ORIGINAL ARTICLE

EVEN A SINGLE, REMOTELY POSITIVE POST-TRANSPLANT ALLOANTIBODY TEST CORRELATES WITH INCREASED CHRONIC ALLOGRAFT NEPHROPATHY AND GRAFT LOSS AFTER KIDNEY TRANSPLANTATION

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Published: 21 July 2009
Ibnosina Journal of Medicine and Biomedical Sciences 2009; 1(1):7-15
Received: 15 May 2009
Accepted: 10 July 2009
This article is available from: http://www.ijmbs.org

Abstract
Background: Chronic renal allograft loss is considered as immunologically mediated when donor-specific alloantibodies are detected. However, remotely detected alloantibodies with lack of detection more proximate to graft loss occurrence may obscure the humoral association with graft damage.
Methods: We retrospectively reviewed 609 patients multiply tested post-transplant for detectable alloantibodies and correlated their results with clinical outcomes.
Results: Most patients had no detectable post-transplant alloantibodies (Group 1, n = 393), some converted from non-detectable to detectable alloantibodies (Group 2, n = 97), some always had detectable post-transplant alloantibodies (Group 3, n = 69), and some demonstrated alloantibodies that subsequently became undetectable (Group 4, n = 50). The incidence of death-censored graft survival for Group 4 patients was similar to Group 2 and 3 patients, and greater than Group 1 patients. Further, interstitial fibrosis/tubular atrophy (IF/TA) free survival was significantly worse (p=0.018) for Group 4 versus Group 1 recipients. Also, Group 4 versus Group 1 IF/TA-free survival was worse when recipients were regrouped based solely on anti-HLA class II (p=0.006), but not anti-HLA class I (p=ns) antibodies.
Conclusions: Detectable anti-HLA antibodies, even remotely, post-transplant identifies recipients at greater risk for IF/TA associated graft loss when compared to patients without detectable alloantibodies.

Keywords: Anti-HLA antibodies, chronic allograft nephropathy, IF/TA, graft failure.

Introduction

It is generally accepted that the cause of chronic graft loss due to interstitial fibrosis/tubular atrophy (IF/TA) following kidney transplantation is multifactorial, with etiologies broadly categorized as either immunologic or non-immunologic in nature (reviewed in (1)). Many clinical studies have found that the incidence of IF/TA and late kidney failure after kidney transplantation are significantly increased in patients that have detectable circulating alloantibodies (2-5). Consequently it is now broadly accepted that alloantibodies can directly cause graft damage leading to late kidney failure. Thus, when graft loss occurs in the setting of detectable circulating donor-

reactive anti-HLA antibodies, immunologic injury can be assumed. More recent availability of immunohistochemical detection of previous peri-tubular endothelial complement deposition (C4d) has been helpful in linking the presence of alloantibodies to graft injury (6-8).

However, ascribing graft loss related to IF/TA as being due to immunologic injury in not always straightforward. For instance, the absence of detectable circulating donor-reactive anti-HLA antibodies does not exclude the possibility that humorally mediated graft injury resulting in graft loss has occurred. There are many reported circumstances where humoral graft damage occurred in the absence of detectable anti-HLA alloantibodies. This can occur for a variety of reasons. Humorally mediated graft damage may occur due to non-HLA donor reactive antibodies (9-11), anti-HLA antibodies that escape detection due to the method chosen for detection (12) or perhaps the presence of the allograft acting as a "sink", making detection in the circulation difficult (13). Alternatively, circulating anti-donor antibodies may be present in titers too low to detect; contributing to graft damage even though they are difficult to identify.

