Humoral Rejection of Organ Allografts

Solange Mol a,b and Manuel Pascual a,b,∗

aGeneva and bLausanne University Hospitals Transplant Network, Réseau Romand Hospitalo-Universitaire de Transplantation, Switzerland

*Corresponding author: M. Pascual, Transplantation Center, CHUV, Lausanne, Manuel.Pascual@chuv.ch

In recent years, the deleterious clinical consequences of recipient de novo alloantibody production against HLA antigens from human organ allografts have been extensively investigated. In kidney transplantation, the identification of the complement C4d fragment in peritubular capillaries as a specific marker for humoral rejection has helped to define and characterize distinct clinical alloantibody-mediated syndromes. This knowledge is relevant for patient management as new therapeutic strategies to remove and control anti-donor antibody production, particularly in the setting of acute humoral rejection, have been reported. For recipients of nonrenal organ allografts such as heart transplant recipients, de novo anti-HLA alloantibody may also be important, although more studies are needed before clear guidelines can be proposed.

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Introduction

The clinical syndrome of hyperacute rejection, due to the presence of preformed donor specific antibodies (DSA) in the sera of kidney allograft recipients (a pre-transplant ‘positive crossmatch’), was observed in the late 1960s. Moreover, in 1970, Jeannet et al. reported that kidney transplant recipients who developed de novo DSA in the early post-transplant period suffered from severe vascular rejection, resulting in poor clinical outcomes (1). However, the precise pathogenic significance of de novo DSA production against kidney allografts remained the subject of controversy for many years among transplant specialists, that is, until distinct clinical syndromes were eventually defined (2).

In landmark observations in the early 1990s, Halloran et al. were the first to identify acute rejection of kidney transplants associated with de novo anti-HLA DSA production as a defined clinico–pathologic entity carrying a poor prognosis (2,3). They described the clinical and pathologic features of this type of rejection and also postulated that the ‘complement-neutrophil pathway’ was probably the major pathophysiological mechanism of antibody-mediated injury under these circumstances (2–8). Interestingly, in a separate study, Feucht et al. detected vascular complementary C4d deposits in kidney allograft biopsies from recipients with early allograft dysfunction (6). They suggested that capillary C4d deposition in biopsies could be an evidence for humoral alloreactivity, although no repeat post-transplant crossmatches were performed and de novo circulating DSA were not assessed (6).

Another subsequent important step, therefore, was the demonstration by the Massachusetts General Hospital’s group in Boston that widespread and diffuse C4d deposits in peritubular capillaries (PTC) indeed correlated with the detection of de novo anti-HLA DSA in recipient’s serum (a de novo post-transplant positive crossmatch) at the time of renal allograft dysfunction (7–9). This work, which extended the observations made previously by Halloran and Feucht, further delineated the clinical syndrome of acute humoral rejection in kidney transplantation. The specificity of capillary C4d staining as a marker of anti-HLA alloantibody-dependent allograft injury was also shown subsequently by the Vienna group in a study analyzing a larger cohort of transplant recipients who had undergone kidney allograft biopsies (10).

C4d (molecular weight about 42 kDa) is one of the split products generated during complement activation of the classical pathway (e.g., triggered by anti-donor antibodies). The activation of C4, a protein possessing an internal thioester group with covalent binding property generates C4b (11). The C4b molecule is then cleaved into C4d and larger fragments such as C4c. Whereas C4c remains in solution, the split product C4d, which contains the reactive thioester group, rapidly binds to the target structures and remains bound for several days or weeks (11).

In kidney allografts with humoral rejection (alloantibody-mediated rejection), deposits of C4d are detected in the peritubular capillaries, indicating that the main target of alloantibodies is probably within the microcirculation (5–13). The C4d molecules are located at the luminal surface of PTC endothelial cells, or between endothelial cells and the basement membrane of PTC (13). Either diffuse (in every capillary) or focal (only in few capillaries) staining can be
detected, possibly reflecting the dynamic course of the C4d deposition (8–15). Although some authors only consider diffuse capillary C4d staining as significant, focal capillary staining might also be clinically relevant, but this remains to be investigated in prospective studies. Recent work indicates that in the setting of acute tubular injury and delayed kidney graft function, C4d is not deposited as a consequence of ischemic injury, but, if present, it indicates an antibody-mediated reaction resulting in local complement activation by the classical pathway (16, 17). Indeed, Haas et al. recently demonstrated that capillary C4d deposition can appear as early as 1 hour post-transplantation and is associated with circulating anti-donor alloantibodies, even when present in low levels (17).

