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REVIEW

The diagnostic significance of C4d deposits, as an immunohistochemical proof of complement activation, in kidney glomerular pathologies and kidney transplantation

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ABSTRACT

C4d, a split product of C4 activation in classical and lectin pathways of the complement system activation, has been regarded as a footprint of tissue damage in antibody-mediated rejection in transplantology. The introduction of C4d staining into daily clinical practice aroused an ever-increasing interest in the role of antibody-mediated mechanisms in kidney allograft rejection. However, this marker of complement activation is also important in other various kidney glomerular pathologies such as immunoglobulin A nephropathy, membranoproliferative glomerulonephritis, lupus nephritis, and others. In routine histopathological practice, C4d staining can be done by two histological methods, specifically by immunofluorescence on frozen tissue using monoclonal antibody to C4d (with the downside of unsteady availability of frozen tissue) or by immunohistochemistry using C4d antibodies on formalin-fixed and paraffin-embedded renal tissue. The aim of this narrative review is to summarize recent knowledge about the complement fragment C4d and its significance in different kidney pathologies, focusing on its immunohistochemical detection in renal tissue biopsies. We have supplemented this review with our experience with our proprietary methodology of preparation and practical use of antibodies such as anti-C4d, on a small national level. Immunohistochemical staining for C4d has revolutionized the field of renal histopathology. Despite being a simple diagnostic test, its utility can be of utmost importance, especially in a resource-poor setting where immunofluorescence and frozen tissue may not be available (*Fig. 2, Ref. 53)*. Text in PDF *www.elis.sk*

KEY WORDS: C4d deposition, immunohistochemistry, kidney glomerular diseases, kidney transplant, renal tubular damage.

Introduction

The complement system is a complex, dynamic and ubiquitous system of more than 50 sequentially arranged proteins. It forms a critical arm of the innate immune system and plays a crucial role in both health and disease (1). Although complement activation occurs in many kidney glomerular diseases as well as in kidney transplant pathology, the exact details of its activation pathway (s) have not been discerned yet. C4d is a split product of C4 activation in classical pathway and lectin pathway of the complement system activation. Owing to its thioester bond, it can covalently bind to cell surfaces and can serve as a marker of complement activation. In summary, the significance of C4d can be described as a degradation product of complement cascade and in different kidney pathologies, it serves as an evidence of complement activation (2). C4d has been regarded also as a footprint of tissue damage in antibody-mediated rejection in transplantology, and the introduction of C4d staining in daily clinical practice aroused an ever-increasing interest in the role of antibody-mediated mechanisms in kidney allograft rejection (3, 4). Antibody-mediated rejection is highly detrimental to the prolonged survival of transplanted kidneys. However, C4d has a much wider application in transplantology, specifically in the detection of antibody-mediated rejection of other diverse organ allografts, including the heart (5), pancreas (6), liver (7), small intestine (8), lungs (9) or more recently, in face tissue allografts (10). Immunohistochemical staining for complement components, including C4d, in different transplant organ biopsies may be useful in understanding how complement is activated in individual cases. In clinical practice, it may help identify patients who could benefit from complement-targeted therapies.

The aim of this narrative review is to summarize recent knowledge about the diagnostic significance of immunohistochemical

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detection of C4d with the focus on kidney glomerular diseases and kidney transplantation. We broadened the present narrative review with our own experience with preparation and practical use of monoclonal C4d antibodies, on a national level.

C4d deposits in some kidney pathologies

Although the importance of deposits of C4d is mentioned most often in relation to kidney transplantation, this marker of complement activation is also important in other various kidney glomerular pathologies (2).

Immunoglobulin A nephropathy is a glomerular disease mostly affecting the mesangium, as prevalent immunoglobulin A deposition associated with mesangial proliferation is the characteristic hallmark of this entity. C4d deposits at glomerular levels have been reported to be associated with an adverse prognosis and may serve as a useful biomarker of disease prediction in immunoglobulin A nephropathy (11). C4d deposits might theoretically be produced by the lectin pathway activation. However, the mechanism leading to C4d production and deposition are not completely clear, as galactose-deficient immunoglobin A1 selectively activates the alternative complement pathway, while lacking activity on the lectin pathway (12, 13). For immunoglobulin A nephropathy patients, the majority of C4d deposited in renal glomeruli and tubules, with less found in the interstitium and peritubular capillaries of renal tissue (14). Jebali et al (15) found positive glomerular C4d staining in 57% of renal biopsies of patients with immunoglobulin A nephropathy. Proteinuria levels were significantly higher in these patients, and according to mentioned studies, C4d may be used as a marker to evaluate the condition and prognosis of adults with immunoglobulin A nephropathy. However, there have been few studies conducted on pediatric populations with immunoglobulin A nephropathy. Zhou et al (16) posited that in children, C4d is found to be associated with proteinuria, segmental lesions, and immunosuppressant treatment. Activation of the lectin pathway may reflect the severity of clinical and pathological manifestations of immunoglobulin A nephropathy in children. Similarly, Fabiano et al (17) conclude that the positivity for C4d in the mesangial area is an independent predictor of progression of kidney disease in pediatric patients with immunoglobulin A nephropathy. Additionally, the recurrence of immunoglobulin A nephropathy limits graft survival in kidney transplantation, and C4d positivity was found to be associated with graft loss in patients with immunoglobulin A nephropathy recurrence (18).

