Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

# Prevalence and significance of anti-HLA and donor-specific antibodies long-term after renal transplantation

Francesca Cardarelli,[1] Manuel Pascual,[2] Nina Tolkoff-Rubin,[3] Francis L. Delmonico,[4] Waichi Wong,[3] David A. Schoenfeld,[5] Hui Zhang,[5] A. Benedict Cosimi[4] and Susan L. Saidman[6]

1 Clinica Medica e Nefrologia, Universita degli Studi di Parma, Parma, Italy
2 Transplantation Center, CHUV, Lausanne, Switzerland
3 Renal Unit, Department of Medicine, Massachusetts General Hospital, Boston, MA, USA
4 Transplant Unit, Department of Surgery, Massachusetts General Hospital, Boston, MA, USA
5 Biostatistics Center, Department of Medicine, Massachusetts General Hospital, Boston, MA, USA
6 Histocompatibility Lab, Department of Pathology, Massachusetts General Hospital, Boston, MA, USA

**Keywords**
chronic rejection, donor-specific antibodies, enzyme-linked immunosorbent assay, human leukocyte antigen antibodies, humoral rejection, kidney transplantation.

**Correspondence**
Francesca Cardarelli, Nefrologia E Dialisi, Arcispedale S. Maria Nuova Viale Risorgimento 80, 42100 Reggio Emilia, Italy.
Tel.: +3305222 36492; fax: +3305222 36770;
e-mail: cardarelli.francesca@asmn.re.it

Received: 24 February 2004
Revised: 2 August 2004
Accepted: 28 October 2004

doi:10.1111/j.1432-2277.2005.00085.x

## Summary

Post-transplant circulating anti-human leukocyte antigens (HLA)-antibodies and C4d in allograft biopsies may be important in chronic rejection in renal transplant recipients (RTR). We determined the prevalence and significance of anti-HLA-antibodies and donor-specific antibodies (DSA). Sera were collected from 251 RTR >6 months post-transplant. Sera were tested using enzyme-linked immunosorbent assay (ELISA) screening for anti-HLA antibodies. Positive sera were retested with ELISA-specific panel for antibody specificity. A 11.2% of patients had anti-HLA antibodies and 4.4% had DSA. Anti-HLA antibodies were significantly associated with pretransplant sensitization, acute rejection and in multivariate analysis, higher serum creatinine ($2.15 \pm 0.98$ vs. $1.57 \pm 0.69$ mg/dl in negative anti-HLA antibodies group). Allograft biopsies performed in a subset of patients with anti-HLA antibodies revealed that 66% had C4d in peritubular capillaries (0% in patients without antibodies). Anti-HLA antibodies were associated with a worse allograft function and *in situ* evidence of anti-donor humoral alloreactivity. Long-term RTR with an increase in creatinine could be screened for anti-HLA antibodies and C4d in biopsy.

## Introduction

Chronic rejection (CR) is a major cause of late allograft loss after renal transplantation [1]. Numerous studies have implicated post-transplant donor-specific antibodies (DSA) in the pathogenesis of chronic renal allograft rejection [2–13]. In addition, DSA have been associated with CR of various other transplanted organs, such as lungs and hearts [2,14,15]. Importantly, some reports indicate that post-transplant detection of anti-HLA antibodies can predate the clinical manifestations of chronic renal allograft rejection, suggesting that allo-antibodies may be the cause of CR [4,9–12]. In a recent study, the development of DSA strongly predicted renal allograft failure [16]. Moreover, the pathogenic role of anti-donor antibodies in chronic vascular rejection has been demonstrated in small animal models [17,18]. Recent data from our institution, in nonhuman primate models, also indicate that production of *de novo* anti-donor antibodies can lead to CR [19]. Overall, these clinical and experimental data emphasize the need for more studies to further delineate the precise role of anti-HLA antibodies – particularly of DSA – in chronic renal allograft rejection in humans.

As recently pointed out by Lee *et al.* [10], the question of anti-HLA antibody specificity in patients with CR and circulating anti-HLA antibodies has not been resolved so far. In many studies, the distinction between 'donor-specific' and 'nondonor specific' anti-HLA antibodies has

not been documented. It remains unclear whether, in patients with CR, DSA may bind to the allograft and thus remain undetectable in serum. A critical question, therefore, is whether screening alone for post-transplant anti-HLA antibodies might be sufficient [10], or whether additional testing for 'donor-specific' anti-HLA antibodies should be routinely performed as well. For example, it can be hypothesized that recipients with detectable levels of DSA in serum may be at higher risk of worsening allograft function (and graft failure), when compared with those patients with 'nonspecific' anti-HLA antibodies alone.

Traditionally, the detection of anti-HLA antibodies has been performed using complement- dependent cytotoxicity assays. Recently, the enzyme-linked immunosorbent assay (ELISA), using mixtures of purified HLA antigens, has been found to be useful as a screening tool to detect and serially measure anti-HLA antibodies both pre and post-transplant [8,10,12,20–22]. Given the ease and relatively low cost of these screening assays, it has been suggested that all post-transplant patients should be regularly tested.

In the present study, we determined the prevalence of both circulating anti-HLA antibodies and DSA in renal transplant recipients (RTR) greater than 6 months post-transplant using two different ELISA assays. The first ELISA was performed for screening purposes in order to determine if HLA antibodies could be detected often enough to justify routine screening. The second ELISA assay was performed using panels of HLA antigens to determine the anti-HLA antibody specificity (i.e. to determine whether the antibodies were 'DSA' or 'nondonor-specific' anti-HLA antibodies). Finally, we correlated the presence of anti-HLA antibodies and DSA with clinico-pathological findings.

