

Capillary C4d Deposition as a Marker of Humoral Immunity in Renal Allograft Rejection

BRUNO WATSCHINGER,* and MANUEL PASCUAL†

*Division of Nephrology, Department of Internal Medicine III, University of Vienna, Vienna, Austria; †Renal and Transplantation Units, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts.

Although the detrimental effect of preformed donor-specific antibodies (DSA) became apparent in the 1960s, the possible pathogenic role of *de novo* DSA production against allografts after renal transplantation remained controversial for many years (1). An interesting initial observation, however, had been reported in 1970 by Jeannot *et al.* (2), who suggested that *de novo* production of DSA against a transplanted donor organ can lead to severe graft injury. In subsequent years, most studies on the mechanisms of renal allograft rejection focused on the central role of T cells and of other cellular mechanisms of tissue injury (3). A critical step that stimulated a renewed interest in the study of anti-donor humoral responses was the work by Halloran *et al.* (4,5) in the early 1990s. These authors established that acute rejection associated with the development of *de novo* anti-HLA DSA in recipient's serum is a defined clinicopathologic entity carrying a poor prognosis. They subsequently described the histopathologic features of acute renal allograft rejection associated with DSA and postulated that the "complement-neutrophil pathway is probably the major mechanism of antibody injury" (6). However, a specific *in situ* diagnostic marker of humoral immunity was not identified, and particularly deposits of immunoglobulins or C3 in biopsies were not found to correlate with alloantibody-positive acute rejections (6). Around the same time Feucht *et al.* (7,8) detected C4d, a split product of C4 of the classical pathway of complement, in capillaries of biopsies from patients at "high immunological risk," and they suggested that capillary C4d deposition was evidence for humoral alloreactivity against the graft. However, DSA detection at the time of allograft dysfunction, by performing repeat posttransplant crossmatches to detect anti-HLA class I and/or class II alloantibodies against the donor, was not reported in these studies (7,8).

Another important step was the demonstration by Collins *et al.* (9) that staining of allograft biopsies for the fragment C4d is a specific and reliable method for identifying lesions due to humoral immunity, *i.e.*, due to alloantibodies against HLA class I and/or class II antigens. In this study, it was found that

widespread and diffuse C4d deposits in cortical peritubular capillaries correlate with the detection of *de novo* anti-HLA DSA in recipient's serum at the time of allograft dysfunction. Contrary to immunoglobulins and most complement proteins, the fragment C4d remains covalently bound to the nearby endothelium or basement membrane collagen, thereby providing *in situ* evidence of complement activation by the classical pathway following alloantibody binding to the peritubular capillaries. The specificity of capillary C4d staining for anti-HLA alloantibody-dependent graft injury was further demonstrated recently by Böhmig *et al.* (10), who analyzed a larger patient population. Interestingly, it appears that capillary C4d deposition can also be used to diagnose antibody-mediated injury due to non-HLA antidonor antibodies, as shown by a report from Japan analyzing rejection patterns in ABO-incompatible renal transplantation (11).

To date, two methods for the detection of C4d in allograft biopsies are available and have already been included in the routine work-up of biopsies in several centers. Depending on the center practice, either a three-step immunofluorescence is performed on unfixed frozen tissue with a monoclonal anti-C4d antibody (9); alternatively, immunohistochemistry with a recently developed polyclonal anti-C4d antibody can be done when only paraffin sections of biopsies are available (12). Both methods allow virtually identical endothelial staining of peritubular capillaries for C4d. Differences between frozen and paraffin sections exist in the results of glomerular or mesangial C4d staining, but these are likely irrelevant for the diagnosis, prediction of transplant outcome, or for the decision of therapeutic interventions.

From a clinical perspective it has become evident that, because acute alloantibody-mediated rejection (acute humoral rejection, AHR) presents most of the time as severe allograft dysfunction (19), its prompt diagnosis and optimal treatment are essential. Indeed, the prognosis of renal allografts demonstrating capillary C4d deposition has been found to be significantly worse than that of allografts without evidence of C4d deposits (8,10,13–16), but recent data suggest that intensified antirejection therapy may improve the long-term outcome of these grafts (17,18). By analyzing capillary C4d deposition in renal allograft biopsies and correlating the results with DSA detection in serum, it has been estimated that the overall incidence of AHR is 3 to 10%, that is approximately 20 to 30% of all acute rejection episodes have a humoral component (15,16,19–21). It should be emphasized that it is not uncom-

