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Original Article

Long-Term Effects of Antibodies against Human Leukocyte Antigens Detected by Flow Cytometry in the First Year after Renal Transplantation

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ABSTRACT

Objective: In this study, we aimed to investigate the incidence, dynamics and profiles of human leukocyte antigen (HLA)-directed antibodies developed after transplantation and their impact on graft rejection and outcome in kidney recipients.

Study Design: Prospective follow-up study.

Material and Methods: A total of 56 kidney recipients were monitored at 1st, 6th and 12th months for the development of anti-HLA antibodies using bead based flow-cytometry assays (Flow PRA tests).

Results: In 21 (37.5%) patients, panel reactive antibodies (PRA) was positive after transplantation, however, in 35 (62.5%) patients PRA was found negative. Twelve (57.1%) patients with post-transplantation HLA-reactive antibodies [PRA (+)] and 8 (22.9%) patients with no detectable alloantibodies [PRA (-)] were developed allograft rejection (p=0.010). In the PRA positive patient group the rates of early period infection and delayed graft function (DGF) were higher than the PRA negative patient group. Serum creatinine levels of PRA positive group at 6. and 12. months after transplantation were significantly higher than the PRA negative group (p=0.015 and p=0.048, respectively). The rejection rates of patients who had class I and II HLA antibodies were significantly higher than the patients who had either class I or II HLA antibodies (p=0.011). Acute rejection rates were significantly higher in patients who had class I and II HLA antibodies at the first month (p=0.007).

Conclusion: Higher occurrence of rejection episodes in PRA positive group may show the importance of anti-HLA antibody monitoring using Flow-PRA after renal transplantation as a prognostic marker in terms of graft survival.

Key Words: Anti-HLA antibodies, flow cytometry, renal transplantation

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Introduction

Renal transplantation is associated with several complications, some of which may cause irreversible loss of graft function. Despite reliable pre-transplant screening methods and improvement of immunosuppression therapy, failures of kidney allografts are still occurred because of cellular and/or humoral mediated rejections (1). Several recent studies evaluated the prevalence of human leukocyte antigen (HLA)-specific antibodies and the clinical importance of these antibodies in acute allograft rejection (2, 3). Chronic rejection is also known to have several immunologic and non-immunologic causes. Acute rejection episode after renal transplantation is also a known risk factor for the development of chronic rejection (4). Antibodies against HLA developed after blood transfusions, pregnancies and graft rejections were generally described as panel reactive antibodies (PRA). After sensitization antibodies appear against to both HLA class I and HLA class II. Class I and Class II HLA antibodies activate different cells, initiate immune response and contribute to rejection. Over the past years many studies reported the relevance of various incidences of alloantibodies detected after transplantation (5-8). This variability can be attributed to the use of different techniques to detect the antibodies and differences in the time after transplantation that samples are collected (6). Post-transplantation detection of HLA antibodies was found to be associated with high rejection rates (7-10). HLA antibodies developed in the early term of transplantation damages allograft more than antibodies developed after 1 year of transplantation (11). Post-transplantation alloantibody development in the early period may be associated with reperfusion and prolonged cold ischemia time [a chief factor leading to delayed graft function (DGF)] induced activation of endothelium and impaired cytokine gene expression, release of proinflammatory cytokines, and upregulation of HLA and adhesion molecules (1, 12, 13). These events lead to stimulation of the immune response in the early post-transplantation period and, as a consequence, to HLA antibody production. However, in some instances even in the absence of detectable pre-transplantation sensitization, reactivation of memory

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B cells from sensitizing events in the patient's history may facilitate the alloantibody production in the early days after transplantation. Rejections may still occur in the absence of detectable lymphocytotoxic antibodies, suggesting that non-HLA antigenic systems may also play a role in renal allograft rejections (10-16). Despite increasing recognition of the role of posttransplantation humoral alloreactivity in graft outcome, there is still debate regarding the clinical relevance of anti-HLA antibodies detected by sensitive solid-phase assays.

In this study, we aimed to investigate the incidence, dynamics and profiles of HLA-directed antibodies developed after transplantation and their impact on graft rejection and outcome in kidney recipients using sensitive and specific flow-cytometry bead-based techniques.

