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Pretransplant Sensitization Against Angiotensin II Type 1 Receptor Is a Risk Factor for Acute Rejection and Graft Loss

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The angiotensin II type 1 receptor (AT_1R) is an emerging target of functional non-HLA antibodies (Ab). We examined the potential of determining the degree of presensitization against AT_1R as a risk factor for graft survival and acute rejection (AR). The study included 599 kidney recipients between 1998 and 2007. Serum samples were analyzed in a blinded fashion for anti-AT_1R antibodies (AT_1R-Abs) using a quantitative solid-phase assay. A threshold of AT_1R-Ab levels was statistically determined at 10 U based on the time to graft failure. An extended Cox model determined risk factors for occurrence of graft failure and a first AR episode. AT_1R-Abs >10 U were detected in 283 patients (47.2%) before transplantation. Patients who had a level of AT_1R-Abs >10 U had a 2.6-fold higher risk of graft failure from 3 years posttransplantation onwards ($p = 0.0005$) and a 1.9-fold higher risk of experiencing an AR episode within the first 4 months of transplantation ($p = 0.0393$). Antibody-mediated rejection (AMR) accounted for 1/3 of AR, whereby 71.4% of them were associated with >10 U of pretransplant AT_1R-Abs. Pretransplant anti-AT_1R-Abs are an independent risk factor for long-term graft loss in association with a higher risk of early AR episodes.

Key words: Acute allograft rejection, allosensitization, angiotensin II receptors, antibody mediated rejection, pretransplant, survival

Abbreviations: ACMR, acute cellular mediated rejection; AMR, antibody-mediated rejection; AR, acute rejection; ATG, antithymocyte-globulin; AT_1R, angiotensin type 1 receptor; AT_1R-Abs, anti-AT_1R antibodies; CDC, complement dependent cytotoxicity; CHO, Chinese Hamster Ovary; DGF, delayed graft function; DSA, donor specific antibodies; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen; HR, hazard ratio; IF, indirect immunofluorescence; IL2, interleukin 2; LCT, micro-lymphocytotoxicity; MHC, major-histocompatibility-complex (MHC); MICA, MHC class I-related molecules A; MMF, mycophenolate mofetil; PRA, panel reactive antibodies.

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Introduction

Substantial developments in immunosuppressive regimes, HLA tissue typing and HLA-antibody detection, have led to continuous improvements in allograft survival. A lower incidence of acute cellular rejection has been pivotal to this progress. Perhaps as a result of this success, acute antibody-mediated rejection (AMR), which develops despite modern drug regimens, is more frequently observed (1). Advantages in detecting HLA sensitization in serum have improved AMR prediction and recognition. However, features resembling AMR occur even in HLA-identical sibling transplants, emphasizing the importance of immune responses against unidentified non-HLA antigens (2). Identification of humoral presensitization leading to AMR would improve the mechanistic understanding of this type of rejection and would facilitate its prediction and potentially its prevention. The identification of new immune targets

that may serve as biomarkers correlating with or predicting AMR could identify recipients at risk. Antibodies (Ab) reactive with non-HLA antigens found in association with rejection in solid organs may be directed against a variety of determinants, including angiotensin II type 1 receptor (AT_1R) (3). Functional AT_1R Abs act as allosteric receptor agonists by binding to the second extracellular loop of AT_1R and initiating biological processes leading to severe graft injury (4,5). We reasoned that the detection of humoral presensitization against AT_1R before kidney transplantation may serve to detect patients at risk of developing acute rejection (AR). We therefore employed a newly developed solid-phase assay for the detection of anti-AT_1R Ab in serum. In this paper we report, for the first time, that the presence of anti-AT_1R-Abs before transplantation is associated with a higher risk of AR and, more importantly, long-term graft failure.

Patients and Methods

Patients

Since 1998, serum samples have been systematically prospectively stored in the DIVAT-biocollection (Données Informatisées et Validées en Transplantation, Inserm N° 02555) for each consecutive patient receiving a transplant at the Nantes University Hospital. Five hundred ninety-nine adult patients who received a kidney transplant between 1998 and 2007 were included in the study. Donor and recipient data were extracted from the DIVAT clinical prospective cohort (www.divat.fr, N°CNIL 891735 version 2, August 2004). Codes were used to ensure donor and recipient anonymity and blinded testing. The data are computerized in real time as well as at each transplant anniversary. The quality of the DIVAT data bank is validated by an annual cross-center audit, systematic verification during data entry and a weekly automatic report on the identification of incoherencies between parameters.

Studied parameters

Data were extracted from the DIVAT data bank. For the donors, the parameters analyzed were age, gender and deceased/living status. For the recipients, the parameters analyzed were age, gender, number of previous transplants, delayed graft function (DGF, defined as time to reach an eGFR $\geq$10 mL/min (6)), pretransplantation anti-HLA immunization (% historical peak of class I or class II PRA determined by complement-dependent cytotoxicity (CDC) on a selected panel of typed HLA donors or by ELISA or Luminex), HLA-A-B-DR incompatibilities, induction therapy with anti-thymocyte globulin (ATG) or anti-IL2 receptor antibody and time of any AR episodes.

