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Invited Comment

Kidney transplants, antibodies and rejection: is C4d a magic marker?

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Abstract

The immunohistochemical detection of the complement degradation product C4d in renal allograft biopsies has gained considerable clinical interest in recent years. The accumulation of C4d along peritubular capillaries is generally regarded as a marker for an antibody-mediated allo-response and is associated with poor graft survival. The aim of this review is to discuss histological findings associated with the deposition of C4d. Emphasis is placed on diagnostic and therapeutic implications. Unanswered questions regarding C4d and graft injury are highlighted.

Keywords: antibodies; C4d; diagnosis; rejection; therapy

Background

The gold standard for the diagnosis of rejection and for guiding patient management is the histological evaluation of a renal allograft biopsy [1]. Over the past decades, morphological criteria of acute and chronic rejection have been defined, and classification schemes of rejection have been introduced, such as the CCTT and the Banff schemes [2,3]. They form the backbone for the clinical decision making, outcome studies and multicentre analyses of the efficacy of new immunosuppressive drugs. However, all current classification schemes of renal allograft rejection have major shortcomings. In particular, the proper identification of humoral rejection episodes after the immediate post-transplantation period causes problems. The difficulties with identifying humoral rejection are due mainly to the lack of typical morphological and immunohistochemical changes characterizing different forms of an antibody response. Hence, antibody-mediated rejection episodes frequently remained undiagnosed and unclassified. Consequently, nearly all acute rejection episodes have been classified as 'cell mediated'. Tubulointerstitial rejection is a prime example [4,5]. This traditional view currently is under scrutiny. Stimulated by the pioneering work by Feucht and colleagues from Munich years ago, C4d has led to major changes in our understanding of kidney transplant pathology [6–11]. C4d is regarded as an immunohistochemical marker for a humoral mediated allo-response [10–14]. The transplant centre in Basel has gained experience with C4d over the last decade. Basel was the first transplant centre in the world which considered C4d to be a very valuable diagnostic tool and incorporated it into the diagnostic decision-making process. At present, many centres use C4d during the work-up of allograft dysfunction. Major attempts are underway to understand 'C4d-positive humoral rejection episodes' better. Classification schemes of renal allograft rejection are being revised accordingly [15].

C4d is the degradation product of the activated complement factor C4, a component of the classical complement cascade which is typically initiated by binding of antibodies to specific target molecules. Following activation and degradation of the C4 molecule, thioester groups are exposed which allow transient, covalent binding of the degradation product C4d to endothelial cell surfaces and extracellular matrix components of vascular basement membranes near the sites of C4 activation. C4d is also found in intracytoplasmic vacuoles of endothelial cells [16]. Covalent binding renders C4d a stable molecule that can easily be detected by immunohistochemistry (Figure 1; see Appendix for details of staining protocols). Detection of C4d is regarded as an indirect sign, a 'footprint' of an antibody response [10–14,17]. This observation marks a 'revolution': for the first time, a general and robust immunohistochemical marker for humoral rejection is identified. Since C4d is practically never detected along peritubular capillaries in the native diseased and inflamed kidney, such as active lupus nephritis, antineutrophil cytoplasmic antibody (ANCA) disease or anti-glomerular basement membrane (GBM) disease

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(personal observation), its detection seems 'transplant specific' [17]. However, it should be kept in mind that apart from the classical antibody-mediated route of complement activation, C4 can also be activated via an alternative, antibody-independent mechanism, the 'mannan-binding lectin' pathway [18,19]. Thus, C4d may also be potentially deposited without prior antibody binding. Currently, it is unknown whether this lectin pathway plays any pathophysiological role in the activation of C4 in renal transplants. Therefore, based on our current understanding, C4d accumulation is considered to be a marker for an 'antibody-mediated allo-response'. The detection of C4d in a graft biopsy ideally should be amended by clinical information on circulating donor-specific antibodies against major histocompatibility complex (MHC) class I or class II [15].

