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Serum analysis after transplant nephrectomy reveals restricted antibody specificity patterns against structurally defined HLA class I mismatches

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Abstract

This study deals with HLA-mismatched kidney transplants that have been removed following rejection. Sera from 27 patients were screened for HLA-specific antibodies by direct complement-dependent lymphocytotoxicity with HLA-typed cell panels. Circulating donor-specific antibodies were detected in 3 cases (11%) before and in 26 cases (97%) after allograft nephrectomy. These findings demonstrate the production of donor-specific antibodies in patients with rejected transplants, but in most cases, they were undetectable before nephrectomy, because the graft had adsorbed them.

With an HLAMatchmaker-based serum analysis program, we observed restricted antibody specificity patterns against amino acid triplet-defined epitopes on donor HLA-A,B antigens. Many donor triplets were non-reactive while others were apparently recognized by antibodies. In some patients, the donor triplet specific antibodies persisted for a long time whereas in many other patients, they became undetectable after a few months.

The characterization of the antibody specificity profiles of post-allograft nephrectomy sera is clinically useful in defining criteria of HLA mismatch acceptability for sensitized patients awaiting another transplant. It provides also opportunities for determining the relative immunogenicity of mismatched triplets.

Keywords: Allograft nephrectomy; Alloantibodies; Kidney transplant; HLAMatchmaker; Triplet; Serum analysis

1. Introduction

Humoral sensitization to HLA antigens is an important cause of kidney transplant failure due to rejection [1]. HLA-specific antibodies cause graft injury through complement-dependent inflammatory mechanisms as evidenced by intragraft immunostaining for C4d [2] and the elution of lymphocytotoxic antibodies from rejected kidneys [3,4]. After transplantation, the development of HLA antibodies is associated with and precedes acute rejection and chronic rejection [5]. Many patients with rejected kidney transplants have HLA antibodies in their serum but it has been noted that these antibodies are often specific for antigens not found on the donor [1]. The most likely explanation is that donor-specific antibodies are undetectable by routine serum screening because the HLA antigens expressed by the graft have adsorbed them. Once this antigen source has been removed, we can expect that donor-specific antibodies become more readily detectable in patient's serum. This concept is consistent with reports showing increased serum HLA antibody reactivity after allograft nephrectomy

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(alloNx) [6–12] and the elution of HLA antibodies from rejected kidneys [8]. Recent studies showed a good correlation between the specificity of anti-HLA antibodies in post-alloNx sera and eluates from rejected transplants [13].

We have examined the serum screening records of patients with failed kidney transplants who had undergone alloNx after being placed on the waiting list for a subsequent transplant. These sera were screened by direct lymphocytotoxicity testing with HLA-typed panels and we have analyzed their reactivity patterns with an HLAMatchmaker-based program that determines antibody specificity against amino acid triplet-defined epitopes. HLAMatchmaker is a structurally based matching algorithm that considers each HLA antigen as a distinct string of polymorphic triplets in antibody-accessible sequence positions [14]. Triplets constitute key elements of immunogenic epitopes that can induce the formation of specific alloantibodies. This study provides further evidence that donor-specific HLA class I antibodies become readily detectable in patient sera following transplant nephrectomy. The specificity of such antibodies was often restricted to a small number of triplets. Analysis of post-alloNx sera provides a better assessment of HLA mismatch acceptability of a subsequent transplant.

2. Materials and methods

2.1. Patient records

We have reviewed the records of patients with failed kidney transplants who after returning to the transplant waiting list, had undergone alloNx between January 1, 1996 and December 31, 2001. Pre- and post-nephrectomy serum screening information was available for 27 adult patients. There were 15 males and 12 females; five patients were African-Americans. Their median pre-transplant Panel Reactive Antibody (PRA) was 2% (range: 0–20%). All cross-matches with donor T-lymphocytes were negative and no reactivity was detected against donor HLA-A,B antigens. Twenty cases were primary transplants and seven were repeat transplants (Table 1). One patient received a simultaneous pancreas transplant. All patients had been on tacrolimus-based immunosuppressive treatment protocols. This study was approved by the University of Pittsburgh Institutional Review Board (IRB protocol 0308107).

The median time of graft failure was 1401 days after transplantation (range 12–4840 days). Because of clinical indications such as pain, fever, and infection, alloNx was done an average of 115 days later (range 0–1951 days). The histopathology of each removed allograft was determined by Banff criteria [15] and considered one or more of the following primary features: chronic allograft nephropathy, acute rejection, thrombosis, and infarction with hemorrhage.