## Materials and methods

### Patient Population

This was an observational, cross-sectional study, which was approved by the Massachusetts General Hospital Institutional Review Board. All patients signed a consent form at the time of enrollment in the study.

Between March 2001 and October 2002, a single serum sample was collected from 251 RTR who came to our outpatient clinic for a routine visit and who were at least 6 months post-transplant. Clinical information was obtained from the transplantation unit medical charts and using the transplantation unit database.

Between 1970 and 2002, a total of 1622 kidney transplants were performed at the Massachusetts General Hospital (1009 deceased, 541 living-related and 72 living-unrelated donors) in 1005 male and in 617 female recipients. There were 243 cases (15%) of nonprimary transplants (second or third transplants).

Before 1984, azathioprine and prednisone were the main drugs used for maintenance immunosuppression. Since January 1984, standard baseline immunosuppression has consisted of cyclosporine and prednisone, with or without azathioprine or mycophenolate mofetil (MMF). Tacrolimus was introduced in 1996 in place of cyclosporine in selected patients, either as rescue therapy for refractory allograft rejection or as primary therapy. The routine use of MMF in place of azathioprine as a third agent began in 1997 [23]. The standard immunosuppressive regimen at the Massachusetts General Hospital since 2002 has included tacrolimus, MMF and steroids, with or without anti-lymphocyte antibody induction therapy.

Delayed graft function was defined as the need for dialysis within the first week post-transplant. Recorded acute rejection (AR) and CR episodes were all biopsy-proven. Humoral rejection (acute or chronic) was defined by the presence of biopsy C4d staining in peritubular capillaries and *de novo* DSA in serum, as previously reported [24–27].

### Detection of anti-HLA antibodies and DSA in serum

Routine pretransplant panel-reactive antibody (PRA) was determined by anti-human globulin (AHG) cytotoxicity for T cells. In some cases, B-cell PRA was determined by standard cytotoxicity. Both assays used local frozen cells, as described previously [28].

For the current study, detection of anti-HLA antibodies and DSA in serum was performed by ELISA (One Lambda Inc., Canoga Park, CA, USA), according to the manufacturer's instructions, with the addition of one additional wash before both the labeled anti-human IgG antibody and the substrate. Assays were read at 630 nm using a Bio-Tek ELX 800 ELISA (Bio-Tek Instruments Inc., Winooski, VT, USA) reader and One Lambda computer software. As a first step, all sera were tested using an ELISA-screening assay for immunoglobulin G (IgG) against class I and class II antibodies (ELISA screen). Subsequently, positive sera (defined as values >10% of the positive control) were then retested with an ELISA against HLA class I and/or class II specific panels (ELISA panel) to confirm the screening result and identify HLA antibody specificity. Specificity analysis was carried out with the assistance of One Lambda software, which determines antibody specificities based on correlation coefficient and chi-square analysis. In cases in which the anti-HLA antibody specificity was unclear, a two-color flow cytometry cross-match against T or B donor cells, or surrogate cells sharing HLA-antigens with the donor, was performed

[28]. Detection of antibodies in the post-transplant samples was also done by AHG, to allow comparison of pre and post-transplant results.

### Statistical analyses

Normally distributed variables are expressed as mean ± SD and non-normally distributed variables are expressed as mean or median and range. Observations between groups were compared using Fisher's exact test for categorical variables. The Cochran–Armitage Trend Test was used for ordinal variable PRA and an unpaired *t*-test for continuous variables. Two-sided *P*-values less than 0.05 were considered significant.

Multivariate linear regression was used to look at the relationship of the log of serum creatinine with anti-HLA antibodies and DSA, adjusted for concerned variables (age, gender, HLA mismatches, previous transplants, delayed graft function, previous AR episodes, previous acute humoral rejections, baseline immunosuppressive regimen and time from the transplant to the study) respectively. A logistic regression was used to determine the relationship between anti-HLA antibodies and concerned variables (age, gender, PRA at the time of the transplant, previous transplants, AR episodes, baseline immunosuppressive therapies and time from the transplant to the study).

## Results

### Baseline clinical characteristics of all patients

A total of 251 kidney transplant recipients who had received a kidney transplant at the Massachusetts General Hospital between 1970 and 2002 were enrolled in the study. Because neither donor typing nor cells were available for two patients who had HLA antibody, data were analyzed only for the remaining 249 patients (Table 1).

Demographics and patient characteristics (Table 1) were representative of the general kidney transplant population of our center. Anti-lymphocyte therapy (ATGAM or thymoglobulin) for immunosuppression induction was used in 18% of patients.