C4d and allo-antibodies

Several lines of evidence clearly support the close link between C4d and a humoral allo-response defined by circulating antibodies. Depending on the testing method used [e.g. crude panel-reactive antibody (PRA) titre testing *vs* more sensitive flow cytometry analyses], antibodies can be detected in 43–90% of C4d-positive *vs* 0–50% of C4d-negative patients [11–14,17]. Most antibodies detected in C4d-positive cases seem donor specific (directed against MHC class I and/or class II antigens). They typically are produced after transplantation, since C4d characteristically is found during the post-transplantational period [17,20]. Only pre-sensitized high-risk transplant recipients with circulating antibodies at the time of surgery can show C4d accumulation immediately after grafting [20]. The dynamics of the mounted antibody response, complement activation and degradation seem to be reflected by the rapid turnover of C4d [17]. Based on the location of the C4d deposits along peritubular capillaries, antibodies are probably directed against peritubular capillary endothelial antigens. Strong evidence of C4d as a marker for a humoral response is also provided by ABO-incompatible transplant recipients, 53% of whom show C4d deposition in graft biopsies [21,22].

However, many factors remain unknown regarding antibodies and C4d. For example, are circulating antibodies not associated with C4d positivity directed against different antigens and what is their clinico-pathological significance? Is persistent C4d positivity over weeks or months associated with a persistent antibody response? How closely does C4d positivity mirror changes in antibody titres? Do all antibodies associated with C4d positivity target the same antigens and do they all have the same clinical significance? Most importantly, is C4d positivity in the absence of detectable antibodies (~10% of patients) due to our current inability to detect those antibodies, or is C4d positivity the result of alternative pathways of C4 activation, such as the lectin pathway? Many questions remain to be answered.

Histological changes

C4d can easily be detected by immunofluorescence microscopy in frozen material (Figures 1, 2C and 3C) or by immunohistochemical techniques in formalin-fixed and paraffin-embedded specimens (Figure 3D; see Appendix for staining protocols). Of diagnostic relevance is the focal or diffuse, strong accumulation of C4d along peritubular capillaries in the renal cortex and/or medulla [11,17]. Immunohistochemistry on formalin-fixed tissue samples often yields weaker staining signals. Only non-fibrotic and non-necrotic parenchymal regions should be evaluated. The minimal threshold level to call a biopsy 'positive' is the detection of C4d in at least 10 capillaries surrounding adjacent tubules [17]. C4d deposits in other locations (e.g. in glomeruli, arterioles with hyalinosis or along atrophic tubules) are regarded to be non-diagnostic. It is important to remember that the accumulation of C4d marks an independent humoral allo-response. Consequently, C4d deposits can be detected in combination with various histological changes (Figure 4). The association between C4d and morphological signs of acute 'cellular' rejection defined by the CCTT criteria is statistically significant [17]. C4d is found in 24–43% of type I rejection episodes (i.e. tubulo-interstitial; Figure 2), in 45% of type II rejection (transplant endarteritis), in 50% of type III rejection (i.e. vascular rejection with fibrinoid vascular wall necrosis or thrombosis) and in 50–60% of glomerular rejection (i.e. transplant glomerulitis or glomerulopathy) [16,17,23]. Tubular MHC class II (HLA-DR) expression, an immunohistochemical marker of acute rejection [24], is found in 85% of C4d-positive biopsies (Figure 2D; [17]). C4d positivity can also be detected in combination with various other histological changes (e.g. interstitial or arterial vascular sclerosis or even cyclosporin toxicity); however, these associations do not reach statistical significance [17]. C4d can be seen in 14% of diagnostic biopsies lacking any morphological evidence of rejection (even Banff 'borderline' changes or polymorphonuclear leukocytes in capillaries), accounting for 13% of all C4d-positive biopsies in our experience [17].