Acute rejection episodes

All AR were biopsy proven (n = 63) and were taken into account for all statistical analyses. All mentioned biopsies were for the purposes of the study retrospectively analyzed by three pathologists according to recent Banff classification (2007) (7) Pathologists were blinded to the antibody status. C4d deposition was evaluated by a two-step indirect immunofluorescence (IF) method with a monoclonal antibody specific for C4d on frozen tissue (Quidel, Santa Clara, CA). All patients with C4d-negative IF and patients with no available frozen tissue had protocol immunohistochemical C4d staining performed on paraffin sections using human polyclonal antibody (Biomedica, Vienna, Austria). AR were classified in acute cellular rejection, acute humoral rejection, mixed humoral and cellular rejection (''mixed rejection'') and finally borderline. Particular attention was paid to the clinical parameters surrounding each AR episode, including blood pressure data and the type of antihypertensive drugs, which were specifically reanalyzed at the time of rejection diagnosis.

Detection of anti-AT_1fcR antibodies by solid-phase assay

Anti-AT_1R Ab were measured with a sandwich ELISA (CellTrend GmbH Luckenwalde, Germany, now One Lambda, Canoga Park, CA, USA). The microtiter 96-well polystyrene plates were coated with extracts from transfected Chinese Hamster Ovary (CHO) cells overexpressing the human AT_1R. Conformational epitopes of the receptor were maintained by addition of 1 mM calcium chloride to every buffer. Duplicate samples of a 1:100 serum dilution were incubated at 4°C for 2 h. After washing steps, plates were incubated for 60 min with a 1:20 000 dilution of horseradish-peroxidase-labeled goat anti-human IgG (Jackson, Bar Harbor, ME, USA) used for detection. In order to obtain a standard curve, plates were incubated with test sera from an anti-AT_1R antibody positive index patient. When compared to the neonatal cardiomyocyte bioassay used in the first study (4), the solid-phase assay had a 96% specificity and 88% sensitivity. The inter-assay variability was 8%; the intra-assay variability was 5%. All sera were coded and sent from Nantes to Berlin for anti-AT_1R antibody assessment by individuals who had no information regarding the patients' characteristics. All tests were done in duplicate. The detection threshold of Anti-AT_1R-Abs was set at 2.5 U.

HLA typing

HLA A, B, DR and DQ typing of transplant recipients and their corresponding donors was performed by CDC (Monoclonal typing trays; One Lambda, Inc.) or by molecular biology (PCR-SSP or PCR-SSO; One Lambda, Inc.).

Pretransplant crossmatches

All pretransplant crossmatches were performed by direct CDC on total and separated T and B lymphocytes, according to National Institutes of Health (NIH) rules. Donor lymphocytes were isolated from spleen cells or lymph nodes. CDC was performed on peak, historical and current sera. Current sera included sera collected within 3 months pretransplantation in immunized patients without a recent sensitization event or within 6 months pretransplantation in nonimmunized patients without a recent sensitization event. T lymphocytes were purified using monoclonal Ab (LymphoKwick T; One Lambda, Inc.), and B lymphocytes were isolated using magnetics beads (Dynabeads, InVitrogen, Saint Aubin, France). All patients were transplanted across a negative prospective crossmatch. In our center, a positive IgG T cell crossmatch on current serum was a contraindication to transplantation, as opposed to a positive IgM CDC. Transplantation across a positive B cell crossmatch either on current or on historical sera was allowed for first grafts only.

HLA Ab testing

During this long study period, pre- and posttransplant immunological status was assessed using different combinations of tests to detect anti-HLA Ab (CDC, ELISA or Luminex Bead Array technique). Screening of anti-HLA-Ab was performed by CDC until 1999, by ELISA until 2005 and by Luminex since 2005. CDC screening was performed according to standards of the NIH with a panel of 36 selected HLA-typed and separated T and B lymphocytes on platelet-absorbed and -unabsorbed sera. For ELISA screening (LAT-M, One Lambda, Inc.) or identification (LAT ID-1288; One Lambda, Inc.) wells were coated with purified HLA antigens derived from human B cell lines. Specific Ab were detected by optical density signal measurement. Cut-offs were calculated as the percentage of the reactivity range of the provided serum control tested in the Positive HLA wells minus the nonspecific background of the test serum analyzed. For Luminex assay screening (LABScreen Mixed

LSM12, One Lambda, Inc.), identification (LABScreen LS1 PRA, LS2 PRA) or single antigen (SA; LABScreen LS1A04, LS2A01; One Lambda, Inc.), Abs were detected by the fluorescent signal for each bead coated with HLA antigen, normalized to the value measured with the negative control serum. All MFI values >500 were taken into account to detect DSA.

Since CDC PRAs were prospectively registered for all patients in our database, we used historical anti-class I or class II CDC PRAs to define pretransplantation immunization in our statistical model. In order to minimize the lack of sensitivity of the CDC PRAs, the cut-off of historical anti-class I and class II PRAs used in the statistical analyses was defined at 0%.

We retrospectively retested, by Luminex technology (single antigen technique) HLA donor-specific antibody (DSA and MFI) for all available sera from patients who developed biopsy-proven AR episode, collected at the time of transplantation and at the time of rejection. Only A, B, DRB and DQB Ab were considered since Cw and DP typing was not performed.