Material and Methods

Patients

A total of 56 patients [35 male, 21 female, mean age 38±10 years (range 15-63)], underwent renal transplantation between 2001 and 2007 at the Istanbul Faculty of Medicine Hospital, were included in this observational prospective study. Information on demography, body mass index (BMI), the etiology of end stage renal disease (ESRD), time on dialysis treatment, viral serology and donor characteristics were collected by reviewing patient files and medical records. Fifty patients underwent living related and 6 patients underwent cadaveric renal transplantation. The living related donors were mother (n=17, 34%), siblings (n=13, 26%), father (n=12, 24%), spouse (n=4, 8%), cousin (n=3, 6%) and maternal aunt (n=1, 2%). Twenty seven (48%) of the patients have a history of pre-transplant sensitization. The sensitizing events were blood transfusion in 21 patients, blood transfusion and pregnancy in 4 patients and solely pregnancy in 2 patients.

The standard immunosuppressive regimen of the patients at the İstanbul Faculty of Medicine included a calcineurin inhibitor, mycophenolate mofetil (MMF) and prednisone. Target blood levels for cyclosporine A (CsA) were 200-300 ng/mL in the first 3 months, 100-200 ng/mL between 3-12 months and 50-150 after the first year of transplantation. Target blood levels for tacrolimus were 10-15 ng/mL in the first 3 months and 5-10 ng/mL after the third month of transplantation. MMF was given as a daily dose of 2 gr in CsA based regimen and 1 gr in Tacrolimus based regimen. All of the patients received prednisone; beginning with an infusion dose of 250 mg per 6 hours on the day before transplantation, 500 mg infusion on the transplant day and 120 mg iv on the next day of transplantation with a rapid taper and reaching to maintenance dose of 10 mg daily within the first month. However, doses were individualized according the patients needs. Induction therapy (ATG Fresenius, 2 mg/kg/day) was used in transplantations from deceased donors.

Delayed graft function (DGF) was defined as the need for dialysis within the first week post-transplant. Recorded acute rejection and chronic rejection episodes were clinical or biopsy proven. Humoral rejection (acute or chronic) was defined by the presence of biopsy C4d staining in peritubular capillaries and de novo donor spesific antibodies (DSA) in serum. Post-transplant acute tubular necrosis (ATN) was defined as exclusion of other causes of DGF such as acute rejection and technical complications. Chronic allograft nephropathy (CAN), infection, cardiovascular disease, malignancy and bone disease in the late post-transplant period were also recorded as long-term post-transplant complications. Our examinations of the patients conformed to good medical and laboratory practices and to the recommendations of the *World Medical Association Declaration of Helsinki: Recommendations Guiding Medical Doctors in Biomedical Research Involving Human Subjects*.

HLA tissue typing and screening of anti-HLA antibodies in serum

Human leukocyte antigen tissue typing was performed in European Federation for Immunogenetics (EFI)-accredited HLA laboratories of Department of Medical Biology. Class I HLA-A,-B typing was performed by complement dependent cytotoxicity (CDC) method, whereas class II HLA-DRB1 typing was performed by low-resolution polymerase chain reaction (PCR)-sequence-specific primer (SSP), as has been described elsewhere (17, 18). In case of an ambiguity in class I typing, PCR-SSP was performed as well. For the current study, anti-HLA antibodies and DSA in serum were monitored at 1st, 6th and 12th months using bead based flow-cytometry assays (FlowPRA™ Screening Test, FL12-60; One Lambda, Canoga Park, CA, USA).

Flow-Cytometric Analysis of Alloantibody

Flow-cytometric detection of HLA-specific antibodies was performed by FlowPRA™ Screening Test (FL12-60; One Lambda, Canoga Park, CA, USA). FlowPRA™ specific tests (FL1SP, FL1SP44, FL1HD, FL2SP; One Lambda, Canoga Park, CA, USA) were used for definition of HLA antibody specificity in the sera. The tests were performed according to the instruction of the manufacturer. In brief, 5 µL of FlowPRA microparticles were admixed with 20 µL of patient serum and incubated for 30 min. at room temperature. Control non-MHC-coated beads were included in each sample to monitor the nonspecific interaction of the testing serum with the beads. After washing, the beads were stained with 100 µL of pretitered FITC-conjugated F(ab)'2 fragment of goat antihuman IgG for an additional 30 minutes. After a final wash, 500 µL of wash buffer was added per tube and analyzed on flow cytometer (EPICS-XL Coulter Corporation, Miami FL, USA). Screening results were recorded as positive when 10% of class I and/or class II beads exhibited fluorescence above the negative control serum and/or a significant change in the histogram architecture compared with the negative serum control. Specificity results were scored according to the scheme included in the kits. HLA specificities were determined by referring to the FlowPRA data sheets and software (HLA-fusion software, version 1.2.1B, One Lambda, Inc. USA).