**Table 1.** Baseline characteristics of all patients ($n = 249$). Kidney transplant recipients ≥6 months post-transplant.

| | |
|---|---|
| *General characteristics* | |
| Mean recipient age at Tx (years) | 42.8 (range 5–82) |
| Recipient gender (% male) | 63% |
| Donor organ source | |
| Deceased | 54% |
| Living related | 34% |
| Living unrelated | 12% |
| Retransplants | 12% |
| Mean months from Tx to study | 66.7 (range 6–392) |
| *Immunologic characteristics* | |
| Median number of HLA mismatches | 3 |
| Class I PRA at Tx | |
| <10% | 88.8% |
| 10–50% | 4.7% |
| >50% | 6.5% |
| *Clinical characteristics* | |
| Delayed graft function | 12.3% |
| AR post-Tx | 32.0% |
| AHR (DSA+, C4d+) post-Tx | 5.3% |
| Baseline immunosuppression | |
| MMF group [CNI (CSA or FK), P, MMF] | 56% |
| Not MMF group (CsA, P ± Aza; P, Aza; FK, P, sirolimus) | 44% |
| Serum creatinine (mg/dl) (mean ± SD) | |
| 1 month after Tx | 1.64 ± 0.85 |
| 6 months after Tx | 1.44 ± 0.38 |
| At the time of the study | 1.64 ± 0.75 |

Tx, transplantation; delayed graft function is defined by the need of dialysis in the first week after transplantation; AR, acute rejection confirmed by biopsy; AHR, acute humoral rejection, defined by the presence of '*de novo*' donor-specific antibodies and peritubular capillary C4d staining in biopsy; MMF, mycophenolate mofetil; CNI, calcineurin inhibitor; CsA, cyclosporine; FK, tacrolimus; P, prednisone; Aza, azathioprine; SD, standard deviation.

### Prevalence and characteristics of patients with anti-HLA antibodies long-term after renal transplantation

Anti-HLA class I and/or class II antibodies were detected in the serum of 11.2% (28 of 249) of the patients by ELISA screen, and subsequently were confirmed by using the ELISA panel. The antibodies were directed against HLA class I antigens in 12 patients (43%), against HLA class II antigens in nine patients (32%), and against both class I and class II antigens in seven patients (25%). Of note, an additional 4% of the total sera (10/249) were found to be positive by ELISA screen, but negative with the ELISA panel, and were considered as negative results.

The mean age at transplant was significantly lower and the time from the transplant to the study was significantly longer in patients with anti-HLA antibodies ($n = 28$) compared with patients without antibodies ($n = 221$). Patients with anti-HLA antibodies less frequently received a baseline immunosuppressive therapy including MMF (Table 2). This difference tended to be significant ($P = 0.05$) after adjustment for the time from transplant to the study, the gender and the PRA at the time of the transplant (Table 3).

Patients with anti-HLA antibodies were more sensitized as measured by PRA (and more often highly sensitized) at the time of the transplant. In addition, the proportion of retransplants was higher in patients with anti-HLA antibodies, and a history of at least one post-transplant

**Table 2.** Baseline clinical characteristics in patients with or without anti-HLA antibodies or DSA at the time of the study.

| | Anti-HLA Abs POS ($n = 28$) | Anti-HLA Abs NEG ($n = 221$) | *P*-value | DSA ($n = 11$) | Anti-HLA Abs POS, not DSA ($n = 17$) | *P*-value |
|---|---|---|---|---|---|---|
| Recipient age at Tx (mean ± SD) | 35.3 ± 11.1 | 43.8 ± 12.7 | <0.01 | 32.7 ± 13.7 | 37.0 ± 9.0 | NS |
| Recipient gender (% male) | 71.4% | 62.4% | NS | 72.7% | 70.6% | NS |
| Donor organ source | | | NS | | | NS |
| Deceased | 71.4% | 51.6% | | 63.6% | 76.5% | |
| Living related | 21.4% | 36.2% | | 18.2% | 23.5% | |
| Living unrelated | 7.2% | 12.2% | | 18.2% | 0% | |
| Baseline immunosuppression | | | 0.1* | | | NS |
| MMF group [CNI (CsA or FK), P, MMF] | 40.7% | 57.4% | | 36.4% | 43.8% | |
| Not MMF group (CsA, P ± Aza; P, Aza; FK, P, sirolimus) | 59.3% | 42.6% | | 63.6% | 56.2% | |
| Months from Tx to study (mean ± SD) | 113.1 ± 93.0 | 60.9 ± 57.1 | <0.01 | 102.5 ± 79.7 | 120.0 ± 102.5 | NS |

DSA, donor-specific antibody; Tx, transplantation; SD, standard deviation; MMF, mycophenolate mofetil; CNI, Calcineurin inhibitor; CsA, cyclosporine; FK, tacrolimus; P, prednisone; Aza, azathioprine.
*The difference in the baseline immunosuppression (CNI, P and MMF versus all the other therapies) between patients with and without anti-HLA antibodies tended to be significantly different in the multivariate analysis (see Table 3).

**Table 3.** Association of anti-HLA antibodies with other variables in a multivariate analysis.

| Variable | *P*-value* |
|---|---|
| Not MMF versus MMF-based baseline therapy | 0.05 |
| Gender | 0.14 |
| PRA class I at transplant ≥50% | <0.01 |
| Time from transplant to study | <0.01 |

MMF, mycophenolate mofetil.
**P*-value is shown for association of anti-HLA antibodies with each variable in the table, after adjusting for the other variables.

AR, or acute humoral rejection, was more common. All were significant at $P < 0.01$ (Table 4).