Statistical analysis

The primary end point of the study was the posttransplantation time to graft failure. If a patient died with a functioning graft, the event was analyzed as a right censoring. The time-to-event distribution was first described by using the Kaplan–Meier estimator. The threshold of anti-AT_1R Ab was statistically determined by maximizing the distance between the corresponding graft survival using the Horthorn and Zeileis method (8). The principle of this method is to determine the metric and the corresponding cut-off that maximizes the difference between both survival curves based on the log-rank graft survival curves (death censored). Using this procedure, the level of anti-AT_1R-Ab with an optimal cut-off of 10 U was determined.

This threshold value was then used for all subsequent analyses. Univariate analysis was first performed using Kaplan and Meier curves and a log-rank test. Variables were included in the multivariate Cox model if their p values were <0.20 (log-rank test). The Cox model was adjusted for risk factors already described in the literature. In the case of nonproportionality, an extended Cox model was performed (9), which simulates different hazard ratios according to the time posttransplantation. Covariates were considered significant if their p values were <0.05. All the analyses were performed using R version 2.13.0.

The same modeling strategy was used for the analysis of time to the first AR episode.

The primary end point of the study was the posttransplantation time to graft failure. If a patient died with a functioning graft, the event was analyzed as a right censoring. The time-to-event distribution was first described by using the Kaplan–Meier estimator. The threshold of anti-AT_1R Ab was statistically determined by maximizing the distance between the corresponding graft survival using the Horthorn and Zeileis method (8). The principle of this method is to determine the cut-off that maximizes the difference between both survival curves based on the log-rank statistic. Using this procedure, the optimal cut-off of anti-AT_1R Ab was determined at 10 U. This threshold value was then used for all subsequent analyses. Univariate analysis was first performed using Kaplan and Meier curves and a log-rank test. Variables were included in the multivariate Cox model if their p values were <0.20. The Cox model was adjusted for risk factors already described in the literature. In the case of nonproportionality, an extended Cox model was performed (9), which allows different hazard ratios according to the time posttransplantation. The posttransplantation time intervals, within the hazard ratio can be considered fixed, were defined according to the log minus log survival function. Covariates were considered significant if their p values were <0.05. All the analyses were performed using R version 2.13.0.

Results

Patient characteristics

Among the 599 kidney recipients enrolled in the study, 521 (87%) had received a first kidney transplant, 59 (10%) a second kidney transplant and 3% more than two transplants. Sixty-one percent were male, with a mean age of 48.9 years (±14.2), and 36.2% were older than 55 years. The majority (94.2%) were recipients of a deceased-donor kidney (63.3% male with a mean age of 46.6 ± 16.4 years and 31.8% older than 55 years). Induction therapy was antithymocyte globulin (Thymoglobulin®, Pasteur Mérieux, France) in 34% of patients or anti-interleukin-2-receptor-antibody (Simulect®, Novartis, Reuil Malmaison, France) in 49.6% patients. Ninety-five percent of patients received calcineurin inhibitors (49.4% tacrolimus; 43.2% cyclosporine A) associated with mycophenolate mofetil (90.1%) and steroids for maintenance therapy. Considering the whole cohort, 197 (32.9%) patients were historically sensitized against HLA before transplantation (PRA >0% for class I or II). The mean historical PRA was 23.7% for class I ([range 0–100%], SD = 31.8) and 29% for class II ([range 0–91%], SD = 33.2) in patients with AR. Only 5% of patients were historically highly sensitized (above 80%) against either HLA class I or class II panel antigens.

The median value of anti-AT_1R-Abs in the pretransplant serum of the whole cohort (599 patients) was 9.5 U (range 2.5–40 U). The first quartile was 6.5 U, and the third quartile was 15.0 U.

The mean follow-up time was 6.9 years with a maximum at 13.3 years posttransplantation. A total of 105 patients (17.5%) presented with graft failure during their follow-up and 50 patients (8.3%) died (the date of deaths was considered as the date of right censoring).

Pretransplant anti-AT_1RAb >10 U is an independent risk factor for graft failure

The threshold value of anti-AT_1R Ab estimated by maximizing the differences in graft survival was determined at 10 U (see Patients and Methods section). Among the 599 patients, 47.2% (n = 283) had anti-AT_1R-Abs levels above 10 U before transplantation. Patient demographic characteristics according to a pretransplant anti-AT_1R antibody level above or below the threshold of 10 U are presented in Table 1.

As shown in Figure 1, a similar proportion of graft failure was observed among the two populations studied during the first 3 years posttransplantation. However, patients with pretransplant anti-AT_1R-Abs above 10 U had a higher risk of late graft loss. Since the risk of graft failure was not proportional over time, an extended Cox model was constructed with two different hazard ratios: before and after 3 years. The multivariate analysis was performed on 575 patients (24 observations were not taken into account

Table 1: Demographic parameters of the study population according to the threshold of anti-AT_1R Abs

