Renal allograft pathology with C4d immunostaining in patients with graft dysfunction

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ABSTRACT

Renal allograft biopsy is the gold standard for diagnosis of rejection. Incorporation of C4d as a marker for humoral rejection is a major addition for Banff Schema, 2005. We evaluated the pattern of C4d staining in indicated renal allograft biopsies from January 2005 to December 2009. Of the 67 biopsies analyzed, 21 were C4d-positive. They were 11 cases of acute rejection, seven chronic rejection and one biopsy each of acute tubular necrosis, BK virus nephropathy and normal biopsy. Morphologic features like peritubular capillary dilatation, tubulitis and interstitial inflammation were seen more frequently in C4d-positive biopsies and this was statistically significant. C4d positivity was noted in 50% of the chronic rejection cases indicating a humoral component in the pathogenesis of chronic rejection. There was no significant difference in the serum creatinine levels of C4d-positive and -negative patients, either at the time of biopsy or during the follow-up. This study supports the role of C4d immunostaining in confirming histologically diagnosed acute and chronic humoral rejections and in detecting histologically unsuspected cases.

Key words: Acute, antibody-mediated rejection, C4d, cellular rejection, chronic rejection

Introduction

Renal allograft failure is caused by a variety of diseases, most of which can only be accurately diagnosed by transplant biopsies. Graft dysfunction is mainly attributed to rejection, calcineurin inhibitor toxicity, infections and recurrent native disease.[1,2] Despite the emergence of molecular techniques, allograft biopsy remains the gold standard for diagnosis of rejection and its distinction from the rest. Most studies on the mechanism of renal allograft rejection have focused on the central role of T-cells and other cellular mechanisms of tissue injury. Antibody-mediated rejection generally has a worse prognosis and requires a different form of therapy.

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Humoral rejection is mediated by antibodies to the endothelium, causes activation of the classical complement pathway, and releases split products of complement (C3a, C3b, C3d and C4b). Plasma factor I inactivates C4b to C4d, which remains bound covalently to the tissue. It is thereby a durable in situ marker of complement activation, in contrast to C1q or antibody.[3]

C4d deposition in peritubular capillaries was first demonstrated by Feuchet *et al*., in biopsies with high immunological risk and poor graft outcome. The role of C4d immunostaining in renal allograft biopsies has been published from India earlier.[4,5] In this study we analyzed the pattern of C4d staining in all renal allograft biopsies done for graft dysfunction.

Materials and Methods

All the allograft biopsies between January 2005 and December 2009 were included. All the biopsies were indicated and performed for the diagnosis of graft dysfunction. Details of primary disease, duration of transplant, type of donor, presenting features and the laboratory data were taken from case files. The light microscopic features were evaluated in every biopsy using hematoxylin and eosin, periodic acid-Schiff and silver methanamine stains. The features were

classified according to the Banff'2005 grading schema.[6] Donor-specific antibodies were not assessed in this study. IF was performed in all biopsies with IgM, IgA, IgG, C3, C1q, kappa, lambda.

Immunostaining with C4d was done on paraffin-embedded sections by polymer-Horse Radish Peroxidase (HRP) technique using polyclonal antiserum to C4d (Biogenex, India). Antigen retrieval was done by citrate buffer followed by peroxidase and power block and incubation with primary antibody for 1 h, secondary antibody- super enhancer for 20 min and HRP complex (Biogenex, India) for 20 min. Antigen antibody complex was visualized using diaminobenzidine (DAB) and counterstained with Harris hematoxylin. Biopsies of minimal change disease with glomerular positivity were taken as positive control and peritubular capillaries in the same were taken as negative control.

C4d was assessed in non-fibrotic and non-necrotic areas. Biopsies with no glomeruli and the ones with only medullary tissue were also included in the study.[7] C4d immunostaining pattern was noted in terms of distribution and intensity in peritubular capillaries. Staining was considered diffuse if more than 50% of peritubular capillaries (PTC) were positive and focal if less than 50% peritubular capillaries were positive [Figure 1]. Intensity was graded subjectively as 1+, 2+ and 3+ [Figure 2].

The association of C4d immunostaining with various histological features was studied by χ^2 test using SPSS software.