The present study was undertaken to determine whether even a single flow bead percent reactive antibody (PRA) positive test identifies patients that are at an increased risk of IF/TA and graft loss. Our aim is not to correlate PRA evidence of detectable circulating alloantibodies with the development of a specific pathologic lesion within the biopsy, but rather to correlate the PRA result with overall chronic graft damage, as evidenced by the presence of IF/TA on biopsy. We first retrospectively examined those patients transplanted in our program who have had multiple, post-transplant PRA testing performed. These patients were grouped according to their test results. Group 1 recipients never had detectable post-transplant alloantibodies, Group 2 recipients converted from non-detectable post-transplant to detectable post-transplant anti-HLA reactivity at last testing, Group 3 recipients always had post-transplant detectable anti-HLA reactivity (with no new demonstrable anti-HLA class I or class II reactivities), and Group 4 recipients had remotely detectable post-transplant anti-HLA reactivity that was non-detectable on subsequent testing. We then compared and contrasted the graft survival and IF/TA incidence for these 4 groups.

Materials and Methods

Patients

Six hundred and nine recipients transplanted between 2/83 and 2/03 had sera tested for the presence of anti-HLA class I and anti-HLA class II alloantibodies at least twice beyond 7 days post-transplant (2.5 ± 0.8 tests/patient, range 2-6). This patient population includes 490 kidney recipients (182 living donor and 308 deceased donor kidney recipients) and 119 simultaneous kidney/pancreas recipients. The mean actual follow-up time is 9 ± 5 years (range 1 month to 24 years). The patient group comprised 502 Caucasians, 97 African-Americans, and 10 recipients of other ethnicity. Three hundred eighty four patients were male and 225 were female. Only 29 (4.8%) are retransplant recipients. The mean recipient age at the time of transplantation was 43.2 ± 12.9 years (range 10 to 77). Initial acute rejection prophylaxis in the majority of recipients consisted of triple immunosuppression using prednisone (Pred), Mycophenolate Mofetil (MMF) (Roche, Nutley, USA), and microemulsion cyclosporine (CsA) (Novartis, East Hanover, USA) (66%, n=401). Twenty nine percent were treated with Pred, azathioprine, and CsA (n=176), and the remaining 5% (n=32) were treated with Pred in combination CsA and/or rapamune (Philadelphia, USA) or FTY 720 (Novartis, East Hanover, USA). All except 47 patients received induction therapy consisting of Simulect (n=273) (Novartis, East Hanover, USA), OKT3 (Ortho Biotech, Inc, Bridgewater, USA) (n=140), Minnesota anti-lymphocyte globulin (ALG) (n=128), Thymoglobulin (Genzyme Transplant, Cambridge, USA) (n=8), or FTY 720 (n=13). For the entire group there were 136 deaths with a functioning graft (22%), 115 kidneys lost to causes other than death (19%), and 24/119 pancreata lost (20%). Of the total group, 131 recipients experienced 1 episode of acute rejection (21.5%) and 73 experienced more than 1 acute rejection episode (12%). Post-transplant sera were obtained from these patients either in the outpatient setting for routine screening purposes or the inpatient setting as part of an evaluation for allograft dysfunction.

Anti-HLA Antibody Analysis

All patients were selected for transplantation based on an immediately pre-transplant negative T cell complement-dependent cytotoxicity (AHG-CDC) assay. B cell AHG-CDC assays were rarely performed and the results were not used to consider candidacy for transplantation. Post-transplant sera were analyzed using flow bead PRA analysis for determining the anti-HLA class I and anti-HLA class II PRA (FlowPRA, OneLambda, Canoga Park, Ca.). The commercially available pool of microparticle beads coated with various purified MHC antigens of known specificity, were used according to manufacturers instructions. Briefly,