Correspondence to Dr. Manuel Pascual, Renal Unit, Box MZ 70, Massachusetts General Hospital, Fruit Street, Boston, MA 02114. Phone: 617-724-6350; Fax: 617-726-3713; E-mail: mpascual@partners.org

1046-6673/1309-2420

Journal of the American Society of Nephrology
Copyright © 2002 by the American Society of Nephrology

DOI: 10.1097/01.ASN.0000029941.34837.22

mon for histopathologic findings of acute cellular rejection to be present in allograft biopsies with AHR (mixed pattern of cellular and humoral rejection). Furthermore, the term “acute vascular rejection” should not be used, as this term can be confusing. Indeed “acute vascular rejection” is not necessarily restricted to an antibody-mediated process, as it can also reflect a T cell–mediated pathology. Particularly endarteritis or endothelialitis, a form of “vascular rejection,” can be exclusively due to cell-mediated immunity in the absence of antidonor alloantibody (1). Acute humoral rejection most frequently occurs in sensitized patients or in those with a history of previously failed allograft(s). Recent studies have shown that donor-specific anti-HLA class I and class II alloantibodies detected by sensitive methods (*i.e.*, flow cytometric crossmatching and/or detection of anti-HLA antibodies using HLA antigen-coated fluorescent microparticles) before transplantation can be associated with the occurrence of severe AHR posttransplant (20,22). Regarding the treatment of AHR, removal of DSA with effective control of alloantibody production has been successfully achieved only recently with regimens that included various combinations of plasmapheresis (or immunoadsorption), mycophenolate mofetil, tacrolimus, and intravenous immunoglobulins (19,20,23–25). These therapeutic strategies have been often initiated to treat “refractory” AHR, that is AHR resistant to both steroid and antilymphocyte therapy, or based on C4d staining in the transplant biopsy as part of a specific initial antirejection therapy (17,18).

Increased interest in the role of humoral immunity in acute allograft rejection has triggered new investigations to determine whether active humoral mechanisms of injury might also play a role in late allograft dysfunction leading to graft loss. In the current issue of *JASN*, Regele *et al.* (12) analyzed capillary C4d deposition in biopsies of patients with chronic allograft dysfunction. The data obtained suggest that humoral immunity indeed contributes to chronic rejection/chronic allograft nephropathy (CR/CAN) in a significant subgroup of kidney transplant recipients. Interestingly, the presence of C4d deposits in peritubular capillaries was associated with chronic transplant glomerulopathy (as evidenced by light microscopy) and with tubular basement membrane multilayering (as evidenced by electron microscopy), that is two pathologic features that have been previously reported to reflect signs of subacute or chronic immunologic damage. In addition, capillary C4d deposition preceded the development of chronic transplant glomerulopathy in most patients in whom serial biopsies were available, emphasizing the role of local complement activation in the development of chronic transplant glomerulopathy in allografts.

An important issue, however, remains: the determination of the precise contribution of humoral mechanisms of tissue injury to the pathogenesis of CR/CAN, which remains an important cause of late allograft loss in the modern era of renal transplantation (26). Indeed, it is now recognized that both antigen-specific immune mechanisms and nonimmunologic factors play an important role in CR/CAN; it is often difficult to ascertain the relative contribution of each factor to the development of tubulointerstitial, vascular, and glomerular le-

sions in allografts (27,28). In an earlier study, Mauiyyedi *et al.* (29) found that 61% of cases with typical chronic rejection (*i.e.*, allograft biopsies with histologic criteria of chronic allograft glomerulopathy and/or transplant arteriopathy) had capillary C4d deposition (chronic humoral rejection), and most of the C4d-positive chronic rejection cases had circulating antidonor HLA antibody. However, Theruvath *et al.* (30,31) reported that the prevalence of chronic humoral rejection in recipients with chronic allograft dysfunction of all causes was only 13%. It is therefore interesting to note that in the current study by Regele *et al.* (12), which included a larger number of cases, capillary C4d deposition was found in 34% of allograft biopsies performed for chronic allograft dysfunction. Clearly, more prospective studies with protocol biopsies will be needed to further delineate the contribution of humoral mechanisms of rejection to late allograft pathology, *i.e.*, beyond the first 6 to 12 mo after transplantation.