Table 1
Graft survivals and allograft nephrectomy findings

Case number	Transplant number*	Pre-transplant PRA	Graft failure (days)	AlloNx (days)	Pathology grades[a]
1	K1	0%	4203	5182	RC5,RA4
2	K1	0%	4086	5115	RC5,RA3,RA7
3	K1	20%	4840	4865	RC5,RA4,IS6
4	K1	0%	1960	2144	RC5,RA4,RA7
5	K1	0%	1324	1450	RC6,RA7
6	K3	3%	945	982	RC6,RC5
7	K1	2%	1236	1548	RC6,RA7
8	K1	0%	3626	3736	RC5,RA1
9	K2	8%	3488	3803	RC6,RA3,RA7
10	K1	2%	1197	1406	RC5,RA7,RA9
11	K1	3%	418	425	RA4,RC6
12	K1	1%	3465	3580	RC6,RA4
13	K1	15%	2383	4334	RA4,RC6
14	K1	2%	692	699	RA4,RC6
15	K1	1%	12	17	RA4,IS6
16	K1	2%	342	342	RA3,RA7,IS6,VA1
17	K2	0%	852	942	RC5,RA4,BK
18	K1	4%	157	362	RA4,RA7,IS6
19	K1	0%	2259	2301	RC5,RC3
20	K1	0%	3732	3729	VA1,RA3,RA7,RC3
21	K1	2%	1620	2009	IN6,RC6,RA4,IS6
22	K2	2%	230	383	RC5
23	K2	4%	394	1212	RC5
24	K1	5%	183	3077	RC5,RA3,D11
25	K1	3%	1523	1616	RC5,RA4,BK
26	K2	1%	23	102	RC5,RA4
27	K1P	2%	1401	1646	RC5,IS6,VA1

[a] Pathology classifications: Acute rejection: RA1=minimal, RA3=moderate, RA4=severe, RA7=with intimal arteritis. Chronic allograft nephropathy: RC3=mild, RC4=moderate, RC5=severe, RC6-chronic rejection. VA1=vascular obstruction/thrombosis, IS6=acute infarct, BK=BK virus nephropathy.

* K=kidney, P=pancreas.

2.2. Tissue typing methods

All patients and donors were HLA-typed by standard serological methods. This study focused on class I compatibility; only HLA-A and HLA-B antigens were considered because reliable HLA-C typing data were not available. The average number of mismatched donor HLA-A and HLA-B antigens was 2.8 (range 1–4). Serum screening for HLA antibodies was done by direct lymphocytotoxicity (modified Amos technique) with 50–60 HLA typed cell panels.

2.3. Structural determination of donor HLA-A,B mismatches

We have used HLAMatchmaker to determine donor-recipient compatibility at the structural level. This algorithm considers each HLA antigen as a string of polymorphic triplets of amino acid residues in antibody-accessible sequence positions [14]. Such triplets are considered key elements of epitopes that can induce the formation of HLA-specific antibodies. The overall triplet repertoire of HLA-A and HLA-B antigens has been described elsewhere [14] and

HLAMatchmaker programs can be downloaded from our website https://tpis.upmc.edu.

2.4. HLAMatchmaker-based serum analysis

The patient's HLA phenotype represents the repertoire of self-triplets and HLAMatchmaker determines for each donor HLA antigen the mismatched triplets in corresponding sequence positions. For each serum, we determined the Panel Reactive Antibody (PRA) as the percentage of panel cells that gave positive reactions. Serum reactivity data were analyzed with a HLAMatchmaker-based serum analysis program that is also available on our website https://tpis.upmc.edu.

After entering the HLA-A,B.C phenotype of the patient, the program identifies non-self triplets for each HLA antigen in the panel. The first step of this analysis is to identify triplets on panel cells that give negative reactions with patient's serum. Such triplets are apparently not recognized by patient's antibodies and can therefore be considered as acceptable mismatches. After recording the negatively reacting antigens, the program generates a list of mismatched HLA antigens with zero/acceptable triplet mismatches. This approach is useful in the Acceptable Mismatch program of Eurotransplant to identify compatible donors for highly sensitized patients [16].

The final step of this analysis is the identification of triplets on reactive panel cells and to determine which ones are present in the HLA antigens of the donor. Often enough, certain reactive panel cells have only one or few mismatched triplets. This suggests that patient antibodies specifically react with these triplets and this information permits the determination of unacceptable HLA antigens that express such triplets. This approach is useful in donor selection strategies that consider the avoidance of unacceptable antigen mismatches.

2.5. Statistical methods

Statistical analyses were done with STATISTICA software from StatSoftTM (Tulsa, OK) and included chi-square testing (Fisher's Exact Test), Student *t*-test, and regression analysis.

3. Results

3.1. Serum PRA changes following alloNx

This study was done on 27 patients with rejected kidney transplants and who underwent alloNx while being on the waiting list for another transplant. Before alloNx, their mean serum PRA was 8.0±2.5%. After alloNx, these patients showed a significant PRA increase to 57.3±5.0% ($p=5\pm10^{-12}$). In 24 cases, the PRA rose between 23% and 84% during the first few months post-alloNx.

3.2. Donor HLA-A,B antibody specificity analysis of post-alloNx sera

Table 2 shows details of the serum analysis results, including the pre- and post-alloNx PRA values. The cases are sorted in ascending order of %PRA after alloNx and their numbers correspond to those shown in Table 1. The PRA increases and antibody reactivity patterns did not correlate with graft survival times and the histopathology findings showed acute rejection and/or chronic rejection of all removed allografts.