	AT_1R Abs ≤10 U (n = 316)	AT_1R Abs > 10 U (n = 283)	p-Value
Male recipient	64.2%	57.2%	0.0797
Recipient age (years, mean ± SD)	49.6 ± 14.1	48.1 ± 14.4	0.1999
First graft	86.4%	87.6%	0.6525
Induction therapy			
ATG	34.3%	33.7%	0.8777
Simulect	52.1%	46.8%	0.1998
DGF > 6 days	36.3%	38.8%	0.5406
HLA incompatibilities (mean ± SD)	3.3 ± 1.4	3.4 ± 1.5	0.5265
Historical peak PRA class II > 0%	33.7%	31.3%	0.5438
Historical peak PRA class I > 0%	21%	27.4%	0.0657
Deceased donor	95.3%	92.9%	0.2268
Male donor	64.6%	61.8%	0.4906
Donor age (years, mean ± SD)	47.8 ± 16.6	45.2 ± 16.1	0.0528

due to missing data). The results are presented in Table 2. During the first 3 years of transplantation, the risk of graft failure seemed similar regardless of an anti-AT_1R antibody level below or above 10 U (HR = 1.02, 95% CI = [0.50, 2.08], p = 0.9471). However, starting from 3 years post-transplantation onward, patients with anti-AT_1R antibody levels >10 U had a 2.59-fold greater risk of graft failure compared to patients with a lower level (95% CI = [1.50, 4.45], p = 0.0005). This was found to be independent of pretransplant PRA anti-class I >0% and II (HR = 1.52, p = 0.1173 and HR = 1.29, p = 0.3275, respectively), DGF (HR = 1.58, p = 0.0282) and donor age >55 years (HR = 1.66, p = 0.0202).

No statistical difference was observed for patient survival alone (returns to dialysis censored; p = 0.3523) or for patient and graft survival (p = 0.2251) according to the 10 U threshold of anti-AT_1R-Abs.

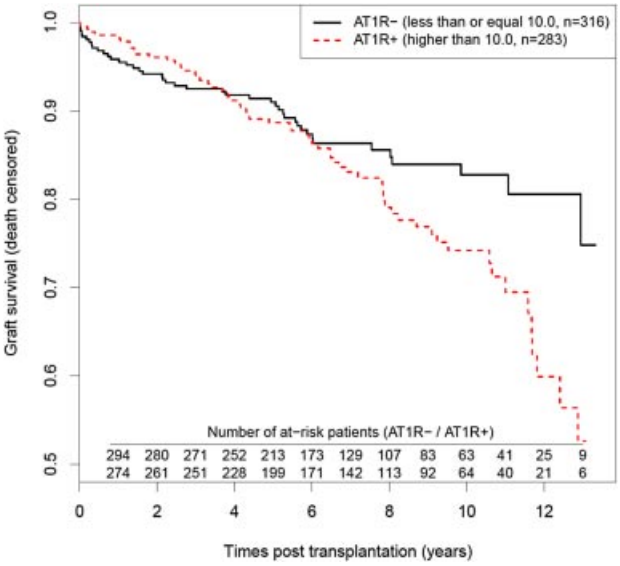

Figure 1: Kaplan–Meier analysis of graft survival according to the anti-AT1R-Abs level (>10 U__, ≤10 U) before transplantation. Patients with anti-AT_1R-Abs >10 U have an increased risk of graft failure during their follow-up.

Pretransplant anti-AT_1R antibody level >10 U and historical pretransplant anti-HLA immunization are independent risk factors for acute rejection

Among the 599 patients, 63 were treated for an AR episode (10.5%); 44 occurred within the first 4 months of follow-up. We observed that patients with a pretransplant level of anti-AT_1R antibody >10 U developed AR, mainly within the first 4 months posttransplantation, as illustrated in Figure 2. Here again, we took into account the nonproportionality of hazards in an extended Cox model, namely before and after 4 months. The results of the multivariate analysis (Table 3) showed that an anti-AT_1R antibody level >10 U before transplantation was a risk factor for AR within the first 4 months posttransplantation (HR = 1.91, 95% CI = [1.03, 3.54], p = 0.0393. Interestingly, the correlation of the pretransplantation anti-AT_1R antibody level >10 U with the occurrence of AR was found to be independent of the historical pretransplant anti-HLA immunization against class I or class II (>0%; HR = 1.63, 95% CI = [0.83, 3.21], p = 0.1519, HR = 0.93, 95% CI = [0.48, 1.78], p = 0.8261, respectively; Figure 2).

Histological features of AR according to pretransplant anti-AT_1R antibody levels

All AR episodes within the first 4 months of follow-up (n = 44) were retrospectively studied in greater detail to describe subsequent rejection phenotypes according to the pretransplant level of anti-AT_1R and DSA-HLA-Abs. Among the 44 AR episodes, 37 biopsies underwent a blind histological re-evaluation according to the 2007 Banff classification (7) in which 62.1% (n = 23) were classified as acute cellular mediated rejection (ACR: 4 borderline, 3 grade I A, 8 grade II A and 8 grade IB. The remaining 31.8% AR episodes (n = 14) were histologically classified as acute AMR or mixed AMR with ACR (n = 7) with both microvascular inflammation, diffuse positive, peritubular capillary C4d deposition (except for one patient for whom

Table 2: Multivariate analysis (extended Cox model) of the risk factors for graft survival shows that a high level of AT_1R-Abs before the transplantation (>10 U) is an independent risk factor for graft loss beyond 3 years of follow-up

	HR	95% CI	p-Value
Anti-AT_1R Abs > 10 U (0–3 years)	1.02	[0.50, 2.08]	0.9471
Anti-AT_1R Abs > 10 U [≥3 years]	2.59	[1.50, 4.45]	0.0005
HLA-A-B-DR inc. >5	1.14	[0.49, 2.65]	0.7658
Historical peak of anti-class II PRA >0%	1.29	[0.77, 2.16]	0.3275
Historical peak of anti-class I PRA >0%	1.52	[0.90, 2.58]	0.1173
Previous transplantation >1	1.13	[0.59, 2.15]	0.7094
Delayed graft function	1.58	[1.05, 2.37]	0.0282
Donor age >55 year	1.66	[1.08, 2.56]	0.0202

this was undetermined for technical reasons). Details of histology are provided in Table 4.