Statistical Analysis

The statistical analysis was carried out by Statistical Package for Social Sciences for Windows ver. 15.0 (SPSS Inc., Chicago, IL, USA). Numerical variables were given as mean±SD,

and were compared by Independent Samples t-test. When distribution was abnormal, non-parametric tests were used. Relationships were determined with Pearson`s correlation coefficient. Correlations between numerical parameters were analyzed by Spearman's rho correlation test. $p<0.05$ was accepted as significant. Survival analysis was carried out using Kaplan-Meier estimates. For differences in survival, a log-rank test was used.

Results

A total of 54 patients were followed up clinically for a mean time of 73.3±26.7 (12-115) months after transplantation. Two patients were lost to follow up after 12 months of the study period. The demographic and clinical features of study patients are shown in Table 1. During the study period, PRAs were detected in the serum of 21 (37.5%) patients by FlowPRA™ Screening Test and remaining 35 patients had negative PRA test. Twelve (57%) of the PRA positive patients and 8 (23%) of the PRA negative patients were developed acute allograft rejection. The rejection rate was significantly higher in the PRA positive group than the PRA negative group (p=0.01) (Figure 1). One of the pre-FCXM (+) patients was developed acute allograft rejection. Rejection was confirmed by allograft biopsy in 7 of the 56 patients. Of these 7 biopsies, 3 showed acute (grade 1, grade 2a and 2b due to Banff classification) and 4 showed chronic rejection. Five (71.4%) of these 7 patients had positive PRA and the other 2 had negative PRA test.

Table 1. The demographic and clinical features of study patients

	Tx recipients (n=56)
Age (years)	38±10
Gender (M/F)	35/21
Donor Characteristics	
Living related	50 (89.3%)
Cadaveric	6 (10.7%)
Donor age (years)	53±15
Donor gender (M/F)	20/36
HLA matching (min-max)	1A-2A2B2DR
Pre-sensitization	27 (48.2%)
Pre-tx FCXM	3 B FCXM (+)
Time of follow up (months)	73.3±26.7 (12-115)
Etiologies of ESRD [n (%)]	
Unknown	23 (41.1%)
Chronic pyelonephritis	15 (26.8%)
Chronic glomerulonephritis	12 (21.4%)
Diabetic nephropathy	3 (5.4%)
Amyloidosis	2 (3.6%)
Hypertensive nephrosclerosis	1 (1.8%)
Pre-tx RRT type	
Preemptive	4 (7.1%)
Hemodialysis	47 (83.9%)
Peritoneal dialysis	5 (8.9%)

M: male, F: female, ESRD: end stage renal disease, RRT: renal replacement treatment

When the PRA results were evaluated with regard to acute rejection episodes in the first month, acute rejection rates in the first month were significantly higher in the PRA positive patient group (47.6%) than the PRA negative patient group (17.1%) (p=0.015) (Table 2).

One patient who had antibodies against HLA class I and II antigens at 1 year after transplantation developed graft failure. Table 2 showed the graft failure rates of PRA positive (14.3%) and PRA negative patients (5.7%) during the clinical follow up period (12-115 months). Kaplan-Meier survival analysis showed overall graft survival rates of 98.2% at 1 year, 94.6% at 5 years and 91.1% at 10 years. During the clinical follow up period, 4 patients died. Kaplan-Meier survival analysis showed an overall patient survival rates of 98.2% at 1 year, 96.4% at 5 years and 92.8% at 10 years. In the PRA positive patient group the rates of early period infection and DGF (47.6% and 20%) were higher than the PRA negative patient group (14.3% and 0). The hospital stay was also longer in the PRA positive group than the PRA negative group. Both groups were similar with regard to immunosuppressive regimens after transplantation (Table 2).