Results

A total 67 biopsies from 56 patients including two graft nephrectomies were analyzed. There were six females and 61 males in the age range 17 to 61 years (mean-32 years). Live related transplant biopsies were 60 [90%] and cadaver transplant biopsies were seven [10%]. The mean serum creatinine at the time of biopsy was 3.1 mg/dl. The majority of grafts showed dysfunction either in the first month (30%) or after one year (30%) of transplantation.

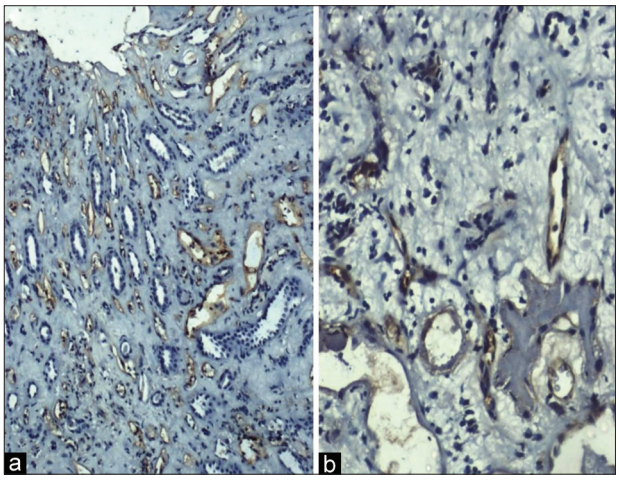

Figure 1: (a) Diffuse positivity in peritubular capillaries (C4d HRP Polymer, ×100) (b) Focal positivity in peritubular capillaries (C4d HRP Polymer, ×200)

Light microscopic features

The light microscopic findings of all the biopsies are given in Table 1. Acute cellular rejection (ACR) was seen in 13[19%] biopsies. In 12 cases histology was Grade I and in one Grade II. Chronic rejection was seen in 14 [20%] biopsies. The biopsies showing interstitial fibrosis and tubular atrophy (IFTA) along with arteriopathy and with or without glomerulopathy features were classified as chronic rejection. One biopsy was diagnosed as acute humoral rejection (AHR) on the basis of morphology due to the presence of acute tubular injury, neutrophils in the glomeruli and endothelitis. There were cases of acute tubular necrosis (ATN) (seven), IFTA (four), calceneurin inhibitor toxicity (CNI) (five) and BK virus nephropathy (one). Two biopsies of BK virus nephropathy with ACR were included in ACR [Table 1].

C4d immunostaining

C4d immunostaining was positive in 21 out of 67 biopsies (31.3%). Nonspecific tubular positivity was seen in some and in only two biopsies glomerular staining was observed. The details of C4d positivity are shown in Table 1.

C4d positivity was noted in 62% of rejection biopsies, one biopsy each of ATN, BK virus nephropathy and normal biopsy. The diagnosis of a single case of AHR on light microscopy was supported by C4d positivity. ACRs showed a significantly high (69%) positivity. Almost 50%

Table 1: C4d immunostaining in 67 renal allograft biopsies

Diagnosis	Number of cases	C4d-positive	Percentage
AHR	1	1	100
ATN	7	1	14
Infarction	2	0	0
ACR	13	9	69
Type 1	12	8	67
Type 2	1	1	100
BR	1	1	100
CR	14	7	50
IFTA	4	0	0
CNI toxicity	5	0	0
BK virus	1	1	100
Normal	17	1	6

AHR = Acute humoral rejection; ATN = Acute tubular necrosis; ACR = Acute cellular rejection; BR = Borderline rejection; CR = Chronic rejection; IFTA = Interstitial fibrosis tubular atrophy; CSA = Cyclosporine toxicity; BK virus = Polyoma virus