Membranoproliferative glomerulonephritis is an uncommon pattern of glomerular injury on kidney biopsy, with characteristic light microscopic changes, including mesangial cell proliferation and structural changes in glomerular capillary walls (thickening of the glomerular basement membrane) (19). Membranoproliferative glomerulonephritis is not a specific disease entity but a histopathologic lesion with two subtypes (immune complex-mediated and complement-mediated known as C3 glomerulopathy). C4d staining may help to differentiate between immune complex-mediated membranoproliferative glomerulonephritis and C3 glomerulopathy. In general, specimens of immune complex-mediated glomerulonephritis show mostly bright C4d staining. Conversely, C4d staining is mostly negative in specimens from patients with C3 glomerulopathy (20, 21).

Systemic lupus erythematosus is an autoimmune disease, and up to 60% of systemic lupus erythematosus patients developed **lupus nephritis** during the course of the disease. Lupus nephritis is an important cause of morbidity and even of mortality in patients with systemic lupus erythematosus. Immune complex deposition and complement activation in the kidney significantly contributed to the pathogenesis of lupus nephritis (22). In lupus nephritis patients, the majority of C4d is deposited in glomeruli, peritubular capillaries and tubular basement membrane, with less found in renal interstitium of the renal tissue. The presence of C4d in renal tissue acted as an independent predictor of relapse for lupus nephritis patients, while arteriolar C4d deposition is associated with renal microvascular lesions and worse renal outcomes (14, 23). Some recent data suggest that renal C4d is a potential biomarker for disease activity and severity not only in adult population, but also in pediatric lupus nephritis patients. Study of Wang et al (24) revealed that in pediatric lupus nephritis patients, glomerular C4d, peritubular capillary C4d, and tubular basement membrane C4d were positively correlated with proteinuria, activity and severity of the disease, and hypertension, respectively.

Membranous nephropathy, also referred to as membranous glomerulopathy, is one of the leading causes of nephrotic syndrome which accounts for 20–30% of nephrotic syndrome cases in adults and 1–9% of cases in pediatric population (25). Membranous nephropathy is defined by the presence of subepithelial immune complex deposits with a spectrum of changes in the glomerular basement membrane. A diffuse continuous C4d staining pattern in the glomeruli is observed in cases with primary membranous nephropathy whereas a discontinuous C4d staining in the glomeruli favors secondary membranous nephropathy (26). The sensitivity and specificity of the differentiation of membranous nephropathy from other glomerulopathies by way of C4d immunohistochemistry is 95% and 87.5%, respectively. In contrast to electron microscopy and immunofluorescence, this method is more practical and cost effective, requiring a lower level of skill and advanced laboratory equipment (25).

C4d immunohistochemistry/immunofluorescence may be a valuable biomarker associated with other kidney diseases histopathologic diagnostic and/or progression, as the thrombotic microangiopathy (27, 28), human anti-glomerular basement membrane disease (29) or post-infectious glomerulonephritis (30).

C4d deposits in kidney transplantation

Transplantation is a widely recognized method of treatment at the terminal stages of many renal, cardiac, hepatic, and pulmonary diseases. Despite considerable advances in that field, graft rejection is still an important clinical problem (31). In August 1991, a group of 12 pathologists and transplant clinicians led by Kim Solez and Lorraine Racusen met in Banff, Alberta (Canada), and established the first widely accepted diagnostic