Among the 37 patients with biopsy-proven AR, 22 patients had pretransplant anti-AT_1R antibody level >10 U. Among all AMR (n = 14), 71.4% (n = 10) had anti-AT_1R antibody level >10 U.

We then studied sera obtained at the time of rejection and just before the transplantation, for both HLA-DSA (Luminex SAB) and anti-AT_1R Ab. Anti-HLA DSA before transplantation were identified in 46% of patients (n = 17) among the 36 out of 37 patients in whom a serum was still available (5 anti-cl1, 8 anti-cl2 and 4 both). In patients with an anti-AT_1R antibody level >10 U, 63% (14/22) displayed DSA before the transplantation (58% of ACR and 70% of AMR) compared with 21% (3/14) in patients with an anti-AT_1R-Abs level ≤10 U (one missing serum; 20% of ACR and 25% of AMR). At rejection time, 14 sera were not available (ND). Among the available sera, 14 displayed anti-HLA DSA in whom 9 were *de novo* (4 out of 12 available in anti-AT_1R antibody level ≤10 U group and 5 out of 11 available in anti-AT_1R antibody level >10 U group).

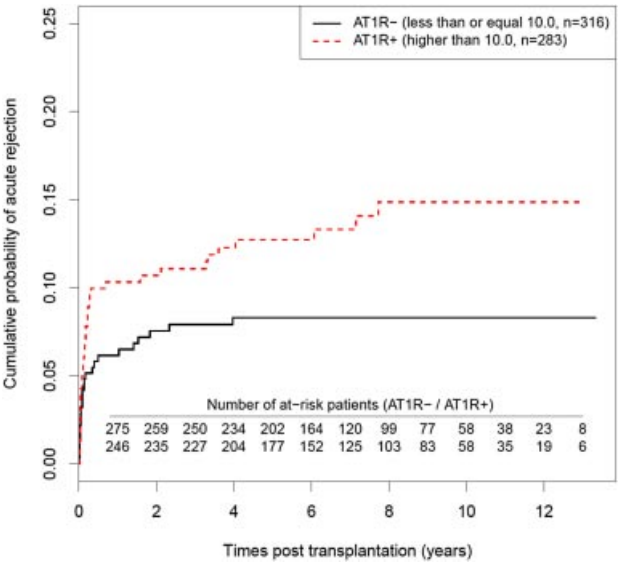

Figure 2: Kaplan–Meier analysis of freedom from acute rejection episodes according to anti-AT1R-Abs levels (>10 U__, ≤10 U) before transplantation. Patients with anti-AT_1R-Abs >10 U have an increased risk of developing an acute rejection episode during their follow-up.

Among the 22 patients with an anti-AT_1R-Abs level >10 before transplantation, only 4 had a level of anti-AT_1R-Abs above 10 U at the time of rejection (2 with ACR and 2 with AMR).

In patients with a pretransplantation level of anti-AT1R-Abs lower than 10, only 2 had an anti-AT_1R-Ab level above 10 (2 with ACR). Except in two patients with AMR, the anti-AT_1R antibody concentrations at the time of rejection were lower (median 8.5 U) than the pretransplant levels (median 10.6 U, p = 0.0010, paired Wilcoxon test).

Histological patterns of AR episodes according to pretransplant status of anti-AT_1R Ab, DSA specificity and mean fluorescence intensity (MFI) are provided in Figure 3 and Table 4.

Blood pressure and use of antihypertensive medications

The initial description anti-AT_1R Ab was reported in preeclampsia and in association with severe hypertension during AR episodes (4,10). We therefore analyzed the frequency of hypertension and the use of antihypertensive therapy before and during the AR episodes. Among the patients with AR during the first 4 months of follow-up, 80% had a past history of treated hypertension. However, no difference in frequency of hypertension was observed between patients with anti-AT_1R Ab >10 U (80.0%) or ≤10 U (83.3%; p = 0.8151). Neither was there a significant difference in the distribution of angiotensin converting enzyme inhibitors and AT_1 receptor blockers, prescribed before and after transplantation, (57.1% when anti-AT_1R Abs >10 U vs. 63.6% when anti-AT_1R Abs ≤10 U, p = 0.7224 and 5% vs. 25% [p = 0.978], respectively) or in the number of antihypertensive drugs prescribed. None of the patients developed malignant hypertension. The median systolic and diastolic blood pressure was 16/8 cmHg in

Table 3: Multivariate analysis (extended Cox model) of the risk factors for AR shows that a high level of anti-AT_1R-Abs before transplantation (>10 U) is an independent risk factor for AR before 4 months of follow-up

	HR	95% CI	p-Value
Anti-AT_1R Abs > 10 U (0–4 months)	1.91	[1.03, 3.54]	0.0393
Anti-AT_1R Abs > 10 U (≥4 months)	1.35	[0.54, 3.45]	0.5178
HLA-A-B-DR inc. >5	2.78	[1.24, 6.21]	0.0127
Historical peak of anti-class II PRA >0%	0.93	[0.48, 1.78]	0.8261
Historical peak of anti-class I PRA >0%	1.63	[0.83, 3.21]	0.1519
Previous transplantation >1	0.92	[0.39, 2.16]	0.8458

patients with high pretransplant anti-AT_1R Ab, compared to 15/9 in patients with low AT_1R Ab (p = NS).