20 ul of recipient sera was incubated with 5 ul of MHC class I plus 5ul of MHC class II microparticle beads for 30 minutes at room temperature (RT). The beads were washed twice with buffer and centrifuged at 10,000 rpm for 2 minutes. The beads were re-suspended in 100 ul of solution containing FITC-conjugated goat anti-human IgG and incubated for 30 minutes at RT. The wash step was repeated and the beads were re-suspended in 500 ul of wash buffer. Negative control serum using pooled sera from non-transfused, non-transplanted males was similarly prepared. Samples were read with the aid of a Beckman Coulter XL2 flow cytometer. The fluorescence profile obtained with negative control sera was used as the baseline fluorescence. MHC class I and class II beads were readily distinguishable since they are fluorescent (excited at 488 nm and maximum emission at 580 nm) and have unique emission spectra. The positive/negative cutoff was empirically determined for each assay by setting a histogram region that excluded 98% of the peak obtained with the negative control serum. The median channel associated with this threshold was recorded for each assay. A test was deemed positive for alloantibody if there was noted a distinct peak or if there was a shift to the right in bead fluorescence of ≥ 6% to the right of the cutoff point.

Acute Rejection

Acute rejection episodes were diagnosed clinically based on significant renal dysfunction as determined by a >= 25% rise in serum creatinine, and biopsy proven prior to treatment. The presence of acute rejection was determined based on the prevailing histologic criteria at the time of biopsy, with the majority of biopsies interpreted using the Banff 1997 classification (14). The acute rejection incidence was determined as the number of episodes occurring prior to PRA testing.

Chronic Allograft Nephropathy

Chronic allograft damage was diagnosed by clinical criteria in 72 recipients and confirmed by biopsy in 64/72 patients. Of the 64 biopsies, 33 revealed grade III, 29 revealed grade II, and 2 revealed grade I interstitial fibrosis/tubular atrophy using the Banff 97 working classification. All diagnosed patients experienced an otherwise unexplained rise in serum creatinine (mean of 6.6 +/- 3.7 mg/dl, range 2.4 to 18.4). All patients were hypertensive at the time of diagnosis, typically requiring treatment with multiple anti-hypertensive medications (mean 3.5 +/- 1.3, range 1 to 6). Significant proteinuria (>500 mg/24 hours) was present in 67/72 patients (mean 2.9 +/- 2.8 grams, range 0.2 to 12.2 grams). It should be noted that 12/72 recipients had a bladder drained pancreas transplant along with the kidney transplant, which will result in significant proteinuria due to pancreatic exocrine secretion into the bladder. However, 11/12 of these patients had biopsy confirmed significant interstitial fibrosis/tubular atrophy.

Statistical Analyses

Student's t test and Pearson chi square test were used for statistical comparison of means (+/– SEM) and proportions between groups, respectively, where appropriate. Comparison of Kaplan-Meier survival curves was made using the Log Rank test for multiple groups where appropriate. Pearson Chi-square test was used to test the overall group difference of categorical response such as: incidence of acute rejection. Logistic regression analysis was used to compare these incidences among each pair of groups. The Bonferroni method was used to control multiple comparison Type I error among the four groups. Statistical analyses were performed using SPSS version 11.0.1 statistical software (Chicago, IL) and STATA version 9.2 (College Station, TX).

Results

Recipient Post-Transplant Alloantibody Characterization

Multiple post-transplant sera analyses for the presence of alloantibodies were available for 609 recipients (1521 sera, 2.5 ± 0.76 sera/patient, range 2 to 6, median 2). To determine whether any detectable alloantibodies post-transplantation identified a group of patients at an increased risk of immunological complications or graft loss, patients were grouped in the following manner. Recipients included in Group 1 had no detectable anti-HLA class I or class II alloantibodies in any post-transplant sera tested (n=393). Group 2 included any recipient that initially lacked detectable post-transplant anti-HLA class I or anti-HLA class II alloantibody reactivity, which subsequently became detectable on repeat sera testing. Detection of these antibodies either persisted in subsequent testing or no subsequent sera were available (n=97). Recipients in Group 3 had detectable post-transplant anti-HLA class I and/or class II antibodies in all post-transplant sera tested and did not demonstrate development of new post-transplant anti-HLA class I and/or class II reactivities (n=69). Finally, Group 4 recipients were those that had detectable post-transplant anti-HLA class I and/or class II antibodies that were not detected in subsequent tested sera (n=50).