When the serum creatinine levels at 1st, 6th, 12th months and 2nd, 3rd, 4th, 5th, 6th, 7th, 8th and 9th years after transplantation were compared between PRA positive and PRA negative groups, serum creatinine levels of PRA positive group at 6th and 12th months after transplantation were significantly higher than the PRA negative group (p=0.015 and p=0.048, respectively). When the serum creatinine levels were compared between patients who had rejection episodes and patients with no rejection episode, patients who had rejection episodes had statistically

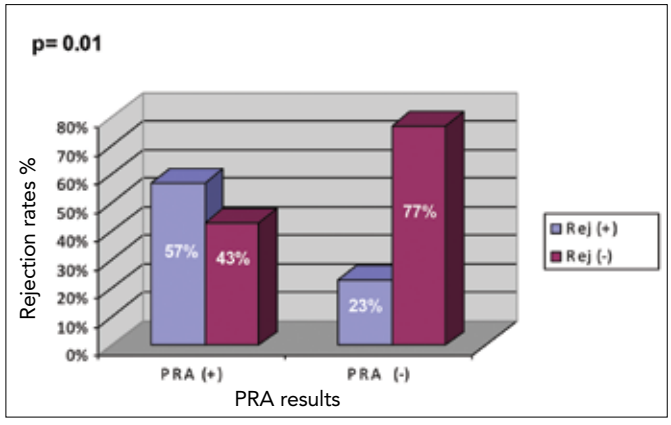

Figure 1. Relation between rejection and the rates of PRA positive and negative groups

significant higher serum creatinine levels at 6th, 12th months and 5th, 6th, 7th years after transplantation (Table 3).

There were no statistically significant differences regarding to presence of anti-HLA antibodies between the patients who received different anti-rejection treatments (Table 4).

Table 2. Post-transplant (post-tx) complications and immunosuppressive treatment regimens of PRA positive and negative patient groups

Posttx Complications	PRA (+) n=21	PRA (-) n=35	p value
Acute rejection	12 (57%)	8 (23%)	0.01
Early term rejection episode	10 (47.6%)	6 (17.1%)	0.015
Graft failure	3 (14.3%)	2 (5.7%)	NS
Mortality	2 (9.5%)	2 (5.7%)	NS
Infection in early period	10 (47.6%)	7 (20%)	0.030
Acute tubular necrosis	4 (19%)	2 (5.7%)	NS
Delayed graft function	3 (14.3%)	-	0.048
Hospital stay (days)	36.76±33.53	21.20±8.99	0.049
Immunosuppressive treatment regimens			
MMF[a] + Tac[b]+Pred[c]	10 (33.3%)	20 (66.7%)	NS
MMF+Cyc[d]+Pred	7 (38.9%)	11 (61.1%)	NS
Azae+ Tac+ Pred	-	3 (100%)	NS
Aza+ Cyc+ Pred	2 (66.6%)	1 (33.4%)	NS
Rapa[f]+ Pred	2 (100%)	-	NS

PRA: panel reactive antibodies, NS: not significant, MMF: mycophenolate mofetil, Tac: Tacrolimus, Pred: prednisolon, Cyc: Cyclosporine A, Aza: azathioprine, Rapa: sirolimus

Twenty seven patients (48%) have a history of pre-transplant sensitization. The demographic features of patients with or without a history of sensitizing events were shown in Table 5. The PRA and acute rejection rates were significantly higher in patients with a history of sensitizing events (p=0.001 and p=0.015, respectively) (Table 5). Of 21 (37.5%) PRA positive patients, 11 (19.7%) patients had class I and II HLA antibodies, 7 (12.5%) had solely class I HLA antibodies and 3 (5.3%) had class II HLA antibodies. Anti-HLA antibodies were not detected in 35 (62.5%) patients. The rejection rates of patients who had class I and II HLA antibodies were significantly higher than the patients who had either class I or II HLA antibodies (p=0.011). The rejection rates were significantly lower in patients with no detected anti-HLA antibodies (p=0.019) (Table 6). The anti-HLA antibody specificity, donors' mismatched HLA antigens, time of antibody detection and rejection rates of post-transplant PRA positive patients were shown in Table 7. Class I DSAs were detected only in one patient's serum in the post-transplant first year.