Altogether, our data suggest that a high level of pretransplant anti-AT_1R Ab is a risk factor for developing an AR episode. Patients with anti-AT_1R Ab alone or in conjunction with preformed DSA were more likely to have AMR. In addition, patients with anti-AT_1R antibody did not exhibit abnormal hypertension within the first week following surgery.

Discussion

Standardized solid-phase assay made pretransplant screening for anti-AT_1R Ab feasible for investigations of its clinical relevance. From the multivariate analysis, we found that patients who displayed an AT_1R level above 10 U developed more AR episodes, independently of traditional immunological risk parameters such as HLA mismatch or panel reactive Ab. Furthermore, we found that such presensitization constituted an independent risk for graft failure, independently from the other standard clinical determinants, such as donor age, panel reactive Ab or DGF. Our data suggest that predicting a given patient's clinical outcome on the basis of HLA presensitization alone is insufficient, in particular in patients without an apparent immunological risk before transplantation. The detection of AT_1R-Ab raises the prospect of improving routine immunological risk stratification.

Presensitization against non-HLA major histocompatibility-complex (MHC) class I–related chain A (MICA) antigens has been associated with worsened graft survival and has been hypothetically considered as important for the rejection process. However, such studies lacked histological data on AR (11). In more recent studies, MICA Ab did not correlate with rejection episodes (12). A potential reason for this discrepancy is that large serum banks containing serum collected over decades fail to provide the individual patient-level data necessary for the reconstruction of outcome surrogates. Furthermore, differences in immunosuppressive regimens in the first, compared to later MICA studies, may also have contributed. Our prospective DIVAT data bank collection enabled us to determine how the anti-AT_1R antibody levels functioned as a risk factor for graft loss and the occurrence of AR in patients not considered as sensitized according to conventional criteria (1,13). In our

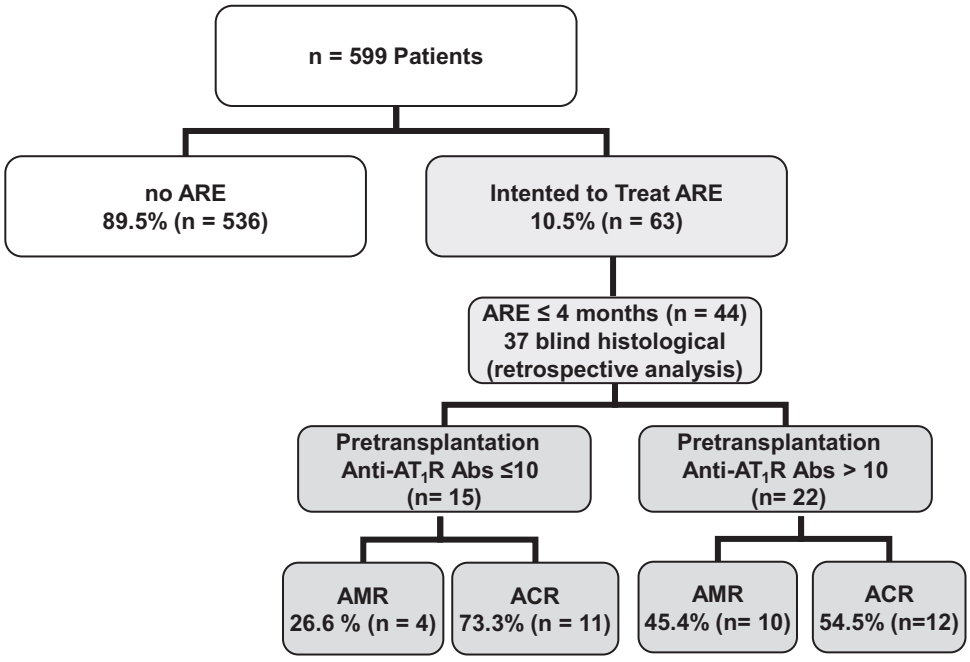

Figure 3: Histological analysis of acute rejection episodes repartition according to the pretransplantation status of anti-AT1R-Abs.