Notably, patients with anti-HLA antibodies had a higher serum creatinine at the time of the study compared to those without anti-HLA antibodies ($2.15 \pm 0.98$ mg/dl vs. $1.57 \pm 0.69$ mg/dl; $P < 0.01$) (Table 4). Moreover, a high serum creatinine was significantly associated with anti-HLA antibodies when adjusted by other variables (age, gender, HLA mismatches, prior transplants, time from the transplant to the study, delayed graft function, AR, acute humoral rejections and baseline

**Table 4.** Immunological characteristics and allograft function in patients with or without anti-HLA antibodies or DSA at the time of the study.

| | Anti-HLA Abs POS ($n = 28$) | Anti-HLA Abs NEG ($n = 221$) | *P*-value | DSA ($n = 11$) | Anti-HLA Abs POS not DSA ($n = 17$) | *P*-value |
|---|---|---|---|---|---|---|
| Number of HLA mismatches | | | | | | |
| Median | 4 | 3 | | 4 | 1.5 | |
| Mean ± SD | 2.8 ± 2.0 | 3.1 ± 1.9 | NS | 3.7 ± 1.4 | 2.1 ± 2.1 | <0.05 |
| % recipients with class I PRA at Tx | | | <0.001 | | | NS |
| <10% | 52% | 94% | | 60% | 47% | |
| 10–50% | 16% | 3% | | 10% | 20% | |
| >50% | 32% | 3% | | 30% | 33% | |
| Retransplants (% of recipients) | 39.3% | 8.0% | <0.001 | 18.2% | 52.9% | NS |
| AR post-Tx (% of recipients) | 61.5% | 28.4% | <0.01 | 90.9% | 40.0% | <0.05 |
| AHR post-Tx (% of recipients) | 30.8% | 2.3% | <0.001 | 54.6% | 13.3% | <0.05 |
| Delayed graft function (% of recipients) | 14.8% | 11.9% | NS | 9.1% | 18.8% | NS |
| Serum creatinine (mg/dl) (mean + SD) | | | | | | |
| 1 month after Tx | 1.66 ± 0.99 | 1.64 ± 0.84 | NS | 1.58 ± 0.76 | 1.78 ± 1.02 | NS |
| 6 months after Tx | 1.36 ± 0.41 | 1.45 ± 0.38 | NS | 1.28 ± 0.47 | 1.45 ± 0.36 | NS |
| At study | 2.15 ± 0.98 | 1.57 ± 0.69 | <0.01* | 2.16 ± 1.00 | 2.15 ± 1.01 | NS |

DSA, donor-specific antibody; Tx, transplantation; SD, standard deviation; AR, acute rejection confirmed by biopsy; AHR, acute humoral rejection, defined by the presence of '*de novo*' donor-specific antibodies and peritubular capillary C4d staining in biopsy.
*Serum creatinine at study was also significantly different between patients with and without anti-HLA antibodies in the multivariate analysis (see Table 5).

**Table 5.** Association of serum creatinine with anti-HLA antibodies and other significant variables in a multivariate analysis.

| Variable | *P*-value* |
|---|---|
| Anti-HLA antibodies | 0.01 |
| Gender | 0.02 |
| Time from Tx to study | <0.01 |
| Delayed Graft Function | 0.02 |
| Acute rejection | <0.01 |

Tx, transplantation.
**P*-value is shown for association of log of serum creatinine at the time of the study with each variable in the table, after adjusting for the other variables. The creatinine was significantly higher in patients with anti-HLA antibodies.

immunosuppression) in a multivariate analysis (data not shown). Table 5 summarizes the association between serum creatinine with anti-HLA antibodies ($P = 0.01$) when adjusted by variables that are themselves significantly associated with serum creatinine (i.e. gender, time from the transplant to the study, delayed graft function, AR).

### Prevalence and characteristics of patients with DSA long-term after renal transplantation

The overall prevalence of DSA, i.e. antibodies specific for mismatched donor HLA antigens, was 4.4% (11/249). Thus, of the 28 patients with anti-HLA antibodies, only 11 (39%) had DSA (Table 6). In 10 cases a pretransplant serum was available and none of them had evidence of DSA before the transplant by ELISA assay (*de novo* antibodies). In the 11 patients with DSA, the antibodies were directed against HLA class II antigens in six patients (55%), HLA class I antigens in three patients (27%), and against both class I and class II antigens in two patients (18%).

Similar to the comparison between the anti-HLA antibody positive versus negative groups, the age at transplant, pretransplant sensitization and history of AR or acute humoral rejection were also significantly different between the DSA positive ($n = 11$) versus DSA negative ($n = 238$) groups (data not shown). However, unlike the HLA antibody positive versus negative groups, differences in time from transplant to study (102.5 vs. 65.1 months) and the number of retransplant patients (18.2% vs. 11.5%) did not reach statistical significance between the DSA versus non-DSA groups (data not shown). Although patients with DSA tended to have a higher serum creatinine compared with those without DSA, this also did not reach statistical significance (2.16 ± 1.00 mg/dl vs. 1.61 ± 0.73 mg/dl).

Finally, we compared clinical and immunological characteristics of patients with DSA ($n = 11$) and patients with nondonor specific anti-HLA antibodies ($n = 17$). There was no difference in the age at transplant, baseline immunosuppression and time from transplant to study (Table 2). Also pretransplant sensitization was not significantly different in the two groups; however, patients with DSA had a higher number of HLA mismatches between the donor and the recipient and more frequently had a history of AR or acute humoral rejection compared with patients with nondonor specific anti-HLA antibodies. Although the number of retransplant patients was higher in the 'nondonor-specific' antibody group, the difference did not reach statistical significance (Table 4). Moreover, there was no significant difference in serum creatinine at the time of the study between patients with DSA and patients with nondonor specific anti-HLA antibodies (2.16 ± 1.00 mg/dl vs. 2.15 ± 1.01 mg/dl; Table 4).