The first step of the HLAMatchmaker-based serum analysis was to identify HLA antigens and triplets on negatively reacting panel cells. Table 2 shows which ones belonged to the transplant donor. In each case, we could generate a list of donor antigens and triplets that did not react with patient's antibodies although the patient had been exposed to these mismatches and had rejected the transplant. These antigens and triplets can be considered as acceptable mismatches.

The second step was to determine what mismatched donor antigens and triplets were present on panel cells that reacted with post-alloNx sera. They are listed in Table 2 and can be considered as unacceptable mismatches because the patient showed specific antibodies apparently elicited by a transplanted organ that was rejected. In many cases, the antibody specificity pattern could be easily determined because it involved one or few triplets. For instance, case 5 was specific for 163dT which is uniquely present on A3 and case 6 was specific 66rKv and/or 74H which are unique for A2. In case 3, the immunizing donor antigen was A25 but the serum reacted also with A26, A34, and A66, all of them share 149tAh with A25. Thus, case 3 had produced anti-149tAh antibodies. Similarly, case 2 had antibodies against 177Dk which is shared between B7 of the donor and B48, B60 and B81 whereas case 9 showed antibodies against 166Dg which is shared by the donor's A1 with A23, A24 and A80.

Serum reactivity with a given donor antigen was often specific for one or few distinct triplet(s) shared with other antigens. For instance, antibodies elicited by A2 reacted frequently with B57 and B58 which share 62Ge (cases 12, 13, 14, and 16 are informative) and with A68 and A69 which share 142T and 144tKh with A2 (cases 10, 14, 16, 24, and 27). There were also four cases whereby a response to B51 involved antibodies to 193Pv which is also present on B35, B52, B53, B58, and B78 (cases 7, 12, 15, and 22). Other examples are the responses to the Bw4-associated triplets 76En, 80rla, and 82aLr (cases 11, 18, 24, 25, and 27). These triplets have high frequencies and antibody responses lead to high PRA values.

It should be noted that the screening results of high PRA sera with 50–60 HLA-typed panels were often incomplete because insufficient numbers of informative cells were available that would give negative reactions. Especially for >85% PRA sera, this may lead to overestimations of the

Table 2
Donor-specific antibody reactivity patterns of post-allograft nephrectomy sera

Case nr.	Pre-alloNx PRA	Post-alloNx PRA	HLA-A,B type patient	HLA-A,B type donor	Mismatched donor antigens and triplets on negatively reacting antigens	Mismatched donor triplets on reactive panel cells*	Reactive donor antigens	Reactive donor triplets
1	0%	0%	A2,A11;B7,B13	A23,A28; B27,B51	A23,A28,B27,B51,9H, 9S,45Te,66qlc,66qlf,76Ed, 80rla,80rTI,82ILr,156W, 163L,166Dg,171H	none	0/4	0/13
2	0%	11%	A2,A24;B13,B55	A2,A24; B7,B62	B62,156W,163L,180E	177Dk	1/2	1/4
3	2%	25%	A3,A23;B7,B49	A25,A29; B7,B18	A29,B18,9T,45Te,76An, 156W,163R,171H,183A, 193Av	149tAh	1/3	1/9
4	2%	30%	A24,A25;B7,B55	A3,A11; B7,B38	B38,9F,62Qe,66qlc,70aQs, 70tNt,74Y,76Vd,80gTl, 151aRv	151aHa,163dT	2/3	2/11
5	2%	30%	A1,A24;B8,B62	A1,A3; B8,B62	66rNv,70aQs,76Vd,151aHe, 156L	163dT	1/1	1/8
6	2%	35%	A3,A23;B49,–	A2,A11; B51,B60	A11,B51,B60,9Y,45Te, 62Ge,62Rn,66qlf,76Es, 80rNl,142T,144tKh, 151aHv,156Q,163E,163R, 171H,177Dk,180E, 183A,193Av	66rKv,74H	1/4	2/20
7	5%	35%	A2,A30:B13,B27	A3,A26; B7,B51	A26,B7,62Rn,66qlf, 66qly,70aQa,76An,76Es, 80rla,80rNl,144tKr,149tAh, 151aHe,156W,163L,163R, 171H,177Dk,180E	45Te,163dT,193Pv	2/4	3/20
8	5%	36%	A2,A5;B7,B27	A1,A2; B7,B8	B8,11Am,62Qe,66qlf, 66rNm,70tNt,76An, 144tKr,156D,177Dt	45kMe,149vHa, 166Dg	1/2	3/12
9	2%	40%	A3,A11;B35,B44	A1,A24; B7,B60	B7,B60,9H,45Ee,66qly, 66rNm,70aHs,70aQa, 76An,80rla,127K,151aHv, 163E,177Dk,180E	45kMe,62Ee, 149vHa,166Dg	2/4	4/16
10	3%	49%	A3,A31;B7,B39	A2,A3; B7,B39	62Ge,127K,151aHv,183A	66rKv,74H,142T, 144tKh	1/1	4/8
11	2%	54%	A1,A31,B7,B35	A1,A32, B49,B61	B61,9H,41T,45Ke, 66qls,183A,193Av	76En,80rla,82alr, 107GrL	2/3	4/10
12	55%	55%	A3,A28;B35,–	A2,A11, B51,B60	A11,9H,41T,45Ke, 66qls,70aHs,76En,80rla, 82aLr,90D,156Q,163E, 163R,171H,180E	62Ge,66rKv,74H, 177Dk,193Pv	3/4	5/20
13	6%	60%	A11,A28;B51,B52	A2,A11; B8,B44	70aHs,76Es,80rNl, 80rTa,151aRv,156D,180E	9D,66rKv,62Ge, 74H,166Es,177Dt, 199V	3/3	7/14
14	2%	60%	A24,A33;B8,B35	A1,A2; B7,B35	B7,B35,9F,62Qe,66qly, 70aQa,76An,163E,163R, 177Dk,183A	45kMe,62Ge,66rNm, 66rKv,74H,142T, 144tKh,149vHa	2/4	8/17
15	5%	60%	A2,A11;B7,B18	A30,A68; B18,B51	A30,A68,9S,11Am, 56R,66qlf,76En,80rla, 82alr,156W,163L	193Pv,	1/3	1/10
16	2%	60%	A24,A30;B7,–	A2,A24; B7,B60	9F,9H,66qls,11Am,70tNt, 74Y,151aRv,183A,193Av	41T,45Ke,62Ge, 66rKv,74H,142T, 144tKh	2/2	7/16
17	3%	65%	A2,A36;B52,B53	A2,A36; B42,B53	45Ee,76Es,80rNL,156D	66qly,70aQa,177Dt, 180E	1/1	4/8
18	3%	70%	A1,A30;B8,B18	A1,A30; B8,B13	9Y,45Ma,163E	41T,76En,80rTa,82aLr, 144tQl	1/1	5/8
19	32%	79%	A2,A3;B8,B56	A1,A2; B8,B45	9H,66q1s,66rNm,74Y, 76An,163R	41T,45Ke,45kMe, 149vHa,166Dg,166Es	2/2	6/12
20	0%	80%	A2,A32;B56,B70	A1,A2; B8,B56	66qlf,90D,144tKr,166Dg, 180E	9D,45kMe,66rNm, 76An,149vHa,156D, 163R,177Dt	2/2	8/13