Table 4: Histological acute rejection classification and details of anti-HLA DSA pre- and posttransplantation according to the anti-AT_1R-Ab level before transplantation

	AR Banff grading	Pregraft anti-AT_1 R-Abs level	Graft rank	CMX at transplant. time	DSA (SBA) at transplant. time CL I (MFI)	DSA (SBA) at transplant. time Cl II (MFI)	DSA (SBA) at AR time CL I (MFI)	DSA (SBA) at AR time CL II (MFI)	AR anti-AT_1 R-Abs level
BED	IA	5.3	1	N		0	ND	ND	ND
CHE	IIA	2.5	2	N	0	DQ2 (2557)	0	DQ2 (5202)	ND
DER	IB	8.0	1	N	0	0	0	DQ5 (1770)	11.3
LE PA	IA	3.3	1	N	0	0	B8 (1881)	0	2.7
MAR	IB	6.6	1	N	0	0	ND	ND	ND
SAN	IIA	6.5	1	N	0	0	0	0	<2.50
SER	IB	8.0	1	N	0	0	0	DQ5 (1905)	18.9
SLA	IIA	8.5	1	N	0	0	0	DQ4 (2044)	<2.50
BOU	IA	5.0	1	N	B51 (1179)	0	0	0	13.5
PIN	Borderline	2.5	1	N	0	0	0	0	<2.50
ROU	Borderline	3.3	1	N	0	0	0	0	ND
DUM	AMR	9.2	2	N	ND	ND	ND	ND	8.9
GUI	AMR and cellular IIA	4.7	1	N	0	0	0	0	<2.5
HER	AMR and Borderline	6.4	1	N	0	0	0	DQ5 (4750), DR14 (2715)	2.8
TOU	AMR and cellular IIA	5.4	1	N	B27 (14 798)	DR7 (4224)	B27 (1124)	DR7 (12 300)	<2.50
ALI	IB	16.5	1	N	0	0	ND	ND	ND
BAR	IB	15.9	1	N	0	DR18 (1576)	ND	ND	ND
BOU	IIA	16.2	1	N	0	DQ 7 (2534)	ND	ND	ND
CLE	IB	14.8	1	N	B7 (686)	0	ND	ND	ND
COS	IIA	11.2	1	B (CDC)	0	0	ND	ND	11.9
DES	IB	11.5	1	N	A 32 (1338)	0	0	0	ND
LOR	IIA	32.7	2	N	0	DR 4 (3988), DQ2 (2276)	B44 (3362)	DQ2 (3258)	15.8
MAR	IB	21.1	1	N	0	0	ND	ND	ND
MAY	IIA	12.2	1	N	A24 (1148)	0	ND	ND	3.2
OUA	IIA	13.7	1	N	0	0	0	0	6.6
CHA	Borderline	19.8	1	N	0	DQ7 (1014)	ND	ND	< 2.5
FOU	Borderline	11	1	N	0	0	ND	ND	3.5
BON	AMR	11.5	1	N	A24 (10 930)	DR14 (3664), DR15 (5139), DR51 (13 870)	A24 (19 995), B44 (4289)	DQ5 (2509), DR51 (2095)	7.8
COL	AMR	27.8	1	N	0	0	ND	ND	ND
DI M	AMR	30.6	1	N	B57 (16 008)	DQ 5 (2972)	ND	ND	9.8
DUR	AMR and cellular IIB	10.3	1	N	0	0	0	0	3.3
ENN	AMR and cellular IIA	22.6	1	N	0	0	0	DR4 (16 677), DQ3 (7980)	8.2

(*Continued*)

Table 4: Continued

	AR Banff grading	Pregraft anti-AT_1 R-Abs level	Graft rank	CMX at transplant. time	DSA (SBA) at transplant. time CL I (MFI)	DSA (SBA) at transplant. time Cl II (MFI)	DSA (SBA) at AR time CL I (MFI)	DSA (SBA) at AR time CL II (MFI)	AR anti-AT_1 R-Abs level
GIR	AMR	10.6	1	N	A2 (8078), B7 (15 751)	DQ6 (1094)	A2 (21 700), A32 (12 328), B35 (16 176), B7 (18 728)	DQ6 (8023)	7.1
ORS	AMR and cellular IA	40	1	N	0	DR18 (1469)	A1 (4416), A2 (8671), B8 (14 063), B44 (5896)	DR7 (2502), DQ2 (14 227), DR53 (7524)	12.4
PEA	AMR	20.4	1	N	A68 (17 598), B50 (3150)	0	A68 (15 880), B50 (17 624)	DQ5 (10 291)	8.6
ROU	AMR	24.8	2	N	0	DR13 (1432)	A23 (4552), B41 (2743)	DR13, DR9, DQ3 (MFI ND)	9.9
VEN	AMR and Borderline	40	2	N	0	DR51 (4403), DQ6 (14 010), DR12 (4784)	0	DR51 (2125)	34.7

Group 1: AT1R-Abs $\leq$10 U; Group 2: $>$10 U. The mean anti-AT_1R-Ab level is 19 U (SD 12.3, median 16 U) in patients with AMR and 12.1 U (SD 9.4, median 11U) in patients with ACR.

study, the overall cumulative probability of AR was 10.5%, which suggests that the cohort enjoyed a relatively low immunological risk. Indeed, the overall incidence of rejection was reported to be below 15% in many recent treatment studies focusing on interleukin (IL) 2 receptor antibody induction and triple drug combinations based on a calcineurin inhibitor and mycophenolate mofetil (14), We are cautious with our interpretations as 1/3 of AR were not histologically reanalyzed and 1/2 of sera at rejection time were unavailable. We observed that one-third of patients with AR during the first 4 months posttransplantation had histologically proven AMR. These findings fit well with the recent observations that AR episodes occurring in the modern era are more severe, thus contributing to the lack of improvement in graft survival (15). AMR accounted for most of these severe rejections. We further found that approximately 70% of patients with AMR had a level of anti-AT_1R Ab $>$10 U before transplantation compared to 40% of patients with ACR. However, this trend did not reach statistical significance. Nonetheless importantly, one-third of patients with a level of anti-AT_1R Ab $>$10 U displayed no HLA DSA before the transplantation or significant part of others had rather low MFIs. Thus, pretransplant detection of anti-AT_1R Ab could be a complementary risk factor for the identification of patients with high immunological risk, who would be otherwise considered as low-risk patients and overlooked based solely on the absence of HLA-sensitization using Luminex criteria.

