

Peritubular capillaries C4d deposits in renal allograft biopsies and anti HLA I/II alloantibodies screening – Incidence and clinical importance

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Received for publication: 21/07/2007

Accepted in revised form: 28/11/2007

ABSTRACT

Aim: To characterise clinically the patients with C₄d in peritubular capillaries deposits (C₄dPTCD) and/or circulating anti-HLA class I/II alloantibodies. To determine the correlation between positive C₄dPTCD and circulating anti-HLA class I/II alloantibodies during episodes of graft dysfunction

Subjects and Methods: C₄d staining was performed in biopsies with available frozen tissue obtained between January 2004 and December 2006. The study was prospective from March 2005, when a serum sample was obtained at the time of biopsy to detect circulating anti-HLA class I/II alloantibodies.

Results: We studied 109 biopsies in 86 cadaver renal transplant patients. Sixteen of these (14.7%) presented diffuse positive C₄dPTCD. There was a 13.5% rate of +C₄dPTCD incidence within the first six months of transplantation and 16% after six months ($p > 0.05$). Half of the +C₄dPTCD in the first six months was associated with acute humoral rejection. After six months, the majority of +C₄dPTCD ($n=7/8$) was present in biopsies with evidence of interstitial fibrosis/tubular atrophy and/or transplant glomerulopathy. The C₄dPTCD was more frequent in patients with positive anti-HCV antibodies ($p < 0.0001$), a previous renal transplant ($p = 0.007$), and with a panel reactivity antibody (PRA) $\geq 50\%$ ($p = 0.0098$). The anti-HCV+ patients had longer time

on dialysis ($p = 0.0019$) and higher PRA ($p = 0.005$). Circulating anti-HLA I/II alloantibodies were screened in 46 serum samples. They were positive in 10.9% of samples, all obtained after six months post transplant. Circulating alloantibodies were absent in 92.5% of the C₄d negative biopsies.

Conclusion: We found an association between the presence of C₄dPTCD and 2nd transplant recipients, higher PRA and the presence of anti-HCV antibodies. The presence of HCV antibodies is not a risk factor for C₄dPTCD *per se*, but appears to reflect longer time on dialysis and presensitisation. In renal dysfunction a negative alloantibody screening is associated with a reduced risk of C₄dPTCD ($< 10\%$).

Key-Words:

Alloantibodies; C₄d; hepatitis C; humoral rejection; kidney transplant.

INTRODUCTION

Allograft rejection has been considered predominantly a T cell centred process over the last five decades. However, in the last few years the importance of antibody mediated mechanism has been rediscovered, partly due to the improved ability to detect antibody activity with C₄d staining, and the

advent of more sensitive methods of detecting circulating antibodies.

At present, four types of antibody-mediated graft injury have been defined: hyperacute rejection, acute humoral rejection, chronic humoral rejection and accommodation¹. In general, antibody-mediated rejection has a worse prognosis than and requires a different form of therapy from the usual T cell-mediated acute rejection.

Within the past decade, reports on the usefulness of PTC C4d staining have emerged. C4d is a fragment of C4b, an activation product of the classic complement pathway. C4b and C4d form a covalent bond with nearby proteins after activation by antibody and C1 complex. C4b/C4d remains bound in the tissue for several days after Ig and C1 complex have been released. No functional role for C4d *per se* has been reported. The use of immunofluorescence with monoclonal antibody in frozen tissue section is the better method for its detection².

The pioneering studies of Feucht *et al.*³ described for the first time the value of capillary deposition of C4d in graft prognosis. Magil *et al.*⁴ were also among those to report that C4d is an important predictor of graft survival, having predictive value independent of several other morphological and clinical factors. They found an association between being positive for C4d, female gender, panel reactivity antibodies (>30%), and resistance to standard anti-rejection therapy.

Colvin *et al.*⁵ were the first to demonstrate a clear correlation between PTC C4d staining with concurrent circulating anti-donor-specific antibody (DSA) and certain pathological features defining the main characteristics of acute humoral rejection: neutrophils in peritubular and glomerular capillaries and vascular and arterial fibrinoid necrosis.

C4d deposition is strongly associated with circulating antibody to donor HLA class I or class II antigens and is currently the best single marker of complement-fixing circulating antibodies to the endothelium¹.

We now know that alloantibodies preferentially react with the endothelium of peritubular and glomerular capillaries, in contrast to T cells which characteristically infiltrate tubules and arterial endothelium.

Overall, the newer and more sensitive techniques for serum antibody screening allow for a clearer understanding of the relationship between antibodies and acute or chronic allograft rejection⁶.