In the PRA positive group antibodies against HLA antigens were detected in 15 (71.4%) patients within the first month, 5 (23.9%) at the 6th month and 1 (4.7%) at the 12th month after transplantation (Table 8). Nine of 15 patients (60%) who had HLA antibodies at the first month after transplantation developed rejection episodes. Three of 5 (60%) patients who had HLA antibodies at the 6th month after transplantation developed rejection episodes. At the 12th month, PRA was positive only in 1 patient and no rejection episode was detected in this patient.

Antibodies against HLA class I and II antigens were detected at the first month in 7 patients who had rejection episodes after transplantation. Acute rejection rates were significantly higher in patients who had class I and II HLA antibodies at the first month (p=0.007). At the 6th month only in one pa-

Table 3. Post-transplantation serum creatinine levels of PRA positive and negative patient groups (mean±SD)

Serum creatinine levels (mg/dL)	PRA (+) n=21	PRA (-) n=35	p value	Rejection (+) n=20	Rejection (-) n=36	p value
Post-transplant 1st year						
1st month	1.67±1.04	1.34±0.42	0.100	1.59±0.58	1.39±0.76	0.342
6th month	1.58±0.51	1.29±0.33	0.015	1.64±0.48	1.26±0.33	0.002
12th month	1.57±0.67	1.31±0.28	0.048	1.62±0.63	1.29±0.32	0.017
Post-transplant 2nd year	1.38±0.43	1.30±0.34	0.507	1.39±1.04	1.30±0.40	0.418
Post-transplant 3rd year	1.35±0.37	1.32±0.38	0.789	1.41±0.39	1.30±0.36	0.321
Post-transplant 4th year	1.37±0.41	1.39±0.46	0.891	1.45±0.41	1.35±0.45	0.491
Post-transplant 5th year	1.47±0.45	1.46±0.48	0.979	1.78±0.55	1.34±0.37	0.010
Post-transplant 6th year	1.35±0.53	1.55±0.62	0.406	1.95±0.85	1.33±0.36	0.006
Post-transplant 7th year	1.85±1.29	1.51±0.61	0.368	2.24±1.27	1.38±0.54	0.022
Post-transplant 8th year	1.37±0.57	1.24±0.28	0.571	1.27±0.25	1.31±0.46	0.886
Post-transplant 9th year	1.70+0.15	1.27±0.20	0.162	-	1.36±0.26	-

PRA: panel reactive antibodies

Table 4. Presence of anti-HLA antibodies between the patients who received different anti-rejection treatments

	PRA (+)/AR(+) 21/12	PRA (-)/AR(+) 35/8	p value
Anti-rejection treatment protocols			
Steroid[a]	3 (30%)	3 (60%)	NS
ATG[b]	3 (30%)	1 (20%)	NS
Steroid+ATG	1 (10%)	-	NS
Steroid+IVIG[c]	3 (30%)	-	NS
Steroid+ATG+IVIG	-	1 (20%)	NS

PRA: panel reactive antibody, AR: acute rejection, NS: not significant
[a]Steroid (500 mg/day for 3 days), [b]Anti-thymocyte globulin (ATG) (2-5 mg/kg/day), [c]Intravenous immunoglobulin (IVIG) [0.5 g/kg (three doses), 0.25 g/kg (4. ve 5. dose) total 5 doses]

Table 5. Comparison of age, gender, positive PRA and rejection rates between patients with and without a history of sensitizing events

	Sensitization (+) (n=27) (48.2%)	Sensitization (-) (n=29) (51.8%)	p value
Age (years)	39.81±10.03	34.41±10.35	NS
Gender (Female/Male)	12/15 (44.4%/55.6%)	9/20 (31%/69%)	NS
PRA (+) [n (%)]	16 (59.3%)	5 (17.2%)	0.001
Acute rejection episode [n, (%)]	14 (51.9%)	6 (20.7%)	0.015

PRA: panel reactive antibody, NS: not significant

Table 6. Rejection rates of patients according to anti-HLA antibodies

Anti-HLA antibody status (PRA)	n (%)	Rejection [n, (%)]	p value
Class I (-)/Class II (-)	35 (62.5%)	8 (22.9%)	0.019
Class I (-)/Class II (+)	3 (5.3%)	1 (33.3%)	0.599
Class I (+)/Class II (-)	7 (12.5%)	3 (42.9%)	0.691
Class I (+) /Class II (+)	11 (19.7%)	8 (72.7%)	0.011

tient with rejection episode class I and II HLA antibodies were detected. In patients with rejection episodes, no anti HLA antibodies detected at the 12th month. At the first month, class I HLA antibodies were detected in 2 patients with rejection episodes and 3 patients with no history of rejection episodes. At the 6th month, class I HLA antibodies were detected only in a patient with a history of post-transplant rejection. At the 12th month, class I HLA antibodies were detected only in a patient without rejection episode. In patients with rejection, class II HLA antibodies were not detected solely at the first month. Only 1 patient without rejection episode developed class II