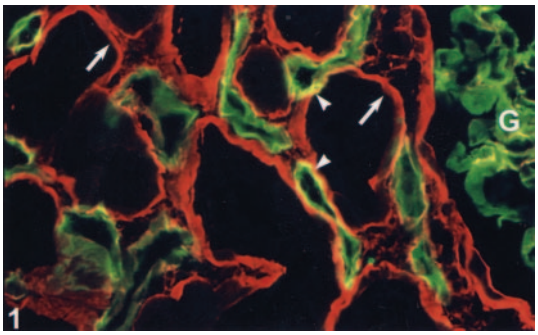

Fig. 1. Immunofluorecence microscopy shows C4d deposits along peritubular capillaries (green, arrowheads) and collagen type IV accumulations along tubular basement membranes (red, arrows). A tangentially cut glomerulus (G) only shows non-specific C4d deposits in mesangial regions. Double incubations performed on fresh frozen tissue sample, original magnification ×125.

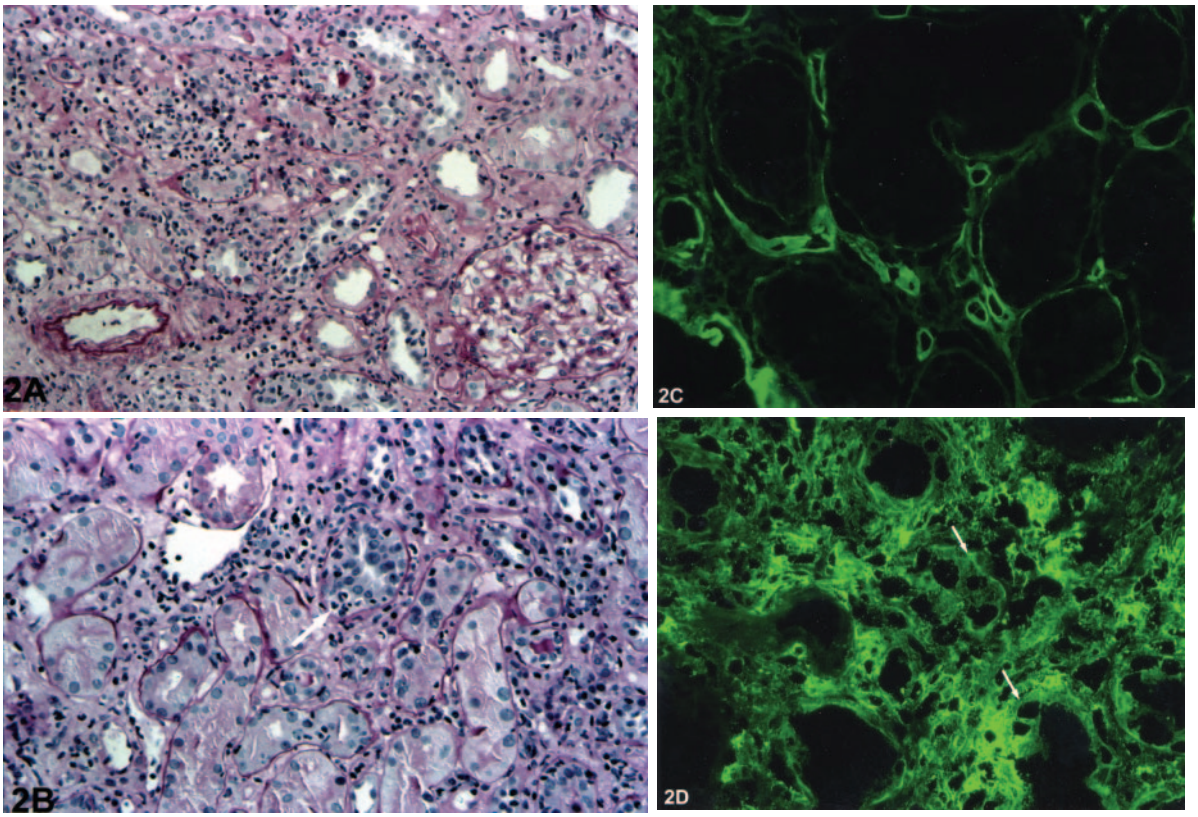

Fig. 2. (A–D) Acute, diffuse cellular tubulo-interstitial rejection (Banff type I B), C4d positive. (**A** and **B**) The interstitial compartment is oedematous and shows a diffuse inflammatory cell infiltrate composed of mononuclear cell elements and plasma cells. There is marked widespread tubulitis (arrow in B) and tubular injury. Periodic acid–Schiff (PAS)-stained sections; original magnifications ×50 (A) and ×80 (B). (**C**) Immunofluorescence microscopy shows bright C4d deposits along peritubular capillaries. Fresh frozen tissue sample; original magnification ×100. (**D**) Immunofluorescence microscopy shows MHC class II (HLA-DR) expression in the cytoplasm of tubular epithelial cells (arrows). Tubular HLA-DR expression is seen typically in cases with cellular rejection and tubulitis. Fresh frozen tissue samples; original magnification ×100. This biopsy illustrates the concurrence of 'cellular' tubulo-interstitial rejection and 'humoral' C4d-positive rejection, seen in 30% of cases in our experience. The biopsy was obtained 3.5 years post-grafting when the serum creatinine levels rose from 150 μmol/l (1.7 mg/dl) to 970 μmol/l (11 mg/dl) due to non-compliance. The patient was treated with a 10 day course of thymoglobulin. Renal function improved (last follow-up: serum creatinine 265 μmol/l, 3.0 mg/dl).