Comparison of Recipient Groups

Recipient demographics were compared between Groups 1 through 4 (Table 1). The Group 4 patients were older and had the highest percentage of African-American recipients. The proportion of female recipients is different among the 4 groups at a significance level of $p<0.05$. Further pair-wised comparison between groups showed that the Group 3 recipients who had persistent post-transplant alloantibodies had a significantly greater percentage of female and retransplant recipients than Group 1 after adjusting for multiple comparisons, suggesting increased previous allosensitization events due to pregnancy and/or previous organ transplantation. There was no difference between groups in regards to the type of transplanted organ received or the immunosuppression administered thereafter.

Comparison of Clinical Outcomes between Groups

The incidence of post-transplant acute rejection, IF/TA, graft loss, death, and follow-up time were compared between the 4 groups (Table 2). The percent of patients who experienced acute rejection episodes and that were diagnosed with IF/TA are similar or identical for Group 2, 3, and 4 patients, and quite different from those who had no post-transplant detectable alloantibodies (Group 1). The overall difference between the 4 groups was statistically significant. Further, the percent of kidneys lost is similar for Groups 2, 3, and 4, and almost twice that for Group 1 patients. When statistically comparing only Group 1 to Group 4 patients (whose most recent sera lacked detectable anti-HLA antibodies) the difference in the incidence of IF/TA, acute rejection, and kidney loss was not significantly different ($p=0.078$ $p=0.08$, and $p=0.74$ respectively). Finally, there was no significant difference between groups in the percent of patients who have died with a functioning kidney or in the time of post-transplant follow-up. Additionally, there were a statistically significantly higher proportion of SKP patients who have lost their pancreas grafts in Group 2 compared to the other groups ($p=0.005$).

Comparison of Kidney Survival between Groups

Death censored Kaplan-Meier survival curves were compared to determine whether graft loss occurs at a different pace within the various groups (Figure 1). The long-term death-censored kidney survival for recipients is similar for Groups 2, 3, and 4 patients, and quite different than the survival for Group 1 patients (who had no post-transplant sera with detectable anti-HLA antibodies). Comparison of long-term death-censored kidney survival

Table 1. Comparison of patient demographics between groups.

Variable	Group 1 (n=393)	Group 2 (n=97)	Group 3 (n=69)	Group 4 (n=50)	P[f]
Age mean ± sd[a] years	44±13	40±13	41±13	46±13	0.006[%]
AA race (n)[b]	13% (50)	20% (19)	22% (15)	26% (13)	0.02*
Female gender (n)	32% (125)	41% (40)	58% (40)	40% (20)	<0.001*
Retransplant (n)	2% (8)	7% (7)	19% (13)	2% (1)	<0.001*
Tx Type (LDK,DDK,SKP)[c]	117/206/70	29/41/27	24/34/11	12/27/11	0.278*
MI (PAC/PMC/other)[d]	106/267/20	30/60/7	29/39/1	11/35/4	0.106*
Induction[e] (none/ALG/ OKT3/Sim/ATG/other)	23/82/87/188/5/7	7/19/27/38/1/5	8/22/14/23/1/1	7/6/12/24/1/0	0.095*

[a]sd = standard deviation
[b]AA = African-American
[c]LDK = living donor kidney, DDK = deceased donor kidney, SKP = simultaneous kidney/pancreas
[d]MI = maintenance immunosuppression, PAC = prednisone, azathioprine, and cyclosporine, PMC = prednisone, mycophenolate mofetil, and cyclosporine
[e]ALG = anti-lymphocyte globulin, OKT3 = monomurab, Sim = Simulect, ATG = anti-thymocyte globulin
[f]P: This p-value is for the testing of overall mean differences=0 or odd ratios=1 among all 4 groups. [%] F test, post hoc analyses found group 2 versus group 4 significant by Bonferroni ($p=0.007$), group 4 versus groups 2 ($p=0.001$) and 3 ($p=0.01$) and group 1 versus group 2 ($p=0.012$). * chi square analysis

Table 2. Comparison of clinical outcomes between groups.