Except for two patients with AMR, the anti-AT_1R antibody concentrations at the time of rejection were lower than the pretransplant levels. Similar phenomena associated with intra-graft antibody adsorption are well established for HLA Ab and could also apply for anti-AT_1R Ab (16). We realize that our study does not provide mechanistic answers to the question of how high pretransplant anti-AT_1R Ab may trigger AR. However, anti-AT_1R Ab may bind to the allograft immediately following transplantation and initiate pathological pro-inflammatory actions on vascular cells, which are well-defined (17). Endothelial cell activation may act as a danger signal and could be a prerequisite for the induction of AMR (18). Interestingly, the molecular weight of endothelial antigen induced by incubation of endothelial cells with pretransplant sera corresponds to the molecular weight of unglycosylated AT_1R (18). There is also increasing evidence for intersections between stimulation of AT_1R and initiation of both innate and adaptive immune responses in a blood-pressure-independent manner (19,20). Similar information has been published on the involvement and predictive capacity of these Ab in systemic autoimmune disease associated with microvascular and macrovascular disease in different vascular beds (21).

Regardless of the specific mechanism and despite a relatively low incidence of AR episodes reflecting modern immunosuppression, our results suggest the importance of increased pretransplant anti-AT_1R Ab as a novel and independent risk factor for AR. This was shown by a

hazard ratio similar to that associated with less-than-ideal HLA matching or the presence of panel reactive Ab. Careful combined pre- and posttransplant monitoring of both HLA and non-HLA humoral responses (22) could help in the detection of recipients at risk and in the initiation of adapted therapy.

Although the initially described AT_1R-Ab-related rejections were accompanied by malignant hypertension, malignant hypertension at the time of rejection was not reported. Nevertheless, our study was not based on patients selected according to specific clinical features. Also, angiotensin II converting enzyme inhibitors and AT_1R blockers were frequently prescribed for our patients and equally distributed among patients with high and low levels of anti-AT_1R Ab.

Our main observation was the correlation between the level of anti-AT_1R antibody before transplantation and the later occurrence of graft loss from 3 years posttransplantation onward. This effect could be directly explained by the higher incidence of AR in patients with a higher level of anti-AT_1R Ab. As common in this type of cohort-based study, there are several limitations to our results. First, the correlation between the pretransplant anti-AT_1R Ab and the long-term graft survival could be the consequence of an increased incidence of AR or an independent risk factor for graft loss. We are not able to prove this hypothesis by the classical approach using a Cox model analysis. The logical approach should be a model with competitive risk between graft failure, death and AR. This is the most frequently used relevant method to test the hypothesis that an AT_1R antibody level above 10 U is initially a risk factor for ARE and then consequently has a deleterious impact on graft failure. In cardiac transplantation, patients with similar values of 10 U or more were more likely to develop AR as well as chronic alloimmune complications such as microvasculopathy (23). However, the low incidence of AR in our cohort (10%) does not allow for a robust study to be performed using this competitive risk method. Second, we decided not to consider patient death as graft failure, since there is no reason to believe that the deaths were related to the pretransplant level of AT_1R. We estimated patient survival according to anti-AT_1R Ab level and observed no difference in the mortality. Third, the cut-off of 10 U for anti-AT_1R antibody concentration was not arbitrarily defined, but instead statistically determined according to the time to graft failure (see Patients and Methods section). Finally, it is unusual that the degree of HLA-A/B/DR mismatch and re-transplantation were not significantly associated with graft failure. Indeed, second transplant recipients are usually considered as a higher risk group for graft failure, mainly due to increased levels of preformed anti-HLA Ab (24). Nevertheless, the putative poor prognosis of second transplant recipients remains a matter of debate, as shown in the study by Coupel et al. (25) where the difference in long-term graft survival was not statistically significant between re-transplantation and first transplantation when an HLA-DR mismatch was avoided. Further studies need to be performed to validate this data-driven cut-off. Although numerous explanatory variables were analyzed, we cannot exclude the possibility that some confounding factors, which were not taken into account in the present study, could have exerted influence.

Nevertheless, our methodological approach manages the issue of nonproportionality by using an extended Cox model (9) and enables different hazard ratios to be modeled according to time posttransplantation. Of note, the time points of 3 years for the graft survival and 4 months for the AR episodes did not correspond to a sudden change in hazard ratio. Thus, we considered this method to be more relevant and the results more valid than those that would have been obtained assuming a constant hazard ratio regardless of time posttransplantation. Given the complexity of pre- and posttransplant antibody responses, mechanisms of HLA and non-HLA pathway intersections should be the focus of future studies.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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