■ SUBJECTS AND METHODS

The study was retrospective between January 2004 and March 2005. From then until December 2006 the study was prospective, with routine assessment of C4d presence in all biopsies performed for allograft dysfunction, and simultaneous screening for circulating anti-HLA I/II antibodies.

■ Patients and biopsies

744 kidney transplantations had been performed in Hospital Curry Cabral, Lisbon by December 2006; 206 biopsies were performed for allograft dysfunction or delayed graft function between January 2004 and December 2006. Allograft dysfunction was defined as unexplained and persistent (more than 3 days) increase of serum creatinine level above 30% baseline. Delayed graft function was defined as failure to achieve a creatinine level under 2 mg/dl at ten days post transplant, or dialysis requirement within the first ten days post transplant.

We evaluated all the biopsies with available frozen tissue in this period (n=109).

The following clinical parameters were registered at biopsy time: 1) patient age; 2) patient gender; 3) race; 4) underlying renal disease; 5) duration of end stage renal failure; 6) duration of post transplantation follow up; 7) previous kidney transplantation; 8) HCV status; 9) panel reactivity antibodies; 10) serum creatinine levels; 11) immunosuppressive treatment.

■ Renal allograft pathology and C4d staining

A needle biopsy core was obtained from each renal allograft for morphological studies. The biopsy core was divided in two parts, one for formalin fixation and one for quick freezing. Haematoxylin/eosin, periodic acid Schiff, Masson's trichrome and silver stains were routinely used for formalin-fixed tissue. Fresh-frozen

tissue was analysed by immunofluorescence (IF) microscopy using antibodies against immunoglobulin (Ig) IgG, IgA, IgM, complement component (C) C3c, C4c, and C1q.

C4d staining was performed on frozen slides, using an indirect 2-step IF technique with a primary affinity-purified monoclonal antibody (mouse anti-human; dilution 1:30; 30-min. incubation at room temperature; Quidel[®], CA 92121, USA) and a fluorescein isothiocyanate-labeled affinity-purified secondary horse anti-mouse IgG antibody (1:50; 30-min. incubation at room temperature; Vector[®], FI 2080, USA).

Diffuse positive C4d staining was defined as bright linear staining along capillary basement membranes, involving over half of sampled capillaries, according to the international consensus criteria based on discussions at the Banff Conference on Allograft Pathology in 2005-2007.

■ Circulating anti-HLA alloantibodies and donor specific antibodies

A serum sample has been obtained at the time of biopsy to detect the presence of circulating anti-HLA class I/II alloantibodies by flow cytometric assay (FlowPRA[®] screening test) since March 2005. In suspected cases of acute humoral rejection biopsies, the circulating donor specific and anti-donor HLA antibodies are determined by flow cytometry.

■ Statistical analysis

Analysis of data was carried out using the MedCalc Software 9.0.1.1. Descriptive data are expressed as the mean ± standard deviation. Fisher's test and independent sample T test are used for comparison between groups. P<0.05 was taken as the statistically significant level.

■ RESULTS

We studied 109 biopsies in 86 cadaver renal transplant patients (61 M, 25 F), with an average age of 47.4±12.9 years old at the time of study (Table I). Overall 16 biopsies (14.7%; n=16/109) presented with diffuse positive C4dPTCD. The incidence of +C4dPTCD (Table I) was 13.5% (n=8/59) in the group within the first six months of transplantation and 16% (n= 8 /50) after six months (p>0.05).

The histological diagnoses are presented in Table II. Half of the +C4d biopsies within the first six months had a histological diagnosis of acute humoral rejection (n= 4/8). Three out of four patients with PTC C4d deposits had a final diagnosis of acute humoral rejection (AHR) confirmed by histology, C4dPTCD and circulating DSA. They were treated with plasmapheresis, intravenous polyspecific immunoglobulin and anti-CD20. One patient lost his graft; two currently maintain renal function with a serum creatinine <2 mg/dl.

Table I

Patient characteristics and PTC C4d status

n=109	Total	C4d+	C4d-	p value
Total	109	16	93	
Gender	81 M/28 F	15 M /1 F	66 M /27 F	0.065
Age (years)	47.4± 12.9	45.9±11.5	47.7±13.1	0.6095
Race	93 C/16 A	10 C/6 A	83 C/10 A	0.013
Duration of dialysis pre-transplantation (months)	59.0±50.0	77.3±53.0	55.8±49.9	0.1142
Follow-up post-transplantation (months)	33.4±46.1	39.9±46.6	32.2±46.0	0.5379
HCV antibody positive	14/109	8	6	0.0001
Previous transplantation	19/109	7	12	0.0070
Serum Creatinine (mg/dl)	3.5±1.8	3.5±1.7	3.4±2.2	0.7621
<6 m Follow up post-transplant	59/109	8	51	
≥6 m Follow up post-transplant	50/109	8	42	0.7896
PRA<50%	98/109	11	87	
PRA≥50%	11/109	5	6	0.0098