Variable	Group 1 (n=393)	Group 2 (n=97)	Group 3 (n=69)	Group 4 (n=50)	P[c] All Grps
AR[a] (n)	29% (116)	40% (39)	42% (29)	40% (20)	0.047*
IF/TA[b] (n)	7% (28)	20% (19)	23% (16)	18% (9)	<0.001*
Kidney loss (n/total)	14% (54)	25% (29)	32% (22)	22% (11)	<0.001*
Pancreas loss (n/total)	17% (12/70)	37% (10/27)	9% (1/11)	9% (1/11)	0.005*
Death (n)	21% (83)	21% (20)	27% (19)	28% (18)	0.475*
Follow-up time (years)	9.0±4.9	8.6±4.6	10.1±6.3	8.4±4.2	0.177%

[a]AR = incidence of acute rejection
[b]IF/TA = interstitial fibrosis/tubular atrophy
[c]P = p-value for overall comparison, * = post hoc analyses by Bonferroni, chi square analysis, % F test

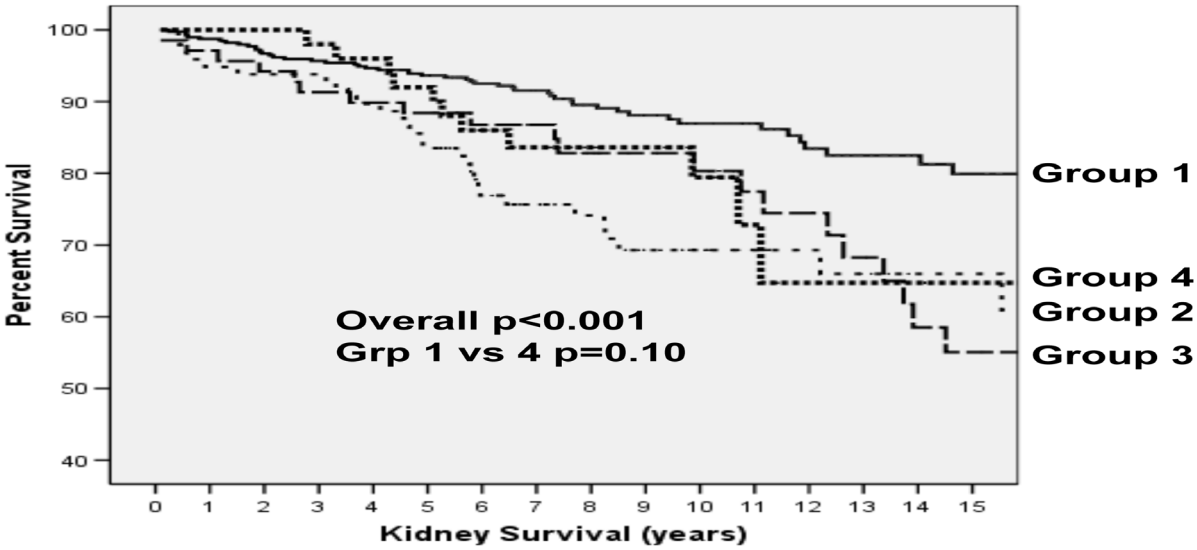

Figure 1: Actuarial death-censored kidney survival for patients in Groups 1-4. Fifteen year Kaplan-Meier survival curves were compared by the log rank test for the 4 patient groups. Group 1 – no detectable post-transplant antibodies, Group 2 – de novo post-transplant development of anti-HLA class I and/or class II antibodies, Group 3 – persistent post-transplant anti-HLA class I and/or class II antibodies, and Group 4 –detectable post-transplant anti-HLA class I and/or class II antibodies that subsequently become undetectable. Overall comparison p<0.001, Group 1 versus Group 4 comparison, p=0.10.

