoto SK: HLA amino acid residue matching. Clin Transplants, 397, 1996.
29. Maruya E, Takemoto S, Terasaki PI: HLA matching: identification of permissible HLA mismatches. Clin Transplants, 511, 1993.
30. Burlingham WJ, Grailer AP, Heisey DM, Claas FH,

Norman D, Mohanakumar T, Brennan DC, de Fijter H, van Gelder T, Pirsch JD, Sollinger HW, Bean MA: The effect of tolerance to noninherited maternal HLA antigens on the survival of renal transplants from sibling donors. N Engl J Med 339:1657, 1998.

31. Smits JM, Claas FH, van Houwelingen HC, Persijn GG: Do noninherited maternal antigens (NIMA) enhance renal graft survival? Transpl Int 11:82, 1998.
32. Claas FH, De Meester J, Witvliet MD, Smits JM, Persijn GG, Doxiadis II: Acceptable HLA mismatches for highly immunized patients. Rev Immunogenet 1:351, 1999.
33. Madden DR, Gorga JC, Strominger JL, Wiley DC: The three-dimensional structure of HLA-B27 at 2.1 A resolution suggests a general mechanism for tight peptide binding to MHC. Cell 70:1035, 1992.
34. Duquesnoy RJ, Marrari M: Determination of HLA-A,B residue mismatch acceptability for kidneys transplanted into highly sensitized patients: a report of a collaborative study conducted during the 12th International Histocompatibility Workshop. Transplantation 63:1743, 1997.
35. Duquesnoy RJ: HLAMATCHMAKER: a molecularly based donor selection algorithm for highly alloimmunized patients. Transplant Proc 33:493, 2001.
36. Lobashevsky AL, Senkbeil RW, Shoaf J, Mink C, Rowe C, Lobashevsky ES, Burke R, Hudson S, Deierhoi MH, Thomas JM: Specificity of preformed alloantibodies causing B cell positive flow crossmatch in renal transplantation. Clin Transplant 14:533, 2000.
37. Shroyer TW, Deierhoi MH, Mink CA, Cagle LR, Hudson SL, Rhea SD, Diethelm AG: A rapid flow cytometry assay for HLA antibody detection using a pooled cell panel covering 14 serological crossreacting groups. Transplantation 59:626, 1995.
38. Piazza A, Poggi E, Borrelli L, Servetti S, Monaco PI, Buonomo O, Valeri M, Torlone N, Adorno D, Casciani CU: Impact of donor-specific antibodies on chronic rejection occurrence and graft loss in renal transplantation: posttransplant analysis using flow cytometric techniques. Transplantation 71:1106, 2001.
39. Sumitran-Holgersson S: HLA-specific alloantibodies and renal graft outcome. Nephrol Dial Transplant 16:897, 2001.
40. Moller E, Soderberg-Naucler C, Sumitran-Karuppan S: Role of alloimmunity in clinical transplantation. Rev Immunogenet 1:309, 1999.
41. Takemoto SK, Cecka JM, Terasaki PI: Benefits of HLA-CREG matching for sensitized recipients as illustrated in kidney regrafts. Transplant Proc 29:1417, 1997.
42. Roelen DL, Stobbe I, Young NT, van Bree SP, Doxiadis II, Oudshoorn M, Morris PJ, Wood KJ, Claas FH: Permissible and immunogenic HLA-A mismatches: cytotoxic T-cell precursor frequencies reflect graft survival data. Hum Immunol 62:661, 2001.
43. van Rood JJ, Lagaaij EL, Doxiadis I, Roelen D, Persijn G, Claas F: Permissible mismatches, acceptable mismatches, and tolerance: new trends in decision making. Clin Transplants, 285, 1993.