Of note, 11 of the 17 patients with non-DSA had antibody screens carried out by the same method pre- and post-transplant and thus could be compared. Only one of 11 had antibodies post-transplant that were not present pretransplant (10 of 11 were not *de novo* antibodies).

### Correlation between serum anti-HLA antibodies and DSA and peritubular capillary C4d staining in biopsy

In the group of patients with anti-HLA antibodies ($n = 28$), nine patients had renal allograft biopsies performed by the clinical team (independent of the current study) within 6 months of the study. Six of the nine biopsies (66%) were C4d positive (i.e. presence of C4d deposits in peritubular capillaries), indicating an active antibody-mediated process (Table 6) [24–28]. All C4d positive biopsies were from patients with DSA. In the group of patients without anti-HLA antibodies ($n = 221$), 11 patients had renal allograft biopsies performed within 6 months of the study by the clinical team, and none was C4d positive, indicating the absence of an antibody mediated process in these biopsied samples (66% vs. 0%, $P < 0.01$).

## Discussion

The role of anti-donor antibodies (particularly anti-HLA antibodies) in the late post-transplant period remains an important issue in renal transplantation and in solid organ transplantation in general. The precise contribution of humoral mechanisms of tissue injury to the pathogenesis of CR, which is an important cause of late allograft loss in the modern era of immunosuppression [1], remains unclear, and needs to be determined.

It is now well recognized that both antigen-specific immune mechanisms and nonimmunologic factors play an important role in CR/chronic allograft nephropathy

**Table 6.** Patients with anti-HLA antibodies (DSA and nondonor-specific anti-HLA antibodies).

| Patient no. | PRA at Tx* | | Donor mismatches | ELISA PRA at study | | Anti-HLA antibody specificity | Flow-XM | | Allograft biopsy† | | Creatinine at study (mg/dl) | Time from Tx to study (months) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Class I | Class II | | Class I | Class II | | T | B | Diagnosis | IF | | |
| Patients with DSA | | | | | | | | | | | | |
| 1 | 0 | 0 | A1, B8, DR3, 12, DQ2 | Neg | 38 | DQ2 | | | CHR and ACR | C4d+ | 2.5 | 7 |
| 2 | 3 | 0 | A23, 28, B44, DR9, 53, DQ2 | Neg | 25 | DR53 | | | HR and ACR | C4d+ | 1.7 | 39 |
| 3 | 0 | 0 | A11, 23, B44, DR7, 53 | Neg | 28 | DR53 | | | CHR and ACR | C4d+ | 1.6 | 40 |
| 4 | 74 | 25§ | A32, B44, DR7, 53, DQ2 | Neg | 13 | DR7 | | | CHR | C4d+ | 2.1 | 133 |
| 5 | 0 | 9 | A33, DR4, 53 | 5 | 53 | DR53 | | | CTG | C4d−‡ | 2.5 | 66 |
| 6 | 0 | 0 | A2, B6, 44, DR7, 8, 53 | Neg | 41 | DR53 | | | | | 1.6 | 170 |
| 7 | 0 | 0 | B7, 18, Cw7, DR2, 3 | 34 | 75 | Cw7, DR3, DQ2 | | | CHR | C4d+ | 2.9 | 155 |
| 8 | 0 | 0 | A1, 68, B8, 60, DR2, 3, DQ2 | 11 | 69 | B48, 60, DR3, DQ2 | | | | | 1.1 | 72 |
| 9 | 14 | | A2, B44, DR16, 51, DQ5 | 2 | Neg | A2 | | | HR and ACR | C4d+ | 4.7 | 246 |
| 10 | 90 | | A3, B35, DR7, 13, 52, DQ2 | 88 | Neg | ? | Pos | Pos | | | 1.5 | 12 |
| 11 | 61 | | A1, B8, DR1, 3 | 93 | Neg | ? | Pos | Neg | | | 1.6 | 187 |
| | | | | | | | | | | | mean ± SD: 2.16 ± 1.00 | |
| Patients with nondonor-specific HLA antibodies | | | | | | | | | | | | |
| 12 | | | A32, B7, 40, DR8, 14 | Neg | 16 | DR4¶ | | | FSGS, IF-TA | C4d− | 4 | 245 |
| 13 | 8 | 88 | A2, 24, DR4, 15, 53, DQ3 | 21 | 47 | A1§, DR3, DQ2 | Neg | Neg | BK nephropathy | C4d− | 1.8 | 12 |
| 14 | 98 | | DR13, 103 | 21 | 16 | A23, 24§, DR7§ | | Neg | | | 1.4 | 66 |
| 15 | 3 | | A1, B35, 44, DR9, 103, DQ5, 9 | Neg | 25 | DR17§ | | | | | 3.1 | 58 |
| 16 | 38 | | 0 | Neg | 47 | DR8§, 52, DQ4 | | | | | 1.2 | 34 |
| 17 | 100 | | 0 | Pos | 97 | ? | | | | | 1.9 | 19 |
| 18 | 6 | 0 | 0 | 4 | Neg | ? | | | | | 1.3 | 45 |
| 19 | 50 | | A30, B18, DR13, 15 | 36 | Neg | B15, 17, 7, 13 | Neg | | | | 2 | 165 |
| 20 | 79 | | 0 | Pos | Neg | | | | | | 0.9 | 20 |
| 21 | 13 | | B35, DR4, 13, 52, DQ3 | 43 | Neg | A66, B7 | Neg | | | | 2.7 | 20 |
| 22 | 0 | 0 | A2, B62, DR6 | 2 | Neg | | Neg | | | | 2.3 | 151 |
| 23 | 0 | | 0 | 36 | Neg | A2, 28, wkA23, 24 | | | | | 1.2 | 263 |
| 24 | 30 | | A24, B27 | 21 | Neg | A2§,69 | | | | | 1.7 | 293 |
| 25 | 0 | | A30; B18, 37; DR4, 17, 53; DQ2, 7 | 9 | Neg | ? | Neg | | | | 1.4 | 15 |
| 26 | | | 0 | Pos | 19 | DR7§ | | | | | 4.3 | 207 |
| 27 | 89 | | 0 | 4 | Neg | A2, 68 | | | | | 3.3 | 188 |
| 28 | 0 | | A2, B15 | Neg | 50 | DQ4, DQ7 | | | | | 2 | 239 |
| | | | | | | | | | | | mean ± SD: 2.15 ± 1.01 | |