by the log rank test shows a difference among the 4 groups at a significance level of $p<0.05$. But further pair-wised comparison between groups does not show significant differences between any two groups. When death censored Kaplan-Meier survival curves where only graft loss related to IF/TA was analyzed and compared between groups (IF/TA-free graft survival), again the survival curve for recipients with detectable anti-HLA class I and/or class II antibodies (Groups 2, 3 and 4) was similar, and significantly worse than that for patients with no detectable anti-HLA antibodies (Group 1) (Figure 2). Comparison of survival curves for only Group 1 and Group 4 recipients (whose most recent sera lacked detectable anti-HLA antibodies) did show a statistically significant difference after adjusting for multiple comparisons ($p=0.018$, by log rank test). This relationship was even more pronounced when recipients were regrouped and compared solely based on their anti-HLA class II alloantibody status ($p<0.006$, by log rank test) (Figure 3), but not when regrouped and compared based solely on their anti-HLA class I alloantibody status ($p=0.84$,

Discussion

Numerous studies have shown that the post-transplant detection of anti-HLA alloantibodies in the sera of patients after kidney transplantation identifies a subset of recipients with a significantly worse short (15, 16) and long-term (17-19) (reviewed in (20) and (21)) renal allograft survival. As a result there has been increasing interest in monitoring kidney recipients for the development of detectable circulating alloantibodies. While recently available solid phase technologies that identify donor-reactive alloantibodies are the preferred methods for alloantibody detection, additional time will be needed to evaluate their ability to identify patients at higher risk of long-term graft loss. Thus this retrospective study was designed using the older flow bead PRA technology to determine whether detectable anti-HLA antibodies by this method, even when

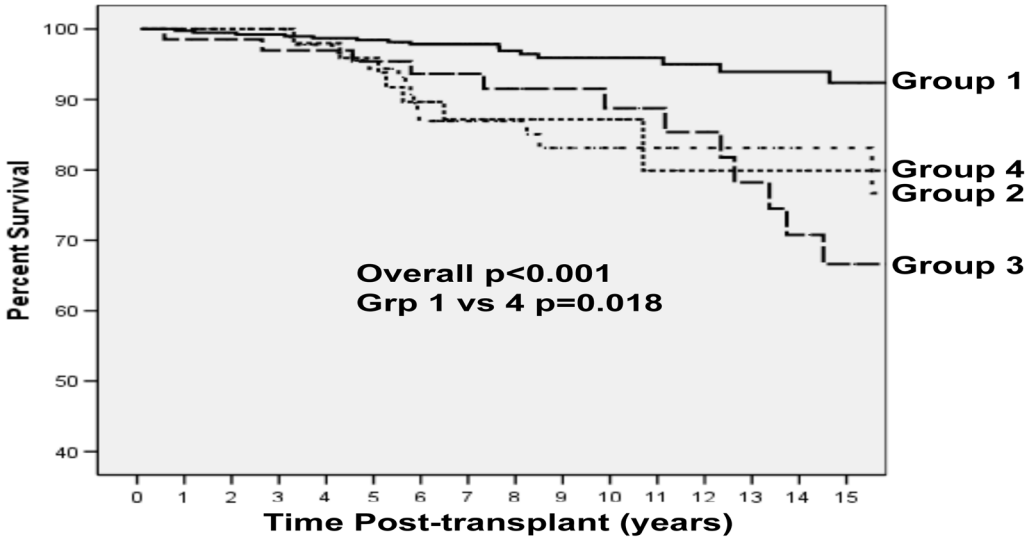

Figure 2: Comparison of graft loss related to IF/TA for Groups 1 through 4. Fifteen year Kaplan-Meier survival curves for the 4 patient groups were compared by the log rank test. Only graft loss related to IF/TA was considered as an event. Group 1 – no detectable post-transplant antibodies, Group 2 – de novo post-transplant development of anti-HLA class I and/or class II antibodies, Group 3 – persistent post-transplant anti-HLA class I and/or class II antibodies, and Group 4 – detectable post-transplant anti-HLA class I and/or class II antibodies that subsequently become undetectable. Overall comparison $p<0.001$, Group 1 versus Group 4 comparison, $p=0.018$.