The recent progress in the understanding of humoral rejection in kidney allografts led the participants of the seventh Banff Conference on Allograft Pathology to standardize the scoring of C4d staining of biopsies with antibody-mediated rejection of kidney allografts (18). First, because antibody-mediated rejection is a significant problem, and because C4d staining is an independent prognostic factor for graft outcomes, it was recommended that C4d immunostaining should be done on all transplant biopsies; moreover, ‘C4d positivity’ may have therapeutic implications (e.g. see ‘Acute humoral rejection’ below). Second, ‘C4d positivity’ was defined as a widespread (typically involving over half of sampled capillaries), and linear circumferential peritubular capillary staining in either cortex or medulla, excluding scarred or necrotic areas. The intensity of the staining should be strong in frozen sections using monoclonal antibody and immunofluorescence, but may be variable in paraffin-embedded sections using polyclonal antibody and immunohistochemistry.

Hyperacute or acute humoral rejection syndromes, due to preformed naturally occurring antibodies against carbohydrates, have also been well described in discordant xenografts as well as ABO incompatible human allografts; however, due to space constraints, these syndromes will not be discussed in this minireview.

**Acute Humoral Rejection in Kidney Transplantation**

Because early acute humoral rejection often presents as severe, refractory allograft dysfunction (resistant to high doses of steroids and to anti-lymphocyte antibody therapy), a prompt diagnosis and optimal treatment are essential (1–12). In the early 1990s, the prognosis of renal allografts demonstrating early post-transplant capillary C4d positivity was found to be significantly worse, with a 1-year graft survival of only 57% and 63% (diffuse and focal C4d deposition respectively), as compared to a 90% 1-year graft survival in allografts without C4d deposits (6). Although this study was performed in the ‘cyclosporine, steroids, azathioprine’ era and may, additionally, contain some selection bias in the way cases were identified (the majority of biopsies were C4d positive), more recent studies performed in the current era of newer immunosuppressive agents have confirmed the association of reduced graft survival and capillary C4d positivity (14, 15, 19, 20). Moreover, capillary C4d deposition has been demonstrated to be a long-term detrimental prognostic factor with a predictive value independent of numerous other morphological and clinical factors (15).

Diagnosis of acute humoral rejection in kidney allografts includes three cardinal features as proposed in the recently updated Banff-2001 classification (18): (i). morphologic evidence of tissue injury; (ii). immunopathologic evidence for antibody-mediated action, that is, C4d deposits and (iii). serologic evidence of circulating antibodies to donor HLA or to other donor endothelial antigens. Acute humoral rejection can occur early or late (e.g. due to noncompliance) after transplantation. Of note, the identification by conventional histopathology of certain morphological features may help in the recognition of acute humoral rejection. Accumulation of polymorphonuclear neutrophils and monocytes/macrophages in cortical PTCs has been observed by several groups, and is suggestive of acute humoral rejection (1–4, 7–13, 18). PTCs are characteristically dilated (18). Other pathological features such as glomerulitis with neutrophils and/or monocyte infiltration, glomerular and arteriolar fibrin microthrombi and severe vasculitis with fibrinoid necrosis have all been described (1–4, 7–13, 18) (Figure 1). However, most of these morphological features alone are not enough specific or sensitive. In contrast, C4d staining was 95% sensitive and 96% specific for circulating anti-donor antibodies in a study of 67 kidney biopsies from patients with acute rejection occurring in the first 3 months (20). Of note, the sensitivity of the different serologic tests (lymphocytotoxic crossmatch assays or flow cytometry testing) can differ. This may explain in part the occasional finding of capillary C4d deposition in DSA-negative patients.