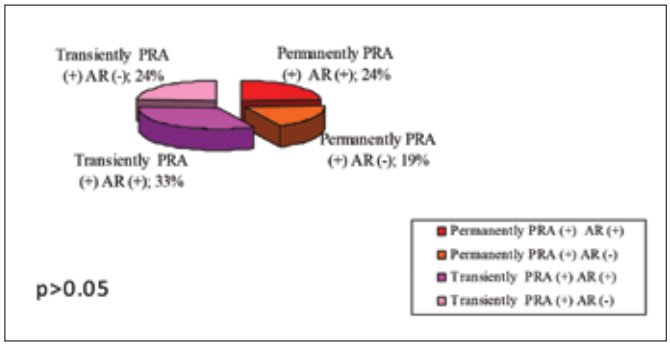

Figure 2. Relation between rejection and the rates of transiently or permenantly positive PRAs

HLA antibody solely at the first month. At the 6th month, class II HLA antibodies were detected in only one patient in each patient groups with and without rejection episodes. At the 12th month, no HLA antibodies were detected (Table 8).

In the PRA positive patient group, 9 (42.9%) patients had HLA antibodies in all three samplings (1st 6th and 12th months). Among these patients, 5 (24%) patients developed rejection episodes, while no rejection was detected in the remaining 4 (19%) patients. Of patients who had positive PRA in one of the three samplings, 7 (33%) patients developed rejection episodes, while no rejection was detected in the remaining 5 (24%) patients (p>0.05) (Figure 2).

Discussion

The role of HLA antibodies in the late post-transplant period remains an important issue in renal transplantation in general. Some reports indicate that post-transplant detection of HLA antibodies can predate the clinical manifestations of chronic renal allograft rejection, suggesting that allo-antibodies may be the cause of CR (4, 19).

The role of sensitive solid-phase assays in the detection of anti-HLA antibodies has been growing recently (1). Here, we investigate the incidence of HLA-directed antibodies developed after renal transplantation and their impact on graft rejection and outcome in kidney recipients using sensitive and specific flow-cytometry bead-based techniques. PRAs were detected in the serum of 21 (37.5%) of the patients by FlowPRA™ Screening Test and remaining 35 patients had negative PRA test. The rejection rate was significantly higher in the PRA positive group than the PRA negative group. Additionally, the high rate of PRA positive patients in patient group with biopsy confirmed rejection (71.4%) may suggest the important role of anti-HLA antibodies in the rejection.

Previous studies suggested that post-transplant monitoring of anti-HLA antibodies is highly useful in predicting patients at risk of acute and/or chronic rejection (4, 16, 19). The results of the present study, which are in conformity with the previous reports, also indicate that development of anti-HLA class I and II antibodies following transplantation is associated with significant rejection (4, 16, 19). Thus the appearance of HLA alloantibody either before or after transplantation is associated with early immunologic complications, which ultimately leads to graft loss.

Table 7. The anti-HLA antibody specificity, donors' mismatched HLA antigens, time of antibody detection and rejection rates of post-transplant PRA (+) patients