Less than 5% of our C4d-positive cases present with so-called 'acute pure humoral rejection' (Figure 3): acute tubular injury, abundant inflammatory cells including polymorphonuclear leukocytes in capillaries, potentially capillary thrombi or fibrinoid arterial wall necrosis and clinical signs of severe graft dysfunction. Evidence of concurrent cellular rejection including tubular MHC class II upregulation is characteristically lacking. These cases differ histologically from 'hyperacute' rejection since thrombi in large arteries are uncommon. 'Acute pure humoral rejection epsiodes' are typically seen during the first weeks following transplantation, most often in ABO-incompatible transplants [21,22]. C4d has helped with accurately classifying 'acute pure humoral rejection', which is now categorized specifically in the most recent revision of the Banff '97 classification scheme of renal allograft rejection [15].

Some authors have suggested a specific association between C4d and so-called chronic rejection: sclerosing transplant vasculopathy, multilayering of peri-tubular capillary basement membranes and splitting of glomerular basement membranes [16,25]. Consequently, the term 'chronic humoral rejection' has been coined [25]. Unfortunately, the studies to support this concept are very limited and lack statistical power due to highly selected case populations. At present, it is undetermined whether the detection of C4d in these biopsies really marks a long-lasting 'chronic' event or, alternatively, an active and acute rejection phenomenon which is superimposed on sclerosing changes. Most of the analysed C4d-positive cases in the chronic rejection category showed well-known morphological signs of activity, i.e. transplant glomerulitis or endarteritis [16,25]. Both transplant glomerulitis and endarteritis are correlated with C4d depositions [17,23], and they are well-defined forerunner lesions of chronic rejection [26]. Thus, it seems likely to us that C4d detection in the setting of 'chronicity/sclerosis' marks an acute/active rejection episode. Such rejection episodes can respond to anti-rejection therapy, underscoring the 'active' component of injury [27]. Whether potentially persistent, long-lasting C4d accumulation/humoral rejection may contribute