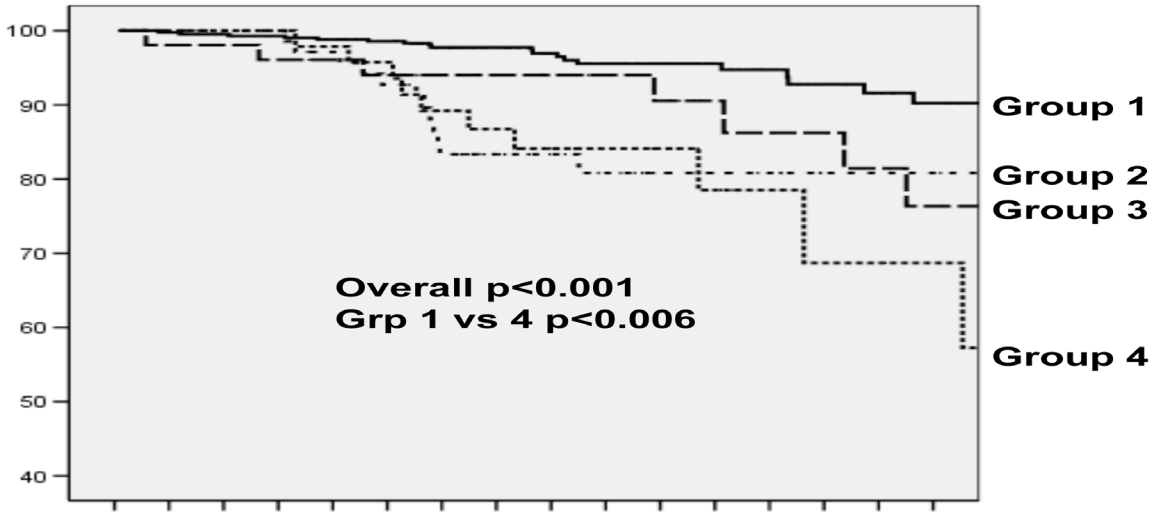

Figure 3: Comparison of graft loss related to IF/TA between recipients regrouped solely by their anti-HLA class II antibody status. Fifteen year Kaplan-Meier survival curves for the 4 patient groups were compared by the log rank test. In this analysis patients were grouped solely on the basis of their anti-HLA class II antibody status. Group 1 – no detectable post-transplant anti-HLA class II antibody, Group 2 –lack of detectable post-transplant anti-HLA class II antibody reactivity that subsequently became detectable, Group 3 - detectable post-transplant anti-HLA class II antibodies in all post-transplant sera tested, and Group 4 - detectable post-transplant anti-HLA class II antibodies that subsequently became undetectable. Overall comparison p<0.001, Group 1 versus Group 4 comparison, p<0.006.

only detected once, identifies a subgroup of alloantibody producing patients at increased risk of long term graft loss in a group of patients with a mean follow-up of about 10 years. Immunohistological detection of peritubular capillary C4d deposition in transplant kidney biopsies would significantly improve the data analyses in this study. Unfortunately, only a few of the most recent allograft biopsies were so treated, thus this data was unavailable.