Mechanistic studies on the processes initiating the production of alloantibodies resulting in *de novo* capillary C4d deposition late posttransplant are important to better understand the fine mechanisms involved in the pathogenesis of CR/CAN. Among these, more investigations analyzing the possible role of the indirect pathway of allorecognition in the production of DSA in patients with CR/CAN will be required. The importance of indirect allorecognition has been acknowledged in acute rejection, but perhaps more importantly in CR/CAN (32,33). In renal transplantation, Vella *et al.* (34) showed that T cells of patients with chronic allograft dysfunction are primed to recognize and respond to specific donor-derived major histocompatibility complex allopeptides, suggesting that T cells primed via the indirect pathway may be critical in initiating mechanisms of CR/CAN. In addition, Baker *et al.* (35) found increased frequencies of indirectly primed T cells in patients with CR/CAN, while these patients exhibited direct pathway hyporesponsiveness. Recently, Najafian *et al.* (36) also reported that patients with a history of acute rejections have evidence of indirect alloreactivity compared with patients with an uneventful posttransplant course. As CR/CAN is also more likely to occur in patients experiencing acute rejection episodes, this suggests that monitoring of indirect alloreactivity may allow for predicting the development of CR/CAN. The contribution of CD4+ cells primed via the indirect pathway on the induction of effector mechanisms of chronic allograft rejection (*e.g.*, production of alloantibodies with subsequent local complement activation) remains to be further studied. It will be interesting to determine whether indirect alloreactivity alone may be sufficient to stimulate humoral responses. Such information may provide more insight into the possible causal relationship between indirectly primed T cells and anti-donor alloantibody production, which is suggested by two studies in animals. Recently, Lee *et al.* (37) showed that lesions of chronic rejection, *i.e.*, cardiac allograft vasculopathy, occurred in an accelerated fashion in miniature swine primed with donor peptides via the indirect pathway of allorecognition. In their study, they were also able to demonstrate the deposition of alloantibodies in vessels. These results are in line with earlier observations in mice indicating that CD4+ cells are essential

helper cells for B cell alloantibody production and that indirect alloresponses are required for IgG switching (38).

It is likely that a better definition of the role of ongoing humoral mechanisms of tissue injury will be important to optimize the treatment and prevention of late allograft loss. In particular, sequential monitoring of intragraft C4d deposition in allograft biopsies with measurement of DSA in serum may be helpful to identify patients at increased risk of late allograft failure. These individuals could also benefit from tailored immunomodulatory interventions. The efficacy of newer immunosuppressants such as tacrolimus, mycophenolate mofetil, and sirolimus (used alone or in combination) in controlling anti-donor humoral responses in patients with chronic allograft dysfunction remains to be determined prospectively in controlled clinical trials. Preliminary data, however, indicate that the combination of tacrolimus and mycophenolate mofetil can effectively suppress antidonor antibody production in recipients with chronic rejection, thereby providing a basis to investigate new means to modulate the humoral limb of the immune response in humans (30). In the future, drug combinations that will control both T cell and B cell responses may improve long-term graft survival, if the immunoregulatory efficacy of such regimens is not hampered by an increase in infectious, neoplastic, or cardiovascular complications.

Taken together, C4d staining of renal allograft biopsies has emerged as an important tool for the diagnosis of alloantibody-dependent allograft injury independent from its occurrence early or late after transplantation. On the basis of several recent studies, it appears that routine C4d staining should now be incorporated in the work-up of all renal transplant biopsies. By analyzing the presence or absence of C4d deposits in peritubular capillaries, the contribution of humoral immunity to the pathogenesis of allograft rejection is currently being clarified. Nevertheless more studies will be needed to further elucidate the role of humoral mechanisms in the late loss of function of renal allografts, and to investigate novel treatment strategies that could be guided, at least in part, by the results of C4d staining in transplant biopsies.