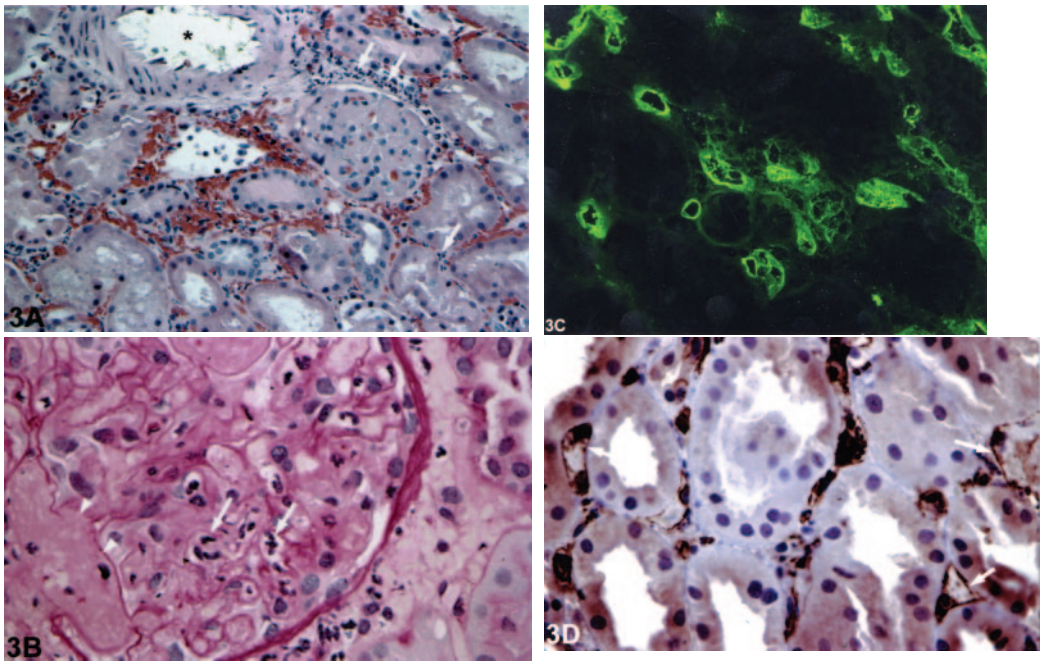

Fig. 3. (A–D) 'Pure' acute humoral rejection without signs of cellular rejection (no upregulation of MHC-class II/HLA-DR in tubules). (**A**) Peritubular capillaries contain numerous polymorphonuclear leukocytes (arrows). Focal haemorrhage is seen in the interstitial compartment. A small artery is unremarkable (asterisk). Glomerular capillaries are occluded due to endothelial cell swelling. Haematoxylin and eosin (H&E)-stained section; original magnification ×62. (**B**) A glomerulus shows polymorphonuclear leukocytes and mononuclear cells in glomerular capillaries (arrows) and intracapillary fibrin thrombi (arrowheads). PAS-stained section; original magnification ×160. (C and D) Detection of C4d. Immunofluorescence microscopy on frozen tissue (**C**) and immunohistochemistry on paraffin-embedded tissue (**D**) show diffuse accumulation of C4d along peritubular capillaries (arrows identify capillary walls in D). (C) Fresh frozen material; original magnification ×100. (D) Formalin-fixed and paraffin-embedded tissue; original magnification ×160. The biopsy was taken on day 6 after implantation of a cadaveric graft, subsequent to induction therapy with anti-thymoglobulin. At the time of biopsy, urine output had decreased from 2 to 0.8 l/24 h. Serum creatinine levels had increased within 2 days from 300 µmol/l (3.4 mg/dl on day 4) to 530 µmol/l (6 mg/dl on day 6). Antibody screening showed 60% anti-HLA antibodies and 28% anti-A2. The patient was treated with five courses of plasmapheresis and a 10 day course of thymoglobulin. Renal function deteriorated and a graft nephrectomy had to be performed 16 days post-surgery, revealing combined cellular rejection (very severe transplant endarteritis, type IIB) and persistent humoral rejection (diffuse C4d positivity) with infarcts (nephrectomy findings not shown).