Patient No	Anti-HLA antibody specificity	Donors' mismatched HLA antigens	Time of antibody detection	Rejection
1	1C, 5C, DR13, DQ5, DQ6	A2, B51, DR13	1st month	+
2	A69, B49	A2, B8, DR03	1st,6th,12th months	+
7	7C	B55, DR01	6th month	+
8	DR1, DR103, DR7	A23, B15, B51, DR14	6th, 12th months	-
9	2C, 5C, DR16, DR14, DR15	A24, B51, DR11	1st, 6th months	+
10	5C, DR16, DQ7	A24, B35, DR4	1st, 12th months	+
11	80% class I	A2, B44, DR07	1st,6th,12th months	+
12	DR7, DR03	A69, B35, DR03	6th months	+
13	A1, DR13, DR03	A3, A68, B7, DR14, DR15	6th months	-
14	DR13, DR14, DR16, DR03, DQ7	A1, B51	1st,12th months	-
15	7C, DR03	A2, B41, DR03	1st,6th,12th months	+
22	12C	A2, B55, DR11	1st,12th months	-
23	A1, DR53	A3, B7, DR04	1st,6th,12th months	-
30	A36, B53, DQ5, DQ6	A1, B57	1st month	+
33	12C	A24, B35	1st,6th,12th months	-
34	A36, B53, B60, B59	-	1st,6th,12th months	-
38	B44	A2, B44, DR16	12th month	-
52	5C, DR11, DR15, DR16	A3, B35, DR13	6th,12th months	+
54	1C, 12C, DR11, DR12, DR8, DQ4	A24, B51, DR14	1st,6th,12th months	+
55	90% class I, DR13, DR10, DR1	A31, A33, B14, B51	1st,6th,12th months	+
56	A2, 5C, DR8, DR16	A24, B51, DR11	1st,6th,12th months	-

1C(10): A25, A26,A34,A66
1C(19): A29, A30, A31, A32, A33, A74
1C: A1, A3, A11,A23, A24, A36, A43,A80
2C:A2, A68, A69, A23, A24
5C: B18, B35, B37, B49, B50, B51, B52, B53, B57, B62, B63, B71, B72, B75, B76, B77, B78
7C: B7, B13, B27, B37, B41, B42, B46, B47, B48, B54, B55, B56, B60, B61, B73, B81.
8C: B8, B18, B38, B39, B59, B64, B65, B67
12C: B41, B45, B48, B49, B50, B60, B61, B82

The effect of the time of anti-HLA antibody development on allograft rejection is not still clear (16). In the present study, anti-HLA antibodies were detected in 71.4% patients within the first month, 23.9% patients at the 6th month and 4.7% patients at the 12th month after transplantation. Our results are in conformity with the study by Mihaylova et al. (1), however Abe et al. (20) found no association between anti-HLA antibodies developed in the first month and allograft rejection in renal transplant recipients. In the study by Abe et al. (20), sensitive solid-phase assays were not used in the detection of anti-HLA antibodies. This may be the reason why all anti-HLA antibodies might not be detected in this study (20). In PRA studies which CDC method was used for detection of anti-HLA antibodies, it is known that some anti-HLA antibodies could not be detected (21). In the study by Mihaylova et al. (1), post-transplant anti-HLA antibodies were detected in 22% of cadaveric kidney transplant recipients. The 81.2% of these anti-HLA antibodies were detected in the post-transplant first week. Rejection and DGF rates were found higher in the post-transplant PRA positive group. In our study, the rate of DGF in the PRA positive patient group was also higher than the PRA negative group which is in conformity with the study by Mihaylova et al. (1). Anti-HLA antibodies in the early period after renal transplantation may harm the graft endothelium and cause DGF (14). In our study, the rate of graft loss was higher in the PRA positive group than the PRA negative group, however the difference did not reach to significance which may be a result of low patient number.

Table 8. Time of Anti-HLA antibody detection in post-transplant PRA positive patients

PRA (+)	Rejection (+)		Rejection (-)				p value
	n	%	n	%	n	%	
1st month							
Class I, II	7	77.8	2	22.2	9	100	0.007
Class I	2	40	3	60	5	100	NS
Class II	0	0	1	100	1	100	NS
			Total: 15				
6th month							
Class I, II	1	50	1	50	2	100	NS
Class I	1	100	0	0	1	100	NS
Class II	1	50	1	50	2	100	NS
			Total: 5				
12th month							
Class I, II	0	0	0	0	0	0	-
Class I	0	0	1	100	1	100	NS
Class II	0	0	0	0	0	0	-
			Total: 1				

The length of hospital stay after transplantation was also longer in our PRA positive patients than the PRA negative patients. High incidence of rejection in PRA positive patients and the need for antirejection and supportive treatments may cause the long hospital stay in these patients (22). Deka et al. (23) also reported a long hospital stay in PRA positive patients which is in conformity with our results.