Table 2 (*continued*)

Case nr.	Pre-alloNx PRA	Post-alloNx PRA	HLA-A,B type patient	HLA-A,B type donor	Mismatched donor antigens and triplets on negatively reacting antigens	Mismatched donor triplets on reactive panel cells*	Reactive donor antigens	Reactive donor triplets
21	2%	80%	A23,A32,B44,B51	A1,A3; B58,B60	B60,9H,70aQs, 76Vd,80gTl, 80rNl,151aHe,163E, 177Dk,180E	45kMe,62Ge,66rNm,70aSa, 76An,144tKr,149aAh, 149vHa,163dT,163R	3/4	10/19
22	2%	86%	A2,A11;B36,B60	A1,A26; B14,B51	B14,45Ee,66qlc,80rla, 82Alr,151aHe,171H	45kMe,66rNm,76An,76En, 149tAh,149vHa,156W, 166Dg,193Pv	3/4	9/15
23	4%	86%	A2,A26;B49,B57	A2,–;B64, B51	B51,45Te,66qlf,80rNl, 171H,193Pv	45Ee,45GeV,66qlc,76Es	1/2	4/9
24	18%	90%	A1,A26;B8,B35	A1,A2, B8,B57	105S,76Vd,149aAh	45Ma,62Ge,66rKv,70aSa, 74H,76En,80rla,82aLr, 127K,142T,144tKh,151aHv	2/2	12/15
25	32%	91%	A2,A68;B18,B72	A29,A33; B38,B58	66rNm,76An,142m1	9T,62Lq,66q1c,70aSa, 74iD,76En,80rla,82aLr,158T, 186R	4/4	10/13
26	7%	91%	A23,–;B7,B8	A3,A31; B55,B63	B55,9F,62Qe,66rNm, 66rNv,74Y,80gTl,131S, 144tKr,149aAh,163L,193Av	9T,45Ma,56R,70aQs,70aSa, 74iD,76Vd,151aHe,163dT	3/4	9/20
27	6%	96%	A31,A34;B14,B60	A2,A30; B13,B57	9F,66rNm,163L	9S,45Ma,56R,62Ge,66rKv, 70aSa,74H,76En,80rla,80rTa, 82aLr,127K,142T,144tKh, 144tQl,149aAh,151aHv	4/4	17/20

numbers of reactive antigens and triplets, although as described elsewhere [17,18], such sera generally have antibodies against small number of high-frequency epitopes.