Various recent case series have reported an overall incidence of early acute humoral rejection of approximately 0–8%, as estimated by systematically analyzing capillary C4d deposition in kidney allograft biopsies and correlating the results with DSA detection in serum (7–10, 18–24). This incidence depends on the type of kidney transplant population which is studied, that is, low versus high immunologic risk patients, as well as possibly the type of induction immunosuppression, but this latter point remains to be studied. It should be pointed out that, not infrequently, acute humoral rejection can coexist with cellular (T-cell mediated) rejection, that is, the findings on biopsy reveal a ‘mixed’ pattern. It will be important to analyze whether the incidence of acute humoral rejection will decrease with the use of modern induction immunosuppressive strategies, as the rates of acute cellular rejection have decreased to less than 20% in many centers with these strategies.
Acute humoral rejection most frequently occurs in sensitized patients or in those with a history of previously failed allograft(s). Most alloantibodies are directed against HLA antigens. In humans, all endothelial cells constitutively express class I antigens. The capillary endothelium, but not the arterial endothelium, can express both class I and II antigens. It should be emphasized that HLA class II-reactive antibodies are not always detected in conventional microtoxicity assays using panel cells, and may require more refined techniques (i.e. flow cytometric crossmatching and/or HLA antigen-coated fluorescent microparticles) (25–27). Recent studies have shown that donor-specific anti-HLA class I and class II alloantibodies detected by sensitive methods before transplantation, even when present at low levels, can be associated with severe acute humoral rejection after transplantation (17,21,26). Therefore, an early post-transplant renal biopsy showing diffuse C4d positive staining in PTCs, associated with suggestive morphological features, can be diagnostic of acute humoral rejection even in the absence of detectable anti-HLA DSA in serum. Of note, non-HLA endothelial antigens have also been suggested in some episodes of acute humoral rejection, although this is probably rare.

From all the above, it has become clear that identification of an antibody-mediated component to an acute rejection episode is very important clinically, and it needs to be performed rapidly as it has immediate therapeutic implications. Removal of DSA with effective control of alloantibody production and acute rejection reversal is now possible, as shown by Pascual et al. and others since the mid-1990s (7,9,12,21–24,28–30). Indeed, therapeutic strategies, including combinations of plasmapheresis (or immunoadsorption), tacrolimus, mycophenolate mofetil, and/or intravenous immunoglobulins (IVIG) have all been employed successfully to treat severe and ‘refractory’ acute humoral rejection, that is, acute humoral rejection resistant to both steroid and antilymphocyte therapy (7,9,21–24,28–30). Anti-CD20 mAb therapy aiming at depleting B-cells and, thereby, possibly suppressing alloantibody production, may be an interesting option and it has also been added as a component of rescue therapy in some isolated cases. However, this approach remains to be further investigated before it can be recommended as a standard therapy of humoral rejection, because of its additional costs and its potential for over-immunosuppression, in patients who are already heavily immunosuppressed.

In the future, it might be also interesting to study the possible usefulness of potent ‘complement inhibitors’ (e.g. soluble CD35, currently in preliminary clinical trials) to block the deleterious pro-inflammatory consequences of in situ...
complement activation triggered by anti-donor alloantibodies. Indeed, specific inhibition of the recipient’s complement system of limited duration (e.g., 7–14 days) may be useful at the time of severe allograft dysfunction, perhaps obviating the need for allograft removal. However, one major problem with investigating any new therapeutic approaches in this field is the relative low incidence of acute humoral rejection, so that well-designed, multicenter studies preferably involving presensitized patients would be required.

Finally, it can be mentioned that plasmapheresis and/or IVIG have also been used to ‘desensitize’ highly sensitized patients in anticipation of living or cadaveric renal transplantation. Such strategies were successful in a number of individuals with positive crossmatch against their potential living donor or, who had been waiting for prolonged periods of time for a suitable cadaveric donor organ (28–32). In general, these high immunological risk kidney transplant recipients remain at higher risk of acute humoral rejection in the early days or weeks after transplantation, so that repeat DSA removal and/or IVIG administration may be necessary in their follow-up.