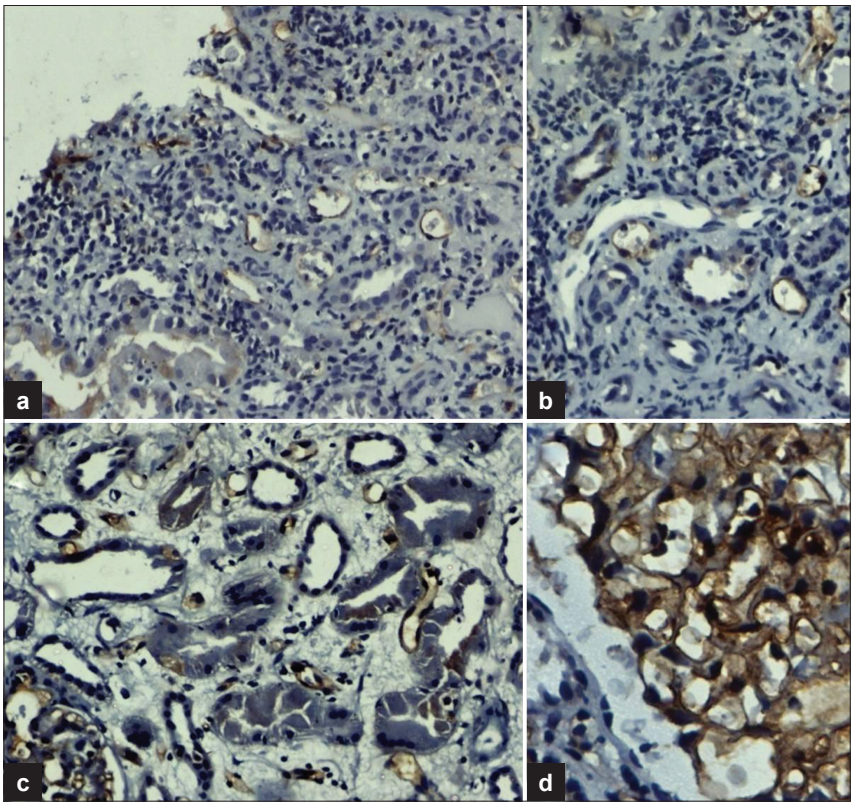

Figure 2: Grades of C4d positivity Grade 1 (a), Grade 2 (b) and Grade 3 (c) (C4d HRP Polymer, ×100) (d) Glomerular positivity for C4d (C4d HRP Polymer, ×200)

biopsies of CRs were C4d-positive. Biopsies showing features of IFTA and CNI toxicity were C4d-negative. Surprisingly, both the cases of graft nephrectomies with infarcts were C4d-negative. Distribution and intensity of C4d are depicted in Figures 3 and 4 respectively.

Focal and Grade 1or 2 positivity of C4d was more common in acute rejection (AR) whereas diffuse and Grade 3 positivity was frequently seen in chronic rejection (CR). And among others BK virus nephropathy showed Grade 2 positivity in 50% of PTC, ATN showed Grade 2 positivity in 20% PTC and the normal biopsy showed Grade 3 positivity in 50% of PTC.

Histological changes in C4d-positive biopsies are shown in Table 2. The association of interstitial inflammation, tubulitis and peritubular capillary dilatation (PTCD) was statistically significant. However, transplant glomerulopathy did not show significant association.

IF was negative in the majority of C4d-positive cases and there was occasional positivity of IgM and C3. C4d was negative in all four cases of mesangial IgA deposition.

Follow-up

Mean serum creatinine at biopsy and at follow-up were 3.0 and 3.3 mg/dl respectively in C4d-positive patients; 2.9 and 3.3 mg/dl respectively in C4d-negative patients. Serum creatinine at biopsy and at follow-up showed no significant association with C4d positivity.

Discussion

Hass *et al.*, demonstrated the utility of C4d staining in the diagnosis of humoral rejection in renal allograft biopsies with minimal morphologic features.[8] In India, there are two published reports on C4d immunostaining.[4,5] The present study is our data of C4d immunostaining in non-protocol-indicated renal allograft biopsies. However, serological donor antibodies (DSA) were not available for any of the cases.