DSA, donor specific antibody; IF, immunofluorescence; C4d+, C4d deposits in peritubular capillary in the biopsy; ACR, acute cellular rejection; HR, humoral rejection; CHR, chronic humoral rejection; CTG, chronic transplant glomerulopathy; FSGS, focal, segmental glomerulosclerosis; IF-TA, chronic interstitial fibrosis and tubular atrophy; Tx, transplant.

*Detected using either lymphocytotoxicity or ELISA.

†Allograft biopsies independently performed by clinical team. Biopsies were considered only if performed within 6 months before or after ELISA measurement.

‡The biopsy in this patient was performed after 3 months of 'rescue therapy' with tacrolimus and mycophenolate mofetil.

§Previous donor/s antigen.

¶Pregnancy.

(CR/CAN). However, it is often difficult to ascertain the relative contribution of each factor to the development of the tubulo-interstitial, vascular and glomerular lesions in the allografts [1,29]. Mauiyyedi *et al.* found that 61% of the cases with typical CR (i.e. allograft biopsies with histologic criteria of chronic allograft glomerulopathy and/or transplant arteriopathy) had capillary C4d deposition (chronic humoral rejection), and most of the C4d positive CR cases had circulating anti-donor HLA antibody in their serum [25]. In another larger study by Regele *et al.*, capillary C4d deposition was found in 34% of allograft biopsies performed for chronic allograft dysfunction (of all causes), further suggesting that humoral immunity indeed contributes to CR/CAN in a significant subgroup of kidney transplant recipients [26].

In the past, various studies have indicated that anti-donor anti-HLA antibodies can be associated with CR of various organs, but their pathogenic (causative) role in the lesions of CR remained controversial [2–16]. It has been shown that *de novo* post-transplant production of anti-donor allo-antibodies can predate the clinical manifestations of CR/CAN, implicating humoral mechanisms of rejection as a cause of CR/CAN [4,9–12]. In addition, Regele *et al.* noted that peritubular capillary C4d deposition preceded the development of chronic transplant glomerulopathy in most patients in whom serial biopsies were available, again emphasizing that evidence of antidonor humoral immunity can be detected before the development of the pathological lesions of CR/CAN [26].

In view of the increasingly recognized pathogenic relevance of anti-HLA antibodies and DSA in late renal allograft pathology, more clinical studies, with different methods/assays, aiming at further delineating the role of humoral immunity in the late post-transplant period are needed, as this may have important therapeutic implications. At our institution, to determine the prevalence of anti-HLA antibodies and DSA in the late post-transplant period, we performed the current study in RTR who were greater than 6 months post-transplant. We also determined if the presence of anti-HLA antibodies in the late post-transplant period correlated with allograft function and other clinicopathologic findings, and investigated whether these data could help to identify a population of patients who should be routinely screened for antibodies post-transplant.

Among the techniques that we used to detect anti-HLA antibodies and DSA, we chose the ELISA screen, because it has been shown to be a sensitive and specific method to detect antibodies directed only against HLA antigens, and it can distinguish between IgG and IgM [8,10,12,20–22]. Also, the ELISA panel assay, with known specific groups of HLA antigens in each well, identifies the anti-HLA antibody specificity, even without donor cell availability. In the ELISA screen assay we used a low positive cutoff (i.e. >10% of positive control) to increase the number of sera with HLA antibodies that could be identified by this technique, but the 'cost' was 4% of 'false positives' that were identified as such only after the panel assay. However, our experience has been that approximately one-third of the screens considered positive with the low cutoff but negative with the higher cutoff do have HLA antibody. These antibodies would be missed if we tried to decrease the frequency of 'false positives' by increasing the positive cutoff (S.L. Saidman, unpublished data). Therefore, given the much lower cost and time required for the ELISA-screen assay when compared with the ELISA panel, we continue to use it as the first step when screening for HLA antibodies.

In the present study, we found that 11.2% of RTR followed in the outpatient clinic had circulating anti-HLA class I or class II antibodies. However, further analysis of the antibody specificity showed that only 4.4% of patients had DSA, i.e. anti-HLA antibodies directed against the donor mismatched HLA antigens. Overall, DSA were directed more often against class II, rather than class I HLA antigens. Interestingly, other authors have reported that anti-donor anti-HLA class II antibodies appear to be preferentially associated with CR/CAN [2–4,9,11,12]. This is in contrast to the syndrome of 'acute humoral rejection', where a greater prevalence of anti-HLA antibodies, and DSA, against class I has been reported [2,3,12,27].