Another finding of this study is the higher serum creatinine levels in PRA positive group at 6th and 12th months after transplantation when compared to PRA negative group. Additionally, when serum creatinine levels were compared between patients who had rejection episodes and patients with no rejection episode, patients who had rejection episodes had statistically significant higher serum creatinine levels at 6th, 12th months and 5th, 6th, 7th years after transplantation. In their study, Fritsche et al. (24) also reported an association between post-transplant serum creatinine levels at the 6th month and graft failure at the 4th year of transplantation. This study enrolled renal transplant recipients who were transplanted between 1981 and 2004. In this 23 years period, various immunosuppressive treatments were used and this variability in immunosuppressive treatment may affect the results (24). Cardarelli et al. (19) also reported similar results with our study in their study which suggested the association of anti-HLA antibodies and high serum creatinine levels.

In the present study, anti-rejection treatment protocols were also similar between PRA positive and negative patients. In the maintenance treatment two different calcineurin inhibitors were not found to be significantly associated with allograft rejection episodes.

The positive PRA and rejection rates were also found significantly higher in patients with a history of sensitizing events in this study. The presence of DSAs after transplantation which were related to pre-transplant sensitizing events is reported to be associated with hyperacute allograft rejection (25, 26). In their study including 4000 patients, Süsal et al. (25) suggested that presensitization of first kidney transplant recipients against either HLA class I or class II is of no clinical consequence, whereas sensitization against both HLA class I and class II results in increased rejection of HLA mismatched grafts (25).

In the present study, 11 of 21 (52.3%) PRA positive patients had class I and II HLA antibodies, 7 (33.3%) had solely class I HLA antibodies and 3 (14.2%) had class II HLA antibodies. The present study also demonstrated that the rejection rates of patients who had class I and II HLA antibodies were significantly higher than the patients who had either class I or II HLA antibodies. In the study by Mihaylova et al. (1), 6 of 16 (37.5%) PRA positive patients had class I and II HLA antibodies, 7 (43.7%) had solely class I HLA antibodies and 3 (18.8%) had class II HLA antibodies (1). Our results were also in conformity with this study (1).

Using the new single-antigen-coated flow-cytometry beads, investigators found that most of the post-transplant anti-HLA antibodies were directly against to cross-reactive groups (CREG) which also included donors mismatched HLAs. This case was similar for both class I and II DSAs (26-28). In the present study, antibodies against CREGs, which also included donor's mismatched HLAs or non-DSAs, were detected during the clinical follow up in most of the patients who developed immunologic complications. The cause of this anti-HLA antibody formation is still not known, however previous studies reported that non-donor specific anti-HLA antibodies were developed frequently during the immunization period (9).

Acute rejection rates were significantly higher in patients who had class I and II HLA antibodies at the first month. Pre-

vious studies also reported that DSA or non-donor specific HLA antibodies developed in the first month after transplantation affects graft survival (1, 29). Although why and how this mechanism developed is still not clear, it was suggested that T cell response develops when patients meet with antigenic epitopes similar to previous sensitizing epitopes such as in blood transfusions and pregnancy (27).

As in the present study, HLA antibodies may not be detected in all samplings, however graft damage still continues in this period. The higher serum creatinine levels in 6. month PRA positive patients may be related to subclinical rejection. The high rejection rate in the PRA positive patients suggests the association of allograft rejection with HLA antibody formation. However, rejection may also occur in cases with no detected or low levels of HLA antibodies. There are many explanations in this issue. DSAs can be held by HLA antigens in the kidney and can not be detected in the circulation. Soluble donor HLA antigens in the serum may also develop a complex with anti-HLA antibodies. This can also prevent the detection of HLA antibodies. Immunological events which were not associated with transplantation may also prevent the detection of anti-HLA antibodies after transplantation (1).

Some patients who did not have pre-transplant anti-HLA antibodies developed anti-HLA antibodies after transplantation. Most of these patients had pre-transplant history of sensitizing events. Non-donor specific antibodies may be developed as a result of non specific triggered memory response by inflammation in the post-transplant period (30-32).

The development of anti-HLA antibodies in the post-transplantation period is a risk for allograft rejection. The higher occurrence of rejection episodes in PRA positive group may show the importance of anti-HLA antibody monitoring using Flow-cytometric analysis after renal transplantation as a prognostic marker in terms of graft survival.

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Informed Consent: *Written informed consent was obtained from patients who participated in this study.*

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