The overall C4d positivity seen in the present study (31%) is comparable to other studies [Table 3].[9,10] The high rate of positivity is mainly because these biopsies are indicated as compared to the protocol biopsies where the reported C4d positivity is only 2%.[11]

The incidence of acute rejection in transplant biopsies varies from 10-60% and it depends on the indication and duration of transplant. Acute cellular rejection (ACR) was seen in 19% of our biopsies. This is comparable to other Western and Indian studies.[4,9] C4d positivity was seen in 69% of the biopsies of ACR. This is significantly higher than that reported by other studies in India and elsewhere.[4,11,12]

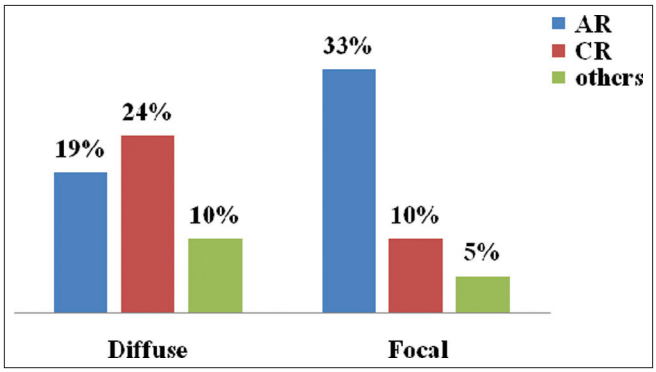

Figure 3: Distribution of C4d immunostaining in peritubular capillaries AR = Acute rejection; CR = Chronic rejection

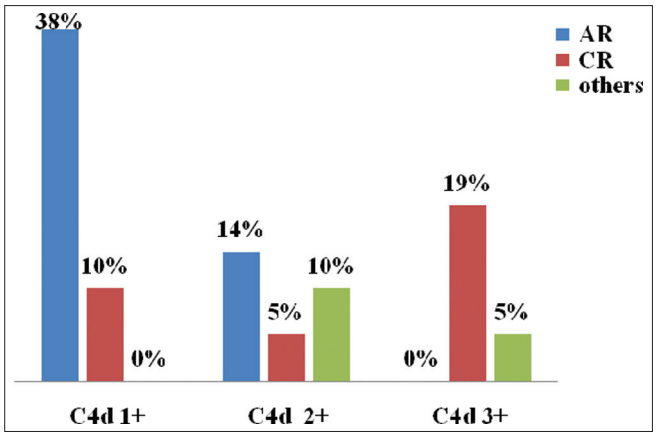

Figure 4: Intensity of C4d staining in peritubular capillaries AR = Acute rejection; CR = Chronic rejection

Table 2: Histological changes in C4d-positive allograft biopsies (*n* = 21)

Histological features	Number (%)	C4d +ve (21) (%)	*P* value
Interstitial inflammation	32 (48)	16 (76)	0.012
Tubulitis	19 (28)	14 (67)	0.001
Vasculitis	4 (6)	3 (14)	0.15
ATN	12 (18)	1 (5)	0.2
PTCD	9 (13)	6 (29)	0.02
Interstitial fibrosis	34 (51)	11 (52)	0.623
Tubular atrophy	36 (54)	14 (67)	0.09
Glomerulopathy	12 (18)	6 (29)	0.14
Arteriopathy	22 (33)	9 (43)	0.09

ATN = Acute tubular necrosis; PTCD = Peritubular capillary dilatation

Table 3: Comparison of C4d positivity of the present study with literature

	Troxell *et al.* (*n* = 60)	Ranjan *et al.* (*n* = 126)	Present study (*n* = 67)
C4d positivity	15% (9)	45% (57)	31% (21)
Focal	-	40% (23/57)	48% (10/21)
Diffuse	100% (9/9)	60% (34/57)	52% (11/21)
AHR	-	81% (13/17)	100% (1/1)
ACR	24% (8/33)	20% (2/10)	69% (9/13)
ACR1A	33.3% (6/18)	20% (2/10)	55.5% (5/9)
ACR 1B	43% (3/7)	-	100% (3/3)
ACR 2A	40% (2/5)	-	100% (1/1)
BR	11% (4/14)	50% (3/6)	100% (1/1)
CAN	-	30% (7/24)	30% (7/23)
Infarction	-	100% (2/2)	0% (0/2)
ATN	-	25% (2/8)	13% (1/8)
BK virus	-	100% (1/1)	100% (1/1)
Normal biopsy	12%	45% (10/18)	5.8% (1/17)

AHR = Acute humoral rejection; ACR = Acute cellular rejection; BR = Borderline rejection; CAN = Chronic allograft nephropathy; ATN = Acute tubular necrosis; BK virus- polyoma virus

AHR can be a part of ACR and the characteristic features include presence of glomerular neutrophils, thrombi or fibrinoid necrosis. None of these were identified in our C4d-positive ACR biopsies. So it is necessary to compare with levels of DSA to identify the humoral component. This can partly explain the higher incidence of C4d positivity seen in our patients with ACR. A single biopsy showing morphologic features of AHR was C4d positive.