Anti-HLA antibodies were significantly associated with pre-transplant sensitization, nonprimary transplantation and post-transplant AR episodes. Moreover, in a multivariate analysis, the presence of anti-HLA antibodies tended to be significantly associated with a baseline immunosuppressive therapy that did not include MMF, compared with MMF-based regimens (Table 3).

Patients with anti-HLA antibodies had a significantly higher serum creatinine at the time of the study, compared with those without antibodies. A higher serum creatinine was significantly associated with anti-HLA antibodies also after adjustment for time from the transplant to the study (as well as other variables) in a multivariate analysis (Table 5).

Moreover, in patients with anti-HLA antibodies, 66% of the renal allograft biopsies performed within 6 months of the study by the clinical team (independently of the current study) were 'C4d positive' (i.e. presence of C4d deposits in peritubular capillaries) and all of the biopsies were from patients with DSA (Table 6). In contrast, in the group of patients without anti-HLA antibodies, 0% of the biopsies were C4d positive, indicating the absence of an antibody-mediated process in these allografts. We acknowledge that although more than half of the patients with DSA had a biopsy tested for the presence of C4d staining,

only a few of the patients with nondonor-specific antibodies or with no anti-HLA antibodies were tested. Thus, although the suggested correlation between C4d and DSA in this study agrees with the significant correlations reported in other studies [24,25], the conclusions that can be drawn from this data are somewhat limited. Moreover, most of the biopsies were performed prior to the patients' enrollment in the study so we cannot comment on the predictive value of DSA for future humoral rejection.

Of note, we did not detect differences in allograft function (as evidenced by serum creatinine) between patients with DSA ($n = 11$) or patients with 'nondonor-specific' anti-HLA antibodies ($n = 17$). Thus, no obvious detrimental effect on the allograft function of DSA versus 'nondonor-specific' anti-HLA antibodies could be demonstrated post-transplant in the current study. As recently suggested by Terasaki [30], patients who are rejecting their graft may have HLA antibodies not directed specifically against donor antigens because DSA could be absorbed by the graft, leaving only nonspecific anti-HLA antibodies circulating in the peripheral blood. However, in our study most (91%) of the patients with non-DSA HLA antibodies who had pretransplant samples available for comparison showed evidence of the same HLA antibody specificities pretransplant, indicating these were not *de novo* antibodies.

Although our data do not support a particularly detrimental effect of circulating DSA on kidney allograft function, this could be the result of the low number of patients found to have circulating DSA. Thus, more prospective, longer studies, involving a larger number of patients will be needed to determine if the detection of circulating DSA might have a particularly detrimental effect.

Recently Nickerson *et al.* [9] reported a 2-year post-transplant prevalence of anti-HLA antibodies of 19% and of *de novo* anti-HLA antibodies of 9%, a finding that is consistent with our findings. Lee *et al.* [10] reported a higher percentage of post-transplant anti-HLA antibodies. However, in the latter study, all patients were treated with cyclosporine and not with newer, more potent drugs, which may explain the difference.

Indeed, in the literature, the frequency of anti-HLA antibodies and DSA detected after renal transplantation is extremely variable [2–13], also probably because of the use of different assays to detect these antibodies or to variable times of sample collection [3]. In addition, some authors measured DSA, whereas others analyzed or reported only PRA. Finally, in some reports only patients with acute or chronic allograft dysfunction are studied, whereas in others a nonselected patient population is considered [3]. This emphasizes the need for standardized conditions in future studies, and precise definitions of the study goals.

In summary, anti-donor humoral responses, as assessed by determination of anti-HLA antibodies, were found infrequently in nonselected kidney allograft recipients, greater than 6 months post-transplant. However, patients with anti-HLA antibodies had a significantly higher serum creatinine at the time of the study, compared with those without antibodies. In a multivariate analysis, serum creatinine was significantly associated with anti-HLA antibodies after adjustment for time from the transplant to the study, gender, delayed graft function and AR episodes. Moreover, most patients with anti-HLA antibodies in serum who underwent allograft biopsies also had *in situ* evidence of anti-donor humoral allo-reactivity. Given these data, we believe that patients greater than 6 months post-transplant with an increase in creatinine should be screened for anti-HLA antibodies, either DSA or non-DSA, and, if these antibodies are present, a biopsy should be considered.

Although we found a low prevalence of DSA in the studied RTR, these data may be important in particular in the current era, as 'minimization' immunosuppression strategies might result in an increased development of anti-HLA antibodies and/or DSA, with possible subsequent CR and allograft dysfunction.

## Acknowledgements

We wish to thank the staff of the Histocompatibility Laboratory at the Massachusetts General Hospital for technical assistance and Ms Marylin Farrell for the Transplant Unit database collection. We also wish to thank Ms Isabelle Wood and the staff of the Brigham and Women's Hospital Tissue Typing Laboratory for the flow cross-matching. Francesca Cardarelli, is a renal research fellow supported by the Clinica Medica e Nefrologia, University of Parma, Italy. Manuel Pascual, is supported by the Leenaards Foundation.