The total number of HLA-A, B antigen mismatches for these 27 alloNx cases was 77 and 51 of them (66%) were found on panel cells that reacted with post-alloNx sera. No antibody reactivity was found against the remaining 26 (or 34%) of these mismatched antigens. This group had a total of 358 mismatched triplets and 211 of them (or 59%) were not recognized by patient antibodies. The remaining 147 triplets were present on panel cells that reacted with patient sera. This analysis could not always identify which triplets on reactive panel cells were actually recognized by patient antibodies–this applies especially to the high PRA sera–and it seems likely that the actual proportion of antibody-reactive triplets is lower. Nevertheless, these findings demonstrate that after removal of a rejected kidney transplant, the serum has a restricted antibody specificity pattern against donor HLA-A,B mismatches.

3.3. Specificity analysis of pre-alloNx sera

These findings demonstrate the impact of alloNx on the detection of circulating donor-specific antibodies in sera from patients with rejected kidney transplants. In 23 cases, such antibodies could not be detected before alloNx. Four cases were exceptions and their pre-alloNx sera had PRA values ranging from 18 to 55% (Table 2). Case 25 had a pre-alloNx PRA of 32% but the sera did not react with any donor antigens or triplets and the antibody specificity was against A10 and A11. In contrast, the other three cases clearly exhibited the presence of circulating donor-specific antibodies.

Case 12 showed that sera before and after alloNx had equally high PRA values (55%) and practically identical antibody reactivity patterns against certain triplets on donor antigens namely 62Ge, 66rKv, and 74H on A2; 193pV on B51; and 177Dk on B60. There was no antibody reactivity against the 15 remaining mismatched triplets on donor antigens.

Case 19 had a mismatch for A1 and B45, and the pre-alloNx sera had a 32% PRA with antibodies reacting with 45kMe and/or 149aVh (these triplets are unique for A1) and 166Es, which is present of the donor's B45 and shared with B44. The PRA increased to 79% after alloNx and additional antibody activity became detectable against 166Dg (expressed by A1, A9 and A80) and 41T and 45Ke (expressed by B45 and several other antigens including B21 and B40).

Case 24 had a mismatch for A2 and B57 and the pre-alloNx sera showed weak antibody reactivity to 62Ge which is uniquely shared between these antigens. After alloNx, the PRA rose from 18% to 90% due to the presence of antibodies reactive with additional triplets including the Bw4-associated 82aLr (present in the immunizing 857) and 127K (shared by A2 with A9 and A28).

Cases 19 and 24 illustrate situations whereby donor-specific antibodies against some epitopes were readily demonstrable in pre-alloNx sera whereas circulating antibodies against other epitopes became detectable only after graft removal. The reason for this is not clear. One possible explanation is that through competitive binding, an antibody

against one epitope of a given antigen expressed by the allograft can block the binding of another antibody against different epitope on the same antigen so that the latter becomes detectable in unbound form.

3.4. Follow-up analysis of post-alloNx serum samples

For 20 of 27 cases, we had serum screening data for 12 or more months after alloNx. Seven cases showed a persistent PRA and antibody specificity pattern but in 13 cases, we noted a progressive decline in antibody reactivity. Fig. 1 shows two examples of antibody persistence and two examples of declining antibody activity. It also shows the strings of mismatched triplets (i.e. triplotypes) of donor HLA antigens and which triplets are shared by serum-reactive panel cells. We also describe the identification of unacceptable antigens for these patients.

Case 7 was a four HLA-A,B antigen mismatched transplant that failed after 40 months (Fig. 1A). The graft was removed 10 months later and the pathology showed severe allograft nephropathy and thrombosis. During a 12-month follow-up after alloNx, the PRA remained in the 35–45% range and the sera continued to react 163dT which is unique to the donor's A3 and two triplets of the donor's B51: 45Te (also present on B18, B35, B37, B52, B53, B58, and B78) and 193Pv (also present on B35, B52, B53, B58, and B78). It should be noted that the 193Pv-carrying antigens are all included in the group of 45Te-carrying antigens. From this antibody reactivity pattern, one can readily conclude that A3, B18, B35, B37, B51, B52, B53, B58, and B78 should be considered unacceptable mismatches.

Fig. 1A shows also which donor triplets did not react with patient serum. They include the entire triplotypes of A26 and B7 and several triplets were present on two or three donor HLA antigens. These triplets can be considered acceptable mismatches and this information can be used to identify acceptable antigens for this patient.

Case 26 was transplanted with a kidney with 4 HLA-A,B antigens; their triplotypes showed a total of 18 different triplets (Fig. 1B). The graft was rejected after one month and alloNx was done 2.5 months later. Shortly thereafter, the PRA increased to more than 90%. The HLA-B antigens of serum-reactive panel cells shared two triplets with the donor's B63, namely 45Ma also present on B13, B46, B57, B62, B75, B76, and B77 and 70aSa also expressed by B57 and B58; these antigens are considered unacceptable HLA-