(M: male; F: female; C: Caucasian; A: African; PRA: panel reactivity antibodies; HCV: Hepatitis C virus)

Table II

Histological diagnosis of PTC C4d positive group

<6 Months of transplantation (4 patients/8 biopsies)	Acute humoral rejection	4/8
	Acute tubular necrosis	1/8
	Thrombotic microangiopathy	1/8
	Calcineurin inhibitor toxicity	1/8
	Normal kidney	1/8
≥6 Months of transplantation (6 patients/8 biopsies)	Interstitial fibrosis/Tubular atrophy and/or transplant glomerulopathy	7/8
	Acute cellular rejection	1/8

patients, two patients subsequently died and four lost their graft.

During the three years of the study we found eight biopsies with a histological diagnosis of transplant glomerulopathy and 25% had deposits of C4d in PTC (n=2/8). Within the TG group we detected one sample (16.6%) positive for circulating alloantibodies (1/6 patients screened).

Table III

Patient characteristics and HCV status

n = 109	HCV positive		P value
	n = 14	n = 95	
Creatinine (mg/dl)	3.6±2.1	3.5±1.7	0.75
Duration of dialysis pre-transplant (month)	97.1±66.8	53.4±44.8	0.0019
Duration of follow-up post-transplantation (month)	48.4±62.5	31.1±43.0	0.1902
First transplantation n = 90	9	81	0.067
Previous transplantation n = 19	5	14	
PRA<50% n = 98	9	89	0.005
PRA≥50% n = 11	5	6	

Biopsies with C4d deposits (Table I) were obtained on average 39.9±46.6 months after transplantation, and presented a median serum creatinine of 3.5±1.7 mg/dl. There was no significant difference in creatinine levels between C4d PTC positive and negative groups. The presence of C4dPTCD was more frequent in biopsies from HCV antibody positive patients (n=8/14; Fisher's test p<0.0001), with a previous renal transplant (n=7/19; p=0.007) and with a PRA level above 50% (n=5/11; p=0.0098). The HCV antibody positive patients (Table III) have longer time on dialysis (p=0.0019) and higher PRA (p=0.005) than the HCV negative group. The small size of the population precludes a multivariate analysis.

After six months, the majority of +C4d biopsies (n=7/8) had evidence of interstitial fibrosis and tubular atrophy (IF/TA) and/or transplant glomerulopathy (TG). In this last group of eight biopsies in six

Circulating anti-HLA I/II alloantibodies (Table IV) were studied in serum samples obtained from 46 biopsied patients (n=20 <6 month; n=26 ≥6 month). Anti-HLA alloantibody were detected in 10.9% of serum samples (n=5; >5% of reactivity), collected six months after transplantation. There were no circulating anti-HLA alloantibodies in 92.25% of the C4d negative (n=37/40).

Table IV

Patient characteristics and anti-HLA class I/II alloantibodies

n=46	Total	Alloantibody positive	Alloantibody negative	P value
Total	46	5	41	
Gender	31 M/15 F	2 M/ 3 F	29 M/12 F	0.311
Age (years)	45.5±14.1	36.6±14.3	46.6±13.8	0.1383
Race	41 C/5 A	5 C / 0 A	36 C/ 5 A	1.000
Duration of dialysis pre-transplantation (months)	57.5±49.3	20.2±15.4	62.1±50.1	0.0722
Follow-up post- transplantation (month)	41.7±47.7	71.4±15.2	38.1±49.2	0.1432
HCV antibody +	5/46	0/5	5/41	1.0000
Previous transplantation	8/46	5/5	3/41	0.0034
Serum Creatinine (mg/dl)	3.3±1.6	2.7±1.7	3.4±1.6	0.3998
<6 month follow- up post-transplantation	20	0	20	0.0592
≥6 month follow up post transplantation	26	5	21	
Positive PTC C4d	6	2	4	0.1199
Negative PTC C4d	40	3	37	
PRA<50%	43	4	39	0.2978
PRA≥50%	3	1	2	

■ DISCUSSION

■ C4dPTCD and acute humoral rejection diagnosis

Since 2001, according to the 6th Banff Conference⁷, being positive for C4dPTC is one of the three cardinal features for AHR.

Our study confirmed that C4d staining is more specific than the morphologic alterations usually present in AHR. Staining for C4d is essential and must be routinely made to allow early diagnosis and timely treatment of AHR. The indirect two-step IF method with the Quidel[®] monoclonal antibody used in our study is the method recommended by the Banff criteria and in the literature².