## References

1. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB. Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 2002; **346**: 580.
2. McKenna RM, Takemoto SK, Terasaki PI. Anti-HLA antibodies after solid organ transplantation. *Transplantation* 2000; **69**: 319.
3. Cardarelli F, Saidman S, Theruvath T, Tolkoff-Rubin N, Cosimi AB, Pascual M. The problem of late allograft loss in kidney transplantation. *Minerva Urol Nefrol* 2003; **55**: 1.
4. Davenport A, Younie ME, Parsons J, Klouda PT. Development of cytotoxic antibodies following renal allograft transplantation is associated with reduced graft survival

due to chronic vascular rejection. *Nephrol Dial Transplant* 1994; **9**: 1315.
5. Abe M, Kawai T, Futatsuyama K, *et al.* Postoperative production of anti-donor antibody and chronic rejection in renal transplantation. *Transplantation* 1997; **63**: 1616.
6. Kerman RH, Susskind B, Kerman DH, *et al.* Anti-HLA antibodies detected in post-transplant renal allograft recipient sera correlate with chronic rejection. *Transplant Proc* 1997; **29**: 1515.
7. Piazza A, Poggi E, Borrelli L, *et al.* Impact of donor-specific antibodies on chronic rejection occurrence and graft loss in renal transplantation: post-transplant analysis using flow cytometric techniques. *Transplantation* 2001; **71**: 1106.
8. Supon P, Constantino D, Hao P, *et al.* Prevalence of donor-specific anti-HLA antibodies during episodes of renal allograft rejection. *Transplantation* 2001; **71**: 577.
9. Nickerson P, Karpinski M, Rush D, Jeffrey J, Pochinco D. De novo anti-HLA antibodies post-kidney transplantation are uncommon, but may be associated with the development of chronic rejection. *Transplantation* 2002; **74**(Suppl.): 273 (A1077) (abstract).
10. Lee PC, Terasaki PI, Takemoto SK, *et al.* All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. *Transplantation* 2002; **74**: 1192.
11. Chan L, Terasaki PI. Class II HLA antibodies after transplantation and chronic allograft failure: a prospective study. *J Am Soc Nephrol* 2002; **13**: 232 SU-FC (abstract).
12. Worthington JE, Martin S, Dyer PA, Johnson RW. An association between post-transplant antibody production and renal transplant rejection. *Transplant Proc* 2001; **33**: 475.
13. Christiaans MH, Overhof-de Roos R, Nieman F, van Hoof JP, van den Berg-Loonen EM. Donor-specific antibodies after transplantation by flow cytometry: relative change in fluorescence ratio most sensitive risk factor for graft survival. *Transplantation* 1998; **65**: 427.
14. Suciu-Foca N, Reed E, Marboe C, *et al.* The role of anti-HLA antibodies in heart transplantation. *Transplantation* 1991; **51**: 716.
15. Jaramillo A, Smith MA, Phelan D, *et al.* Development of ELISA-detected anti-HLA antibodies precedes the development of bronchiolitis obliterans syndrome and correlates with progressive decline in pulmonary function after lung transplantation. *Transplantation* 1999; **67**: 1155.
16. Worthington JE, Martin S, Al-Husseini DM, Dyer PA, Johnson RWG. Post-transplantation production of donor HLA-specific antibodies as a predictor of renal transplant outcome. *Transplantation* 2003; **75**: 1034.
17. Russell PS, Chase CM, Winn HJ, Colvin RB. Coronary atherosclerosis in transplanted mouse hearts 2. Importance of humoral immunity. *J Immunol* 1994; **152**: 5135.
18. Russell PS, Chase CM, Colvin RB. Alloantibody-and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis. *Transplantation* 1997; **64**: 1531.
19. Kawai T, Poncelet A, Sachs DH, *et al.* Long-term outcome and alloantibody production in a non-myeloablative regimen for induction of renal allograft tolerance. *Transplantation* 1999; **68**: 1767.
20. Moore SB, Ploeger NA, DeGoey SR. HLA antibody screening: comparison of a solid phase enzyme-linked immunoassay with antiglobulin-augmented lymphocytotoxicity. *Transplantation* 1997; **64**: 1617.
21. Susal C, Opelz G. Kidney graft failure and presensitization against HLA class I and class II antigens. *Transplantation* 2002; **73**: 1269.
22. Dyer PA, Taylor C, Martin S. HLA and Clinical Solid Organ Transplantation: Recent Publications and novel approaches. In: Gjertson DW, Terasaki PI, eds. *HLA 1998*. Los Angeles, CA: UCLA Tissue Typing Laboratory, 1999: 13–45.
23. Pascual M, Tolkoff-Rubin N, Farrell ML, *et al.* The kidney transplant program at the Massachusetts General Hospital. In: Cecka JM, Terasaki PI, eds. *Clin Transpl 2001*. Los Angeles, CA: UCLA Immunogenetics Center, 2002: 123–130.
24. Collins AB, Schneeberger EE, Pascual MA, *et al.* Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999; **10**: 2208.
25. Mauiyyedi S, Della Pelle P, Saidman S, *et al.* Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 2001; **12**: 574.
26. Regele H, Bohmig GA, Habicht A, *et al.* Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: a contribution of humoral immunity to chronic allograft rejection. *J Am Soc Nephrol* 2002; **13**: 2371.
27. Feucht HE, Opelz G. The humoral immune response towards HLA class II determinants in renal transplantation. *Kidney Int* 1996; **50**: 1464.
28. Crespo M, Pascual M, Tolkoff-Rubin N, *et al.* Acute humoral rejection in renal allograft recipients: I. Incidence, serology and clinical characteristics. *Transplantation* 2001; **71**: 652.
29. Halloran PF, Melk A, Barth C. Rethinking chronic allograft nephropathy: the concept of accelerated senescence. *J Am Soc Nephrol* 1999; **10**: 167.
30. Terasaki PI. Humoral theory of transplantation. *Am J Transplant* 2003; **3**: 665.