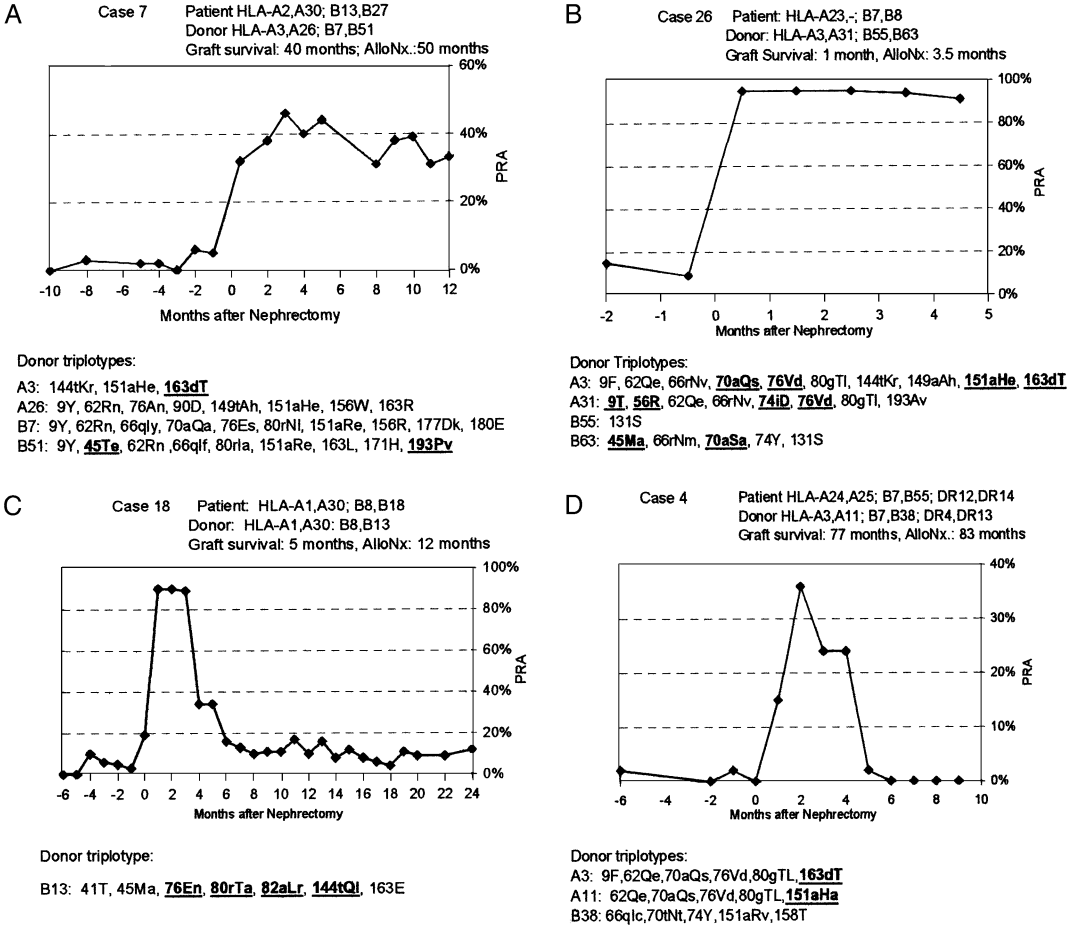

Fig. 1. Four examples of serum reactivity patterns following allograft nephrectomy. The triplotype of each donor HLA antigen shows what triplets are mismatched for the patient. Triplets on serum-reactive panel HLA antigens are underlined in bold font.

B mismatches. The serum-reactive HLA-A antigens shared four triplets with the donor's A3: 70aQa (also on A11, A29, A30, A34, A66, A68, and A69), 76Vd (also on A2, A11, A30, A31, A33, A34, A66, A68, A69, and A74), 151aHe (also on A25, A26, A34, and A66), and 163dT (unique for A3). The donor's A31 had three additional triplets present on serum-reactive antigens: 9T (also on A29 and A33), 56R (also on A30), and 74iD (also on A33). It is apparent that these triplets are expressed on overlapping groups of antigens. While our analysis of this very high PRA serum could not establish which triplets were recognized by patient's antibodies, these findings readily indicate that A2, A3, A11, A30, A31, A33, A34, A66, A68, A69, and A74 and possibly A25, A26, and A29, should be considered as unacceptable mismatches.

The next two cases showed declining serum reactivity. Case 18 was a transplant with a mismatched B13, its triplotype consisted of 7 triplets (Fig. 1C). The graft failed after 5 months and was removed 7 months later. After alloNx, the PRA increased to 90% and the serum showed antibody reactivity against 144tQl (uniquely found on B13) and its Bw4-associated triplets 76En, 80rTa, 82aLr (also on A23, A24, A25, A32, B5, B17, B37, B38, B44, B49, B53, B59, B63, and B77) as well as against A2 not found on the donor. This antibody reactivity persisted for a few months but declined afterwards. The PRA stabilized at about 12% and the sera continued to react with the 144tQl triplet of B13. Thus, although recent serum screening results identified B13 as an unacceptable mismatch, the early post-alloNx data indicate that the Bw4-associated antigens (and A2) should be considered unacceptable mismatches for this patient.

Case 4 was a three-antigen mismatched transplant that failed after 6 years due to chronic rejection (Fig. 1D). After alloNx, the PRA went to 35% and specific antibody reactivity was detected against the donor's A3 (which has a unique 163dT triplet) and A11 (which has a unique 151aHa triplet). No reactivity was seen against B38 and the other triplets of the donor. Subsequent testing showed declines in PRA values and after 6 months, the sera became completely negative. These findings indicate that A3 and A11 were unacceptable mismatches for this patient. All other donor triplets were considered acceptable mismatches.