■ C4d/alloantibodies and transplant glomerulopathy (TG)

In our study, the majority of biopsies from patients with more than six months of transplantation and positive C4dPTCD (n=7/8) had evidence of interstitial fibrosis/tubular atrophy and/or TG. This group has a worse outcome (two patients subsequently died and four lost their graft).

We found an association of 25% between TG and + C4dPTCD (2/8 biopsies), the same as previously described in the literature^{8,9}.

Originally classified as a variant of chronic allograft nephropathy of unknown aetiology, TG is now recognised with increased frequency in patients with a prior history of humoral rejection, and is associated with deposit of C4dPTC and circulating alloantibody, suggesting that TG may be one form of antibody-mediated injury⁸. In addition, capillary C4d deposition preceded the development of chronic TG in most patients in whom serial biopsies were available, emphasising the role of local complement activation in the development of chronic TG in allograft¹⁰.

The association of TG with severe peritubular capillary basement membrane multilayering (PTCBMML) had also been reported¹¹. At present, the emerging picture is of an association between the presence of alloantibody, capillary C4d deposition, TG and PTCBMML. This combination of alloantibody (A), basement membrane multilamination (B), C4d (C) and duplication of the

glomerular basal membrane (D) has been termed the “ABCD tetrad” by Halloran and colleagues⁹.

It is known that TG is associated with poor kidney allograft survival and prior humoral rejection. The risk of TG was demonstrated to be associated with younger recipients, re-transplants, high panel reactive antibodies pre-transplant, hepatitis C and acute rejection, especially humoral rejection¹².

This issue could be of particular importance, as at the present time there is no established treatment for TG, and the presence of C4d deposits or anti-HLA alloantibodies may indicate a need for alternative treatment strategies using intravenous Ig or anti-CD 20 (as shown by several recent running trials – indicated at www.clinicaltrials.gov).

■ Circulating anti-HLA class I/II alloantibodies

Development of new anti-HLA antibodies after kidney transplantation is associated with higher rejection rates, severity of rejection and graft loss, mainly when these antibodies are donor specific¹³.

We found that in patients with renal dysfunction a negative alloantibody screening is associated with a reduced risk of C4dPTC deposits (<10%). We concur with other authors¹⁴ that testing for post-transplant HLA antibodies is a critical diagnostic tool for graft dysfunction. It seems that antibody mediated rejection is very unlikely when the test is negative, allowing other diagnostic possibilities to be pursued early on, and mainly avoiding the need for invasive tests such as allograft biopsies. Even so, these results need to be established in a larger population of patients.

■ HCV and antibody mediated rejection

We found an association between C4dPTCD and HCV antibody. This link has been previously described but is not totally explained. Cosio *et al.*¹⁵ in 1996 found “acute vascular rejection” in 60% of HCV positive recipients compared with 28% in those without HCV who underwent renal allograft biopsy. They confirmed the previously reported association of HCV and acute transplant glomerulopathy, but they do not investigate the presence of deposition of C4d in PTC

and so it is possible that they were in reality describing antibody-mediated rejection.

Our study shows that HCV patients have longer time on dialysis and higher PRA, both risk factors for presensitisation and antibody-mediated rejection.

Similar results was been previously published by Forman *et al.*¹⁶. They found like us that patients with positive hepatitis C serology had higher rates of acute humoral rejection (AHR), an association which lost statistical significance after adjustment for higher PRA, a known risk factor for AHR.

A connection between the use of interferon-alpha (IFN) in hepatitis C with acute rejection and graft loss was been reported by Baid *et al.*¹⁷. Their study may explain the high rate of graft loss reported previously in renal recipients receiving IFN, but cannot explain our results as none of our patients were treated with IFN.

CONCLUSIONS

Our data suggest an association between +C4d PTC deposits, 2nd transplant recipients, and the presence of anti-HCV antibodies. The presence of antibody to HCV does not seem to be a risk factor *per se* for the appearance of C4d deposits, but reflects longer time on dialysis and presensitisation with higher PRA.

In patients with renal dysfunction, a negative allo-antibody screening is associated with a reduced risk of C4dPTC deposits (<10%). These data suggest that monitoring anti-HLA antibodies may be a useful tool for following-up antibody-mediated responses after kidney transplantation.

It is still not known if a positive alloantibody screen test without allograft dysfunction indicates an increased risk of antibody-mediated pathology and if graft biopsy is required. We believe that prospective monitoring of anti-HLA alloantibody will permit diagnosis of subclinical acute humoral rejection and a better knowledge of the importance of humoral components in chronic allograft pathology.

Conflict of interest statement. None declared.

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