References

1. Crespo M, Delmonico F, Saidman S, Tolkoff-Rubin N, Williams W, Colvin RB, Cosimi AB, Pascual M: Acute humoral rejection in kidney transplantation. *Graft* 3:12–17, 2000
2. Jeannot M, Pinn VW, Flax MH, Winn HJ, Russell PS: Humoral antibodies in renal allotransplantation in man. *N Engl J Med* 282: 111–117, 1970
3. Sayegh MH, Turka LA: The role of T-cell costimulatory activation pathways in transplant rejection. *N Engl J Med* 338: 1813–1821, 1998
4. Halloran PF, Wadgymar A, Ritchie S, Falk J, Solez K, Srinivasa NS: The significance of anti-class I antibody response. Clinical and pathologic features of anti-class I mediated rejection. *Transplantation* 49: 85–91, 1990
5. Halloran PF, Schlaut J, Solez K, Srinivasa NS: The significance of anti-class I antibody response. II. Clinical and pathologic features of renal transplants with anti-class I-like antibody. *Transplantation* 53: 550–555, 1992
6. Trpkov K, Campbell P, Pazderka F, Cockfield S, Solez K, Halloran PF: Pathologic features of acute renal allograft rejection associated with donor-specific antibody. *Transplantation* 61: 1586–1592, 1996
7. Feucht HE, Felber E, Gokel MJ, Hillebrand G, Nattermann U, Brockmeyer C, Held E, Riethmuller G, Land W, Albert E: Vascular deposition of complement-split products in kidney allografts with cell-mediated rejection. *Clin Exp Immunol* 86: 464–470, 1991
8. Feucht HE, Schneeberger H, Hillebrand G, Burkhardt K, Weiss M, Land W, Albert E: Capillary deposition of C4d complement fragment and early graft loss. *Kidney Int* 43: 1333–1338, 1993
9. Collins AB, Schneeberger E, Pascual M, Saidman S, Williams W, Tolkoff-Rubin N, Cosimi AB, Colvin RB: Complement activation in acute humoral renal allograft rejection: Diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 10: 2208–2214, 1999
10. Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayer J, Saemann MD, Horl WH, Watschinger B, Regele H: Capillary C4d deposition in kidney allografts: A specific marker of alloantibody-dependent graft injury. *J Am Soc Nephrol* 13: 1091–1009, 2002
11. Onitsuka S, Yamaguchi Y, Tanabe K, Takahashi K, Toma H: Peritubular capillary deposition of C4d complement fragment in ABO-incompatible renal transplantation with humoral rejection. *Clin Transplant* 13 (Suppl 1): 33–37, 1999
12. Regele H, Böhmig GA, Habicht A, Gollwitzer D, Schillinger M, Rockenschaub S, Watschinger B, Kerjaschki D, Exner M: Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: A Contribution of humoral immunity to chronic allograft rejection. *J Am Soc Nephrol* 13: 2371–2380, 2002
13. Regele H, Exner M, Watschinger B, Wenter C, Wahrmann M, Osterreicher C, Saemann MD, Mersich N, Horl WH, Zlabinger GJ, Böhmig GA: Endothelial C4d deposition is associated with inferior kidney allograft outcome independently of cellular rejection. *Nephrol Dial Transplant* 16: 2058–2066, 2001
14. Lederer SR, Kluth-Pepper B, Schneeberger H, Albert E, Land W, Feucht HE: Impact of humoral alloreactivity early after transplantation on the long-term survival of renal allografts. *Kidney Int* 59: 334–341, 2001
15. Herzenberg AM, Gill JS, Djurdjev O, Magil AB: C4d deposition in acute rejection: An independent long-term prognostic factor. *J Am Soc Nephrol* 13: 234–241, 2002
16. Mauiyyedi S, Crespo M, Collins AB, Schneeberger EE, Pascual M, Saidman SL, Tolkoff-Rubin NE, Williams WW, Delmonico FL, Cosimi AB, Colvin RB: Acute humoral rejection in kidney transplantation: II. Morphology, immunopathology and pathologic classification. *J Am Soc Nephrol* 13: 779–787, 2002
17. Baid S, Saidman SL, Tolkoff-Rubin N, Williams WW, Delmonico FL, Cosimi AB, Pascual M: Managing the highly sensitized transplant recipient and B cell tolerance. *Curr Opin Immunol* 13: 577–581, 2001
18. Bohmig GA, Exner M, Watschinger B, Regele H: Acute humoral renal allograft rejection. *Curr Opin Urol* 12: 95–99, 2002
19. Crespo M, Pascual M, Tolkoff-Rubin N, Mauiyyedi S, Collins AB, Fitzpatrick D, Delmonico FL, Cosimi AB, Colvin RB, Saidman S: Acute humoral rejection in renal allograft recipients: Incidence, serology and clinical characteristics. *Transplantation* 71: 652–658, 2001