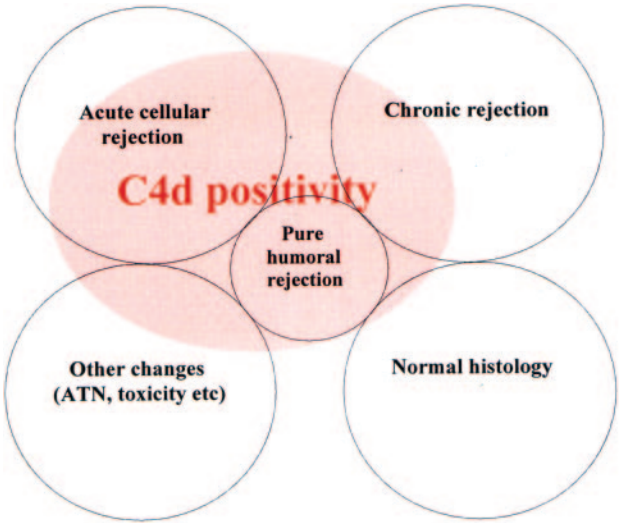

Fig. 4. Histological changes and the detection of C4d along peritubular capillaries. In renal allograft biopsies, C4d can be detected in association with different histological changes and even in the setting of normal histology. Statistical significant is the correlation between C4d and 'acute cellular rejection', in particular transplant glomerulitis. Only a minority of C4d-positive biopsies represent 'pure humoral rejection'.

to the development of chronic rejection currently is undetermined.

Although histological signs of 'acute/active' rejection and, in particular, glomerular rejection (transplant glomerulitis/glomerulopathy) correlate most significantly with C4d accumulation [16,17], it is important to emphasize that there is no specific morphological change defining C4d positivity on light microscopical grounds. In some instances, pronounced polymorphonuclear leukocytes or mononuclear inflammatory cells in dilated capillaries may indicate a C4d-positive humoral rejection episode. These changes can be diagnostically helpful [28]. However, in many transplant biopsies, obvious histological clues suggesting C4d positivity and/or an antibody response are lacking [17,23].

Clinical observations and prognosis

During the post-transplantational period, C4d is detected in 30% of all diagnostic graft biopsies (35% of all biopsied patients). It is typically seen early after transplantation (median: 38 days post-grafting).

Occasionally, C4d can also be detected years after grafting (in our experience, as late as 15 years). C4d is a dynamic marker since it can accumulate and disappear within days (~4–8 days). Occasionally, C4d is detected persistently over many months [17].

At present, the clinical significance of C4d deposits in renal allografts is incompletely understood (Table 1). As a general rule, C4d positivity in the setting of cellular rejection or significant allograft dysfunction (in our experience, serum creatinine levels $>200\,\mu$mol/l, 2.3 mg/dl) indicates serious rejection episodes requiring aggressive treatment [9,17,23,29]. Often, long-term prognosis is poor. Feucht *et al.* initially reported an overall 12 month graft failure rate of 40% in C4d-positive cases, in contrast to only 10% in C4d-negative controls [9]. C4d was found to be the strongest independent predictor of poor graft outcome [11,23]. The survival rates of C4d-positive tubulo-interstitial (Banff type I) and vascular (transplant endarteritis, Banff type II) acute rejection episodes are poor when compared with corresponding C4d-negative controls.

In contrast, the clinical significance of C4d positivity in grafts with only mild dysfunction (in our experience, serum creatinine levels $<155\,\mu$mol/l, 1.75 mg/dl) and without morphological signs of rejection is unclear. We have made this observation in ~15% of C4d-positive cases [17]. Approximately 3% of surveillance protocol biopsies taken from stable grafts without subclinical 'cellular' rejection are C4d positive [30]. The long-term outcome of these transplants appears favourable, and patients do not seem to benefit from immediate anti-rejection therapy [17]. Very little is known about continuous C4d deposition in stable grafts over a period of many months. Such extended accumulation of C4d can be seen in surveillance biopsies taken from well functioning transplants subsequent to successfully treated C4d-positive rejection episodes (personal observation). C4d has also been detected in stable grafts following the successful transplantation across ABO barriers [22]. Whether these findings potentially indicate 'subclinical humoral rejection' with detrimental effects on long-term graft survival or, alternatively, represent 'accommodation' remains to be determined in future studies [31].

Table 1. Different forms of humoral responses seen in kidney transplants/transplant recipients

	Graft dysfunction	Antibodies (donor specific)	C4d	Morphological changes
Form I	No	Yes	No	No
Form II[a]	No	Yes	Yes	No
Form III[b]	No	Yes	Yes	Yes
Form IV[c]	Yes	Yes	Yes	Yes

[a]These cases potentially represent subclinical pure humoral rejection or accommodation.
[b]Most of these cases probably represent subclinical rejection, often humoral and cellular.
[c]Form IV represents cases of humoral rejection, either in the acute pure form or in combination with cellular rejection, chronic sclerosing changes or other alterations.
These forms of humoral responses are descriptive in nature; they do not define treatment groups. Adapted from the NIH-sponsored 'Consensus conference to analyze humoral rejection in solid organ transplantation', Bethesda, MD, April 23–24, 2003.