Chronic allograft nephropathy (CAN) is a nonspecific term. Reliable identification of the various conditions that lead to this morphologic and functional damage is a prerequisite for specific therapeutic interventions. The 2005 Banff schema replaced CAN with definitive terms. Interstitial fibrosis and tubular atrophy (IFTA) is one such clearly descriptive one.[6] Similarly, biopsies with transplant glomerulopathy and arteriopathy are classified as chronic cellular rejection (CCR). Based on their recommendations 14 of CAN biopsies were classified as CCR and C4d was positive in 50% of these biopsies. C4d has been proved to be a marker with 50% positivity in CHR patients. These findings are comparable to that reported by MaYuddie *et al.*[13]

The rest of the CAN biopsies were reclassified as IFTA and CNI toxicity based on morphology. None of them showed C4d positivity. This finding further stresses the role of C4d in sub-classifying the cases of CAN into definite chronic rejection which can have a humoral component.

Significantly high C4d positivity in CR cases is explained by the probable underlying humoral component. This may be the reason for a greater number of CR cases in this study and may be related to more number of unidentified and untreated humoral components of ACR.

A biopsy of BK virus nephropathy with no morphologic evidence of rejection was C4d-positive. The patient was treated with Laforamide along with immune suppression and showed a good response with a serum creatinine

of 1.6 mg/dl at the end of one year. C4d positivity in this patient indicates a possible additional component of rejection.

One case each with features of normal biopsy and ATN showed C4d positivity. This can be explained by the phenomenon of accommodation.[7]

The patient of ATN showing C4d positivity died within one week of biopsy and C4d positivity indicates a possible underlying rejection in this patient with poor outcome. The other patient of normal biopsy features is doing well after four years of follow-up.

C4d negativity in biopsies with infarction in the present study is in contrast to 100% positivity in another Indian study and was possibly a vascular complication. And the point to note is that in contrast to the other Indian study the present study had only 5% positivity in normal biopsies.

Amongst the morphologic features peritubular capillary dilatation (PTCD), tubulitis and interstitial inflammation had significant correlation with C4d positivity. Ranjan *et al*., reported PTCD as a positive correlate; but tubulitis and inflammation have not been mentioned.[4] However, other studies have seen positive correlation with tubulitis and active inflammation.[6,14,15] These findings further emphasize that C4d positivity is associated with the active rejection process and helps to detect presumptive cases of AHR. Transplant glomerulopathy is identified by the characteristic duplication of glomerular basement membrane (GBM) observed by light microscopy, as recommended by the Banff working group.[14] Contrary to other studies, transplant glomerulopathy did not show significant positivity in our series. However, patients with transplant glomerulopathy and positive C4d had poor graft outcome.

Diffuse positivity was seen in 52% biopsies and it was more commonly seen in biopsies with chronic rejection. Modified Banff criteria 2003 requires >50% of peritubular capillary involvement (diffuse) to label the biopsy as C4d-positive.[16] However, the significance of the focal staining is controversial.

Some of the authors have mentioned that there are no significant differences between patients with focal and diffuse PTC C4d staining with respect to histological features on biopsy and graft outcome.[17] In the present study also there was no difference in graft outcome between these two categories.

In the present study, CR showed strong intensity in the majority of the cases. In comparison, ACR and AHR showed low intensity. However, Banff 2005 is silent about the intensity of C4d.

To conclude, C4d is useful in confirming histologically presumed acute humoral rejection. It is possible to pick up humoral rejection in histologically unsuspected cases. Positivity in AR may indicate associated humoral rejection. These cases may have to be treated to prevent possible CR. Hence the study emphasizes the role of C4d as a routine marker in all transplant biopsies.

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