Humoral Rejection As a Cause of Late (Chronic) Kidney Allograft Dysfunction

Chronic allograft nephropathy (CAN) remains an important cause of late allograft dysfunction and loss in the modern era of kidney transplantation. It is well recognized that both immune and nonimmune mechanisms play important roles in the development of CAN. However, it is often difficult to ascertain the relative contribution of these various factors to pathological changes, such as interstitial fibrosis and tubular atrophy, observed in allograft biopsies (33,34). The term ‘chronic rejection,’ which can be confusing or misleading (11), should be used only in those cases of late allograft dysfunction due to CAN where an alloimmune mechanism of injury can be identified (cellular and/or humoral component to the injury process).

The classic morphological features of CAN associated with an ongoing (or previous) alloimmune mechanism of injury include arterial intimal fibrosis with the presence of intimal mononuclear cells (allograft arteriopathy), duplication of the glomerular basement membranes (transplant glomerulopathy) and multilayering and/or lamination of basement membranes of the peritubular capillaries (35–38) (Figure 2). However, all of these findings are not necessarily present in a given allograft biopsy. Recent data do suggest that anti-donor humoral immunity contributes to the development of these histopathological lesions and to the progressive deterioration of allograft function in a subset of kidney transplant recipients (35–40). In particular, transplant glomerulopathy was found to be associated with C4d glomerular deposits and with peritubular capillary basement membrane multilayering (36). The term ‘chronic humoral rejection’ was proposed by Mauvyeyi et al., who showed that 61% of ‘typical’ chronic rejection cases with arterial and/or glomerular pathological changes had capillary C4d deposition (38).

The frequency of capillary C4d positivity in recipients with late (chronic) allograft dysfunction of all causes was 17% in one small study (37), but in a larger retrospective study from the Vienna group, capillary C4d was detected in 34% of kidney allograft biopsies (13). In this study, C4d also correlated with basement membrane injury in the glomerular and PTCs and with accumulation of mononuclear cells in PTCs (13). Moreover, C4d deposition preceded the development of transplant glomerulopathy in most patients in whom serial biopsies were available, suggesting an important role of anti-donor antibodies and local complement activation in the development of transplant glomerulopathy (13,36–40).

In a recently published study, 11% of stable outpatient renal transplant recipients, more than 6 months after transplantation, had circulating anti-HLA antibodies. However, only 4.4% of patients had DSA. A higher serum creatinine was significantly associated with anti-HLA antibodies after adjustment for confounding variables (39). Of note, circulating de novo anti-HLA antibodies can precede renal allograft loss by 6 months to 8 years (40). However, more prospective studies with serial protocol biopsies should be performed to more precisely delineate the contribution of humoral mechanisms of rejection to late renal allograft pathology, that is, beyond the first 6–12 months after transplantation. In that regard, it is important to mention a very recent study in which it was found that ‘protocol biopsies’ (independent from allograft function) show a significantly lower incidence of C4d deposition than ‘indication biopsies’ (41). In protocol biopsies, a diffuse C4d stain was only found in 2% and a focal C4d stain only in 2.4%, whereas diffuse and focal C4d deposits were present in 12.2% and 8.5%, respectively, of indication biopsies.

Mechanistic studies on the processes initiating the production of ‘de novo’ alloantibodies with capillary C4d deposition will also be important to better understand the pathogenesis of progressive immunologic injury leading to late allograft loss in some patients. For example, the role of indirect allorecognition, acknowledged in acute rejection, might be also important in patients with late allograft dysfunction (42–44). When studying alloantibody production, it should be emphasized that the detection of immunoglobulin class-switched alloantibody is inevitably associated with the clinical expansion of T-cells with indirect allospecificity, that is, the only indirect pathway T-cells can provide ‘help’ for allospecific B-cells (45). Thus, de novo anti-HLA antibody production with its characteristic associated pathologic features may be the culmination of an undesired, early and specific T and B-cell cooperation. These mechanistic insights will obviously be relevant at the time of designing new therapeutic strategies.
Figure 2: Chronic humoral rejection. (A) and (B) Chronic transplant glomerulopathy with reduplication or ‘double contours’ of the basement membranes of the capillary loops. Methenamine silver staining. Original magnification ×250 (A) and ×500 (B). (C) Electron microscopy. Multilayering of the basement membrane of a peritubular capillary. Presence of mononuclear cells in the capillary lumen with adhesion to the endothelial cell (Original magnification ×2800). (D) Electron microscopy. Chronic transplant glomerulopathy. Widening of the subendothelial space with basement membrane-like material and some mesangial expansion. (Original magnification ×2200).