20. Bohmig GA, Regele H, Exner M, Derhartunian V, Kletzmayer J, Saemann MD, Horl WH, Druml W, Watschinger B: C4d-positive acute humoral renal allograft rejection: Effective treatment by immunoadsorption. *J Am Soc Nephrol* 12: 2482–2489, 2001
21. Nicleleit V, Zeiler M, Gudat F, Thiel G, Mihatsch MJ: Detection of the complement degradation product C4d in renal allografts: Diagnostic and therapeutic implications. *J Am Soc Nephrol* 13: 242–251, 2002
22. Karpinski M, Rush D, Jeffery J, Exner M, Regele H, Dancea S, Pochinco D, Birk P, Nickerson P: Flow cytometric crossmatching in primary renal transplant recipients with a negative anti-human globulin enhanced cytotoxicity crossmatch. *J Am Soc Nephrol* 12: 2807–2814, 2001
23. Pascual M, Saidman S, Tolkoff-Rubin N, Williams WW, Mauiyyedi S, Duan JM, Farrell ML, Colvin RB, Cosimi AB, Delmonico FL: Plasma exchange and tacrolimus-mycophenolate rescue for acute humoral rejection in kidney transplantation. *Transplantation* 66: 1460–1464, 1998
24. Montgomery RA, Zachary AA, Racusen LC, Leffell MS, King KE, Burdick J, Maley WR, Ratner LE: Plasmapheresis and intravenous immune globulin provides effective rescue therapy for refractory humoral rejection and allows kidneys to be successfully transplanted into cross-match positive recipients. *Transplantation* 70: 887–895, 2000
25. Schweitzer EJ, Wilson JS, Fernandez-Vina M, Fox M, Gutierrez M, Wiland A, Hunter J, Farney A, Philosophe B, Colonna J, Jarrell B, Bartlett ST: A high panel-reactive antibody rescue protocol for cross-match-positive live donor kidney transplants. *Transplantation* 70: 1531–1536, 2000
26. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB: Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 346: 580–590, 2002
27. Pascual M, Swinford RD, Ingelfinger JR, Williams WW, Cosimi AB, Tolkoff-Rubin N: Chronic rejection and chronic cyclosporine toxicity in renal allografts. *Immunol Today* 19: 514–519, 1998
28. Halloran PF, Melk A, Barth C: Rethinking chronic allograft nephropathy: The concept of accelerated Senescence. *J Am Soc Nephrol* 10: 167–181, 1999
29. Mauiyyedi S, Pelle P, Saidman S, Collins AB, Pascual M, Tolkoff-Rubin N, Williams W, Cosimi AB, Schneeberger E, Colvin RB: Chronic humoral rejection: Identification of antibody mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 12: 574–582, 2001
30. Theruvath TP, Saidman SL, Mauiyyedi S, Delmonico FL, Williams WW, Tolkoff-Rubin N, Collins AB, Colvin RB, Cosimi AB, Pascual M: Control of antidonor antibody production with tacrolimus and mycophenolate mofetil in renal allograft recipients with chronic rejection. *Transplantation* 72: 77–83, 2001
31. Theruvath TP, Saidman S, Mauiyyedi M, Rubin N, Williams WW, Collins B, Colvin RB, Delmonico F, Cosimi AB, Pascual M: Prevalence of chronic humoral rejection in chronic renal allograft dysfunction. *Am J Transplant* 1(Suppl 1): 360A, 2001
32. Watschinger B: Indirect recognition of allo MHC peptides—Potential role in human transplantation. *Nephrol Dial Transpl* 14: 8–11, 1999
33. Sayegh MH: Why do we reject a graft? Role of indirect allorecognition in graft rejection. *Kidney Int* 56: 1967–1979, 1999
34. Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, Carpenter CB, Sayegh MH: Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. *Transplantation* 64: 795–800, 1997
35. Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, Lechler RI: Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: Implications for the pathogenesis of chronic allograft nephropathy. *J Immunol* 167: 7199–7206, 2001
36. Najafian N, Salama AD, Fedoseyeva EV, Benichou G, Sayegh MH: Enzyme-linked immunosorbent spot assay analysis of peripheral blood lymphocyte reactivity to donor HLA-DR peptides: Potential novel assay for prediction of outcomes for renal transplant recipients. *J Am Soc Nephrol* 13: 252–259, 2002
37. Lee RS, Yamada K, Houser SL, Womer KL, Maloney ME, Rose HS, Sayegh MH, Madsen JC: Indirect recognition of allopeptides promotes the development of cardiac allograft vasculopathy. *Proc Natl Acad Sci USA* 98: 3276–3281, 2001
38. Steele DJ, Laufer TM, Smiley ST, Ando Y, Grusby MJ, Glimcher LH, Auchincloss H Jr: Two levels of help for B cell alloantibody production. *J Exp Med* 183: 699–703, 1996

See related article, “Capillary Deposition of Complement Split Product C4d in Renal Allografts Is Associated with Basement Membrane Injury in Peritubular and Glomerular Capillaries: A Contribution of Humoral Immunity to Chronic Allograft Rejection,” on pages 2371–2380.