3.5. Relative immunogenicity of mismatched triplets

The analysis of post-alloNx sera provides opportunities to determine the relative immunogenicity of epitopes that can induce humoral immune responses. Although this cohort of 27 patients is rather small, this analysis has yielded some informative data. Twenty triplets were selected because they were mismatches in five or more transplant cases. Table 3 shows a wide range in the frequencies of

Table 3
Serum reactivity frequencies against mismatched donor triplets

Triplet	Triplet-carrying HLA-A,B antigens	Number of cases	% Positive reactions	Relative immunogenicity[a]
144tKh	A2,A28	5	100%	11.7
76En	A9,B5,B13,B17,B38,B44,B49,B53, B59,B63,B77	6	83%	2.7
62Ge	A2,B17	9	78%	6.6
166Dg	A1,A9,A80	6	67%	1.5
82aLr	A9,A25,A32,B5,B17,B38,B49,B53, B59,B63,B77	8	63%	2.7
41T	B12,B13,B21,B40,B41,B47	5	60%	1.4
66rNm	A1,A36,B17,B63	11	45%	1.2
80rla	A9,A25,A32,B5,B13,B17,B38, B44,B49,B53,B59,B63,B77	10	40%	1.7
66qlc	B14,B16,B27,B71,B73	5	40%	0.1
76An	A1,A26,A29,A36,A43,A80	10	30%	0.7
177Dk	B7,B48,B60,B81	7	29%	2.4
163R	A1,A11,A25,A26,A43,A66	8	25%	1.3
144tKr	A1,A3,A11,A24,A36,A80	5	20%	1.4
76Vd	A2,A3,A11,A28,A30,A31,A33, A34,A66,A74	5	20%	0.2
76Es	A25,A32,B7,B8,B14,B18,B22, B35,B39,B40,B41,B42,B45,B48,B50, B62,B67,B7O,B75,B76,B78,B81,B82	5	20%	1.1
156W	A10,A43,B46,B62,B76	6	17%	0.6
180E	B7,B8,B41,B42,B48,B60,B81	9	11%	0.8
66qlf	B8,B35,B51,B53,B59,B78	7	0%	0.2
163E	A80,B7,B13,B27,B40,B47,B48,B73,B81	6	0%	1.6
80rNl	B7,B8,B14,B18,B22,B35,B39,B40, B41,B42,B45,B46,	6	0%	0.9

[a] Assessed as the radio of frequencies of positive and negative correlations between triplets and the reactivity patterns of high PRA sera as previously described [18].

positive reactions for these triplets. Highly reactive triplets were 144tKh, 76En, 62Ge, 82aLr, and 41T; all of them are associated with well-known public epitopes. Other triplets such as 66qlf, 180E, and 156W showed low frequencies of serum reactivity. When comparing triplets in the same sequence position, we noted that 66qlf seemed considerably less immunogenic than 66rNm and 66qlc (0% in 7 cases versus 44% in 16 cases; p=0.05).

Table 3 also compares these findings with previously published data on the relative immunogenicity of triplets [19]. That study assessed the relative immunogenicity as the ratio of frequencies of positive and negative correlations between triplets and the reactivity patterns of sera from 127 highly sensitized transplant candidates. For these 20 triplets, the frequencies of positive reactivity in the post-alloNx sera showed a significant correlation with relative immunogenicity ratios (r=0.71, p<0.0005 by regression analysis). These findings provide further support that triplet-defined epitopes have different degrees of immunogenicity.

4. Discussion

This study addressed the detection of lymphocytotoxic antibodies against antigens encoded by the HLA-A and HLA-B loci. These class I antigens are widely expressed on many tissues and since a transplanted kidney is a rather large antigen source, one might expect that donor-specific antibodies are readily adsorbed during a humoral response to the allograft. Our findings are consistent with previous reports that in most patients, donor-specific antibodies become detectable in sera following surgical removal of rejected kidney transplants [6–12]. Without nephrectomy, a patient's serum may show an incomplete antibody reactivity pattern and this may interfere with the determination of HLA mismatch acceptability and cross-match outcome when a new donor is being considered. This may help to explain why repeat transplants have lower success rates. For instance, a recent report from the UNOS Scientific Registry showed that for HLA-A mismatches in whites, the 5-year graft survival rate of first transplants is 82% but only 69% for repeat transplants [20]. For African-Americans, these rates are 81% and 64%.

Our findings on this group of 27 patients with rejected grafts showed circulating donor-specific antibodies in 3 cases (11%) before and in 26 cases (97%) after alloNx. All sera had restricted antibody specificity patterns and most patients showed progressive decreases in serum reactivity during follow-up periods of 1–2 years. We could not find any apparent relationship between the serum reactivity or antibody patterns and graft survival times and all removed grafts showed acute and/or chronic rejection. About one-third of the grafts were long-term (>5 years) survivors and almost all of them had donor-specific antibodies that were detectable only after alloNx.