The efficacy of immunosuppressants such as tacrolimus, mycophenolate mofetil, sirolimus and everolimus, used alone or in combination, in controlling anti-donor humoral responses in patients with late allograft dysfunction, remains to be investigated prospectively (46). To date, data obtained from relatively small published series indicate that the combination of tacrolimus and mycophenolate mofetil, if tolerated without adverse effects, can effectively suppress short- and long-term anti-donor antibody production in recipients with acute or late alloantibody-mediated allograft dysfunction, (7,11,28,37). However, no formal controlled clinical trial has demonstrated the superiority of this combination over others (e.g. tacrolimus–sirolimus, cyclosporine–mycophenolate). In the future, drug regimens that will control both T-cell and B-cell responses may prevent late allograft loss and further improve long-term graft survival, provided that the immunoregulatory efficacy of such regimens is not hampered by an increase in infectious, neoplastic or cardiovascular complications. Obviously, defining the best type of long-term immunosuppression to kidney transplant recipients undoubtedly represents the major challenge for the upcoming years (46).

It seems also important to note that, ideally, appropriate immunomodulatory interventions should occur before the arterial or glomerular lesions of CAN develop. Future prospective studies with protocol biopsies and sequential monitoring of C4d deposition, as well as with concomitant measurement of anti-donor antibodies in serum are needed; the results may be helpful to propose guidelines on the precise time intervals to screen for an anti-donor humoral response, in order to identify patients who might be at increased risk of late allograft failure. At our institution, we screen for anti-donor anti-HLA antibodies by ELISA pre-transplantation and at 1 week, 6 months, 12 months and yearly after transplantation. An extra measurement is performed in cases of kidney allograft dysfunction or at the time of an allograft biopsy.

Diagnosing late alloantibody-mediated rejection (acute, subacute or chronic) may become extremely important, if not mandatory, in the current era of ‘minimization’ strategies which aim at lowering immunosuppression drugs in kidney transplant recipients. Although such strategies may sometimes be tempting to reduce long-term drug-associated adverse effects, they carry a significant risk of
insufficient immunosuppression that may culminate in clinical (or subclinical) cellular and/or humoral rejection.

Finally, another interesting issue is the condition termed ‘accommodation,’ in which under certain rare circumstances an allograft can acquire resistance to humoral injury. Circulating anti-blood group antibodies or anti-HLA alloantibodies can, in some transplant recipients, be present without apparent deleterious consequences \((47,48)\). In cases of ‘accommodation,’ early complement fragments such as C4d may be present on biopsies, but full complement cascade activation appears to be interrupted, possibly by molecules such as heparan sulfate \((47)\). Thus, local vascular cytoprotective mechanisms may play an important role in ‘accommodated grafts.’ Recent observations also point toward changes in the function or quality of anti-donor antibodies produced by ‘accommodated recipients’ \((49)\). These observations emphasize that the relationship between alloantibodies and transplant rejection is not always as simple as ‘cause and effect.’