Treatment (Figure 5)

Treatment strategies to manage C4d-positive humoral rejection episodes currently are poorly defined. Some encouraging therapeutic attempts have been reported with different protocols, either alone or in combination. Those include high dose tacrolimus and mycophenolate mofetil, immunoabsorption, plasmapheresis, i.v. immunoglobulin or anti-lymphocytic preparations, which are often given in cases of concurrent cellular rejection [27,29,32–34]. Response to therapy appears to be poor if thrombi are identified. At present, case numbers are too low to render general therapeutic recommendations.

The transplant centre in Basel, Switzerland traditionally has regarded C4d positivity as an indicator for clinically severe rejection episodes (i.e. transplant endarteritis). In most cases, as an immediate response, anti-lymphocytic preparations (i.e. ATG or OKT3) had been administered, especially for C4d-positive tubulo-interstitial rejection episodes and cases of transplant glomerulitis. In our opinion, this therapeutic approach explains the favourable and unique Basel outcome data: neither allograft function nor 1 year graft survival differed significantly between C4d-positive and corresponding C4d-negative groups [17]. Thus, aggressive treatment with anti-lymphocytic preparations may be a practical strategy to manage at least some C4d-positive rejection episodes. Since the Basel data were collected retrospectively and represent the experience of only a single centre, prospective multicentre studies are needed for further validation.

C4d positivity in the setting of normal or only minimally altered allograft function and 'normal' histology is poorly understood. Preliminary data suggest that C4d accumulation under these conditions does not indicate poor outcome and patients do not seem to benefit from immediate anti-rejection therapy [17,30,31].

C4d in other solid organ grafts

Little is known currently about C4d accumulation in other solid organ allografts. Preliminary data suggest that heart allografts are comparable with kidney transplants [35,36]. C4d was found in early post-transplant endomyocardial biopsies and was associated with poor graft survival [37]. In dysfunctioning lung transplants, C4d could be detected in septal capillaries [38]. C4d has also been found in liver allografts carrying a diagnosis of antibody-mediated rejection [39].