Overall, when comparing the clinical outcomes between the 4 groups of patients, we found that those patients with only a remote serum sample demonstrating detectable anti-HLA antibodies (Group 4) had outcomes that were not significantly different from patients who developed (and maintained) de novo post-transplant anti-HLA class I or class II antibodies (Group 2) and those that always had post-transplant anti-HLA antibodies (Group 3) (Table 2). In fact, the Group 1 recipients appear to be the outlier group, with clinical outcomes that were significantly better than those seen in the other Groups. Specifically, the incidence of acute rejection, development of significant IF/TA, and kidney loss in Group 1 were much lower than in any other group. Comparison of only the Group 4 to the Group 1 patients did not demonstrate a significant difference in these outcomes, but significance might have been achieved with larger patient numbers. Comparison of the Kaplan-Meier kidney survival curves demonstrated an overall significant difference among the 4 groups; however the difference between only Groups 1 and 4 was not statistically significant. However, further survival analyses using only kidney loss related to chronic allograft damage as evidenced by significant IF/TA did demonstrate a significant difference between the 2 groups. We also regrouped the entire cohort of recipients using the same criteria as before except we only considered detectable post-transplant anti-HLA class I and not anti-HLA class II, and vice versa. Survival analysis of patients grouped solely

by detectable anti-HLA class II, but not anti-HLA class I, demonstrated a significant difference when comparing all groups ($p<0.001$, figure 3), and also when comparing only Group 1 and Group 4 ($p<0.006$). The relationship between anti-HLA class II antibodies and "chronic rejection" and graft loss has been previously reported by our program (5).

These data suggest that even a single positive flow bead PRA alloantibody test after kidney transplantation identifies a recipient at significantly increased risk for long term graft loss related to chronic allograft damage. Based on previous studies (5), we presume that in many cases where post-transplant anti-HLA antibodies are detectable, that donor-specificity is present. Unfortunately, we could not verify donor-specificity in this study cohort. If donor-specific alloantibodies are prevalent in these study recipients, the loss of flow bead detectable anti-HLA antibodies could result from a drop in serum titer below that which is detectable by this method due to adsorption by the graft. Martin, et al. (13) demonstrated that frequently alloantibodies were present but undetectable in the circulation because they were adsorbed by the renal allograft, as evidenced by eluting them from nephrectomized kidneys. These donor-specific antibodies were detectable in the peripheral circulation following renal allograft removal. Also, we cannot exclude the possibility that circulating anti-HLA antibodies only act as a biomarker for the post-transplant development of either non-HLA, graft-reactive antibodies (10, 11) or alloantigen-driven cellular immunity that causes tissue pathology resulting in chronic allograft damage (21). Of note, 5/50 Group 4 patients had a kidney transplant biopsy performed more recently, when immunohistochemical detection of C4d was available. Only 1/5 showed evidence of peritubular capillary C4d deposition, a finding consistent with humoral rejection, raising the possibiliy that ongoing graft anti-HLA antibody deposition and complement activation is not the usual pathway towards IF/TA-related graft damage in these patients. Whatever the pathway might be that results in allograft damage and eventual loss in patients with a remote or single serum sample with detectable alloantibodies, that these patients exist suggests that the true incidence of alloantibody-associated chronic graft damage may be underestimated.

The worse clinical outcomes in the Group 4, as compared to Group 1 recipients, suggests that prospective monitoring of patients post-transplant for the development of anti-HLA antibodies to identify those at increased risk of IF/TA needs to be performed routinely. Infrequent, random, or single point-in-time testing, when no alloantibodies are detected, may fail to identify a certain percentage of at-risk patients. Conversely, once anti-HLA antibodies are detected, the recipient should be considered at great risk of developing IF/TA, even if subsequent testing fails to re-demonstrate the presence of alloantibodies. This issue will become more important in the future as we devise successful therapeutic strategies designed to obviate the development of IF/TA after kidney transplantation.

Acknowledgements: This study was supported by National Institutes of Health grants PO1-AI/HL40150, RO1-HL61966, and P01-HL70294. We would like to thank Irene DeAndero RN, BSN, CCTC, Melissa Knox LPN, CCTC, Becky Miller LPN, CCTC, and Carol Wheeler RN, BSN for their help in sample acquisition. Also, we would like to thank Celia Ridgeway and Claretha Jones for assistance in preparing this manuscript, and Barbara Heckendorn for her clerical assistance.

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