**Humoral Rejection in Other Solid Organ Allografts**

Antibody-mediated rejection is not confined to only kidney allografts. In heart allografts, the clinical and morphological manifestations of antibody-mediated acute rejection have been described for many years \((50)\). However, only a few studies have evaluated capillary C4d deposition in cardiac biopsies. In a series of 56 heart transplant recipients, Behr et al. showed that C4d deposits correlated with patient death early after transplantation \((51)\). Similar data were found by Michaels et al. in their study of over 300 heart biopsy specimens \((52)\). These authors demonstrated a significant mortality (14%) as a result of C4d positive-acute humoral rejection. In addition, 86% of patients with early acute humoral rejection subsequently developed chronic rejection (transplant coronary artery disease). In another recently published study, Poellzi et al. confirmed that capillary deposition of complement C4d was associated with cardiac allograft vasculopathy as assessed by intracoronary ultrasound at 1-year post-transplantation \((53)\). It was suggested that C4d immuno-histochemical evaluation of serial cardiac allograft biopsies may identify patients at risk for development of coronary vasculopathy. If confirmed, such studies would be of great importance as transplant coronary artery disease after heart transplantation remains a major obstacle to long-term success. According to a recent report from the UCLA heart transplantation program, in contrast to the significantly decreased incidence of acute cellular rejection observed in recent years, the incidence of noncellular, and therefore presumably humoral, rejection has remained unchanged from 1990 to 2000 \((54)\). More prospective studies with serological correlations are required in order to better appreciate the diagnostic and prognostic relevance of capillary C4d deposition after heart transplantation. Of note, complement C4d or C3d deposition in early cardiac transplant biopsies may also be associated with ischemic injury \((55)\).

C4d immunostaining analysis was also reported in lung transplantation \((56)\). Magro et al. evaluated 52 transbronchial biopsies of 23 lung transplant recipients with recurrent acute and chronic rejection. In acute rejection, the amount of C4d deposition was positively and significantly correlated with the degree of parenchymal injury and with the clinical status. In chronic rejection with bronchiolitis obliterans syndrome (BOS), deposits of C4d were detected in the bronchial wall as opposed to the rarity of this finding in non-BOS patients. Although no association with anti-donor HLA Class I/II alloantibodies was demonstrated in a complementary study of the same group, these data likewise suggest that C4d analysis in transbronchial biopsies may have some diagnostic value after lung transplantation \((57)\). In an experimental study, anti-HLA antibodies were demonstrated to induce proliferation of airway epithelial cell proliferation and thus to play a potential role in BOS \((58)\). Furthermore, clinicopathologic studies are needed to define the role of anti-HLA antibodies and, more specifically, the potential diagnostic and prognostic value of C4d deposition in lung transplant biopsies.

In contrast to other solid organs, the liver allograft appears to be relatively resistant to antibody-mediated rejection. To date, however, no comprehensive analysis of capillary C4d staining in liver allograft biopsies has been performed. In a recent preliminary study, Krukemeyer et al. found C4d deposits along the portal capillaries of biopsy specimens from patients with acute liver allograft rejection \((59)\). However, no correlation with anti-donor antibodies was performed. Therefore, whether analyzing C4d deposits in liver allograft biopsies might be useful remains speculative. If capillary C4d deposits are present as a result of acute liver rejection, but not in other causes of liver dysfunction (such as hepatitis C virus recurrence), C4d immunostaining could also become an interesting diagnostic marker in liver allograft biopsies.

**Conclusion**

In the last 15 years, the contribution of humoral immunity (alloantibody and complement-mediated) to allograft rejection has been clarified to a large extent. The results of recent studies indicate that C4d staining of kidney allograft biopsies has emerged as an important tool to diagnose antibody-mediated allograft injury, independent from its occurrence early or late after transplantation. On the basis of several recent studies, it is suggested that routine C4d immunostaining should now be incorporated in the work-up of all kidney transplant biopsies. More work, however, is still needed to further and precisely elucidate the role of humoral mechanisms of rejection, notably in the late loss of function of kidney allografts, and to investigate novel treatment strategies that could be guided, at least in part,
by the results of biopsy C4d. For recipients of heart, lung or liver transplants, new studies will help to determine if the analysis of C4d in allograft biopsies is also useful to reliably detect alloantibody-associated tissue damage. Preliminary data indicate that, at least in heart transplant recipients, biopsy C4d monitoring may become an important diagnostic tool as well.

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