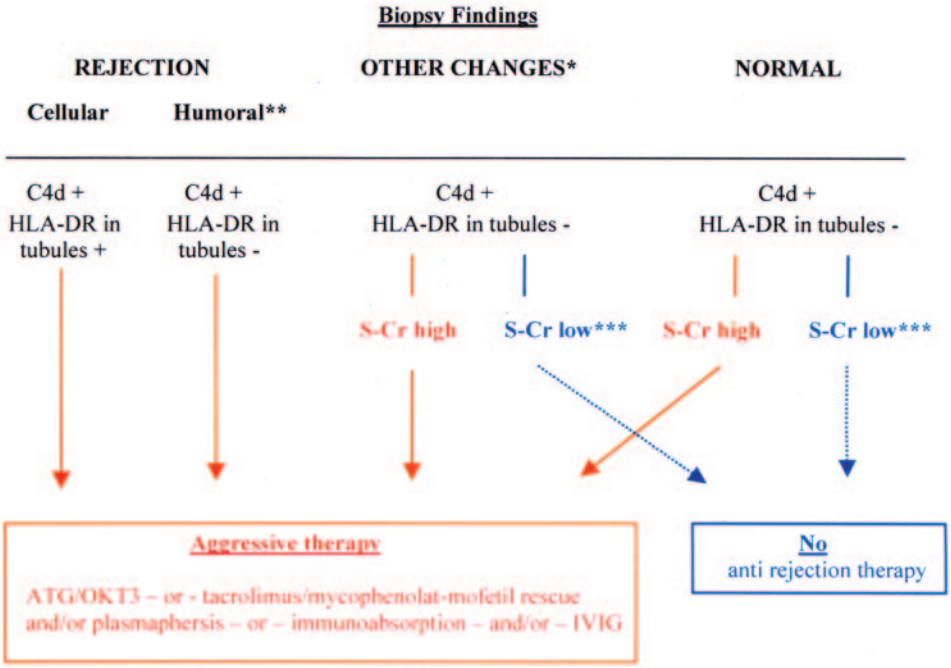

Fig. 5. Clinical implications of C4d-positive renal allograft biopsies. S-Cr, serum creatinine. *Other changes include chronic rejection, calcineurin inhibitor toxicity, etc. **Histological findings of pure humoral rejection include polymorphonuclear leukocytes in capillaries and/or thrombi, sometimes haemorrhage or necrosis; MHC class II (HLA-DR) is not expressed in tubules. This category has been newly defined. ***These C4d-positive biopsies should be thoroughly examined in order to rule out 'focal, smouldering rejection', in particular in the setting of tubular HLA-DR expression. Such cases require therapy. The remaining patients should be closely followed. A subsequent rise in S-Cr may indicate rejection, often transplant endarteritis.

Future perspective

A major change of our philosophy explaining renal allograft dysfunction currently is occurring. C4d has 'magically' enabled us to detect humoral mediated allo-responses in histological sections and to re-focus our attention on antibody-induced graft injury. Thus, C4d seems indeed to be a 'magic' marker. We have learned that humoral mediators of rejection are not limited to rare forms of hyperacute rejection episodes. Rather, humoral and cellular rejection episodes often concur. At present, most of these humoral mediators (potentially antibodies directed against MHC class I and/or class II antigens) and their direct impact on patient management and graft survival are poorly understood. Future research should address several issues. First and foremost, we need to clarify whether the accumulation of C4d is always initiated by the deposition of allo-antibodies or whether alternative pathways, such as the lectin pathway, may be involved. What antibodies lead to activation of C4 along peritubular capillaries and are all of these antibodies clinically relevant? Of utmost practical significance are two questions: (i) what is the significance of C4d deposits in stable grafts with normal histology; and (ii) is persistent C4d accumulations over months pathophysiologically important? We must define treatment strategies in order to manage C4d-positive rejection episodes better. In the spring of 2003, the NIH (National Institute of Health, USA) specifically addressed some of these questions during an expert panel meeting which focused exclusively on humoral rejection and C4d accumulation.

Since many aspects regarding C4d and antibodies remain to be determined in upcoming studies, it seems premature to introduce new classification schemes of humoral rejection at present. Therefore, we, the authors, currently rather recommend to report C4d staining results in pathology reports as 'qualifiers', amending traditional, histology-based diagnostic categories [40]. Thus, for example, tubulo-interstitial rejection, transplant endarteritis or glomerulitis can be 'C4d positive' or 'C4d negative'. In addition, we recognize rare forms of pure, C4d-positive acute humoral rejection episodes characterized by polymorphonuclear leukocytes and thrombi. This approach ensures an adequate clinico-pathological correlation at the present time. Conventional diagnostic categories remain unaltered, facilitating future multicentre comparative trials.

Conflict of interest statement. None declared.

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Appendix

Detection of C4d

The complement degradation product C4d can be detected easily in fresh frozen tissue samples by immunofluorescence microscopy. We use a mouse monoclonal antibody which is commercially available from Quidel (San Diego, CA) according to a previously published protocol [17].

C4d can also be detected in formalin-fixed and paraffin-embedded tissue sections employing a rabbit polyclonal antibody (Biomedica Gruppe, Vienna, Austria). We use the steam antigen retrieval technique (30 min), followed by a 30 min incubation with the primary antibody at 37°C (dilution 1:50) and subsequent avidin/biotin histochemical staining procedures.

Detection of tubular MHC class II (HLA-DR) expression

The upregulation of MHC class II in tubular cells is evaluated by direct immunofluorescence microscopy on frozen tissue samples using a fluorescein isothiocyanate-conjugated mouse anti-human monoclonal antibody (DAKO A/S, Glostrup, Denmark; dilution 1:40; 30 min incubation at room temperature).