This serum analysis addressed the antibody specificity patterns against donor HLA-A,B antigens and more importantly, structural polymorphisms defined as amino acid triplets in antibody-accessible sequence positions. HLAMatchmaker determines the array of mismatched triplets of the donor and the serum reactivity patterns with HLA-typed panels can identify many donor triplets that are non-reactive while others are apparently recognized by antibodies. The latter should be avoided in a subsequent transplant even if they are present on other HLA antigens not previously encountered by the recipient. This approach permits a more complete listing of unacceptable antigens for sensitized transplant candidates. HLAMatchmaker-determined mismatch compatibility has been validated as a highly efficient predictor of cross-match results with potential donors [16,21–23].

Two explanations can be forwarded for the restricted antibody patterns: antigenic competition and anti-idiotypic immune responses. The phenomenon of antigenic competition has been known for many decades [24–26]. The general idea is that individual antigens can evoke antibody formation by themselves, but in combination they will compete with each other and often enough, the immunodominant antigen will suppress the response to the other antigen. Antigenic competition can occur between epitopes on the same or different molecules. The exact mechanisms have remained elusive but they appear to be related to helper T-cell-dependent positive and negative selection of antigen-reactive B-cells undergoing somatic hypermutation and affinity maturation [27,28].

It seems likely that antigenic competition occurs also during humoral immune responses to HLA antigens. Following exposure to one or more HLA mismatches, certain triplets are immunodominant in eliciting antibodies, whereas other less immunogenic triplets induce immunological unresponsiveness at the humoral immune level. Table 3 provides examples of triplets that often or infrequently reacted with patient's antibodies and these findings are consistent with previously reported data on the relative immunogenicity of triplets [19].

The restricted antibody patterns might also be due to a down-regulation of humoral alloimmunization by anti-idiotypic antibodies [29]. Such antibodies may appear after any sensitizing event including transfusion, pregnancy, and transplantation and they can be demonstrated by their blocking effect of the lymphocytotoxic activity of HLA-specific antibodies [29–33]. Anti-idiotypic antibodies appear to enhance graft survival rates of mismatched transplants [34,35]. Our analysis dealt with irreversibly rejected kidney transplants and practically every case showed lymphocytotoxic antibodies against certain donor mismatches. While we could not measure anti-idiotypic responses, it has been reported that anti-idiotypic responses can be elicited against antibodies specific for one antigen but not against antibodies to another antigen on the same immunizer [36].

The identification of mismatched donor triplets that did not induce specific antibodies responses during a previous transplant might be clinically relevant for defining criteria of mismatch acceptability for sensitized patients awaiting another transplant [16]. One might even identify a subgroup of "preferred" acceptable mismatches because these antigens contain mismatched triplets towards which the patient might have developed some type of non-responsiveness. In case of a subsequent transplant, re-exposure to such triplets might be preferable over the introduction of new triplet mismatches on other donor antigens that may or may not induce antibodies.

Kinetic analyses have shown that serum reactivity had the highest levels within a few months after alloNx. Several patients showed a persistent PRA and antibody specificity pattern but many others showed progressive decreases and often enough the sera became non-reactive. Two explanations can be offered for the declines in donor-specific antibody activity in post-alloNx sera and they imply opposite interpretations regarding HLA mismatch acceptability.

First, the removal of the antigen source may cause a cessation of antibody production and a conversion to memory B-cells which can be re-activated to produce antibodies following subsequent antigenic exposure. In this situation, such antigens should always be considered as unacceptable mismatches. It also supports the argument to screen sera soon after altoNx to optimize the detection of HLA-specific antibodies.

The second explanation relates to the development of anti-idiotypic antibodies that block HLA-specific alloantibodies [29,30,37]. In view of the enhancing effect of anti-idiotypic responses on graft survival [31,34], one might speculate that donor antigens and epiopes inducing specific antibodies might turn out to be acceptable mismatches if such antibodies had elicited subsequent anti-idiotypic responses. No sera were available from this cohort of patients to conduct informative studies about anti-idiotypic responses.

The present study has obvious limitations since the serum analysis did not utilize other methods for antibody detection such as ELISA and Flow Cytometry, and we did not consider HLA-C and class II antigens encoded by HLA-DR and HLA-DQ. While studies with these are still in progress, preliminary data (not shown) are consistent with the notion that alloNx permits a better assessment of HLA-specific antibody responses of transplant recipients.

Serum antibody analyses on a large number of alloNx cases can determine how often a mismatched triplet will induce specific antibodies. This report identifies certain triplets that are immunodominant whereas others have low immunogenicity and these findings are consistent with previously reported data on the relative immunogenicity of triplets [19]. Under auspices of the 14th International HLA and Immunogenetics Workshop, a multi-laboratory collaborative project on alloNx cases is underway to determine the immunogenicty of structurally defined epitopes. Such information will be useful in designing new structurally based matching strategies that avoid high-risk HLA antigen mismatches with immunodominant epitopes.

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