criteria for kidney transplant rejection and other lesions seen on kidney allograft biopsies, the so-called **"Banff Classification of Kidney Allograft Pathology"** (32). Since that time, Banff conferences have been held every 2 years at sites around the world, resulting in several major additions and revisions to the classification and network extensions, from criteria based purely on histopathology to the later involvement of physicians and surgeons, geneticists, immunologists, and other basic scientists, including biostatisticians and data scientists. This multidisciplinary and international approach has allowed the Banff classification to gain overwhelming international acceptance as the main reference used for the scoring of kidney allograft biopsies in research studies, routine practice, and clinical trials. Banff meeting reports have been among the most cited papers in the field of transplantation medicine (33). The first edition of the Banff working classification of kidney transplant pathology in 1993 was focused primarily on T lymphocyte–mediated rejection with only a minor mention of antibody-mediated rejection. The first version lacked the detection of C4d in transplant organ biopsies (32). Our understanding of the pathology and clinical consequences of acute antibody-mediated rejection was greatly aided by the introduction of complement C4d immunostaining, first described in renal allograft biopsies in 1993 (34). It was in this context that the initial Banff classification for acute and chronic active antibody-mediated rejection was first developed at the 2001 Banff conference. This classification, published in 2003 (35), subsequently underwent minor modifications at the 2007 conference (36). The basis of this classification was that three separate pieces of evidence were needed to diagnose antibody-mediated rejection, i.e., histologic evidence (microvascular inflammation and injury), serologic evidence and immunohistologic evidence, usually of C4d deposition in peritubular capillaries (37). The latest Banff classification (38) included one important revision regarding C4d staining, specifically replacing the requirement for C4d staining for antibody-mediated rejection diagnosis with the "evidence of current / recent antibody interaction with vascular endothelium," which includes but is not limited to C4d deposition. This revision was elicited by the fact that multiple studies supported the existence of antibody-mediated rejection with negative or minimal C4d deposition. A new entity was recognized and included into the antibody-mediated rejection classification as C4d-negative antibody-mediated rejection (33). C4d-negative antibody-mediated rejection has a low incidence rate. It usually presents early after transplantation but carries better outcome than C4d-positive antibody-mediated rejections (39).

Histological methods of C4d staining

The detection of peritubular capillary C4d deposition in tissue sections of renal allograft biopsies became an important aid in the diagnosis of antibody-mediated rejection. Staining renal allograft biopsies for C4d has become a routine histopathological practice for pathologists in many major transplant centers. Two histological methods can be routinely employed: **immunofluorescence** on frozen tissue using monoclonal antibody to C4d (with the downside of unsteady availability of frozen tissue) or **immunohistochemistry** using C4d antibodies on formalin-fixed and paraffin-embedded renal tissue (2). Data from numerous studies (40–42) indicate that both widely used methodologies to detect C4d in peritubular capillaries of renal allograft biopsies are reliable and provide adequate and comparable results. In renal transplantation, C4d staining by immunofluorescence still appears to be more sensitive although it is more complicated in routine practice due to the need of frozen renal tissue in contrast to easier use of fixative solution, the formalin. It is worth noting that in healthy (normal) kidneys, the C4d depositions in the mesangium, along the glomerular capillaries, and in some renal arteriolar walls have been observed in frozen tissue. The results of Zwirner et al (43) suggest that the C4d fragment in normal human glomeruli is indicative of a continuous, local complement activation via the classical pathway induced by the physiological deposition of IgM-containing immune complexes. Not only does glomerular deposition of complement C4d indicate activation via the classical pathway, but it also represents a general phenomenon of renal homeostasis and seems to be involved in the physiological clearance of immune complexes (44). Nowadays, the immunohistochemical technique using polyclonal anti-human C4d antibodies is so sensitive that it can detect mesangial positivity for C4d in more than one-third of normal (healthy) renal tissue (45).

Practical example of anti-C4d antibodies preparation

Several monoclonal and polyclonal antibodies against the complement component C4d are currently available on the diagnostic antibody market. These antibodies target several different epitopes of the C4d protein. Using our proprietary method, we designed a specific linear immunogenic peptide to produce antibodies against complement component C4d.

The process consists of 18 to 22 steps, commencing with a demanding structural analysis of the antigen (protein) molecule to identify possible linear epitopes. These potential linear epitopes are considered in the context of the higher protein structure and then analyzed using special techniques and software ("Epitope Design and Analysis System" or "EDAS"). The EDAS epitope mapping process is unique and far surpasses traditional epitope mapping software and processes.

The analysis of the protein aims at determining its naturally occurring form, structure, hydrophobic and hydrophilic sites, intracellular and extracellular sites, possible interactions with other proteins within related cell signaling pathways, and potential interactions with nucleic acids (DNA/RNA). The final output of the analysis identifies three or four linear epitopes that will be accessible to antibody binding under any circumstances, and this accessibility is not affected by conformational changes of the target protein (marker) itself.

Target linear epitopes usually consist of 4 to 7 amino acids. By carefully selecting the adjacent amino acids at the beginning and end of the amino acid sequence of the epitope, an immunogenic peptide is selected. A rabbit is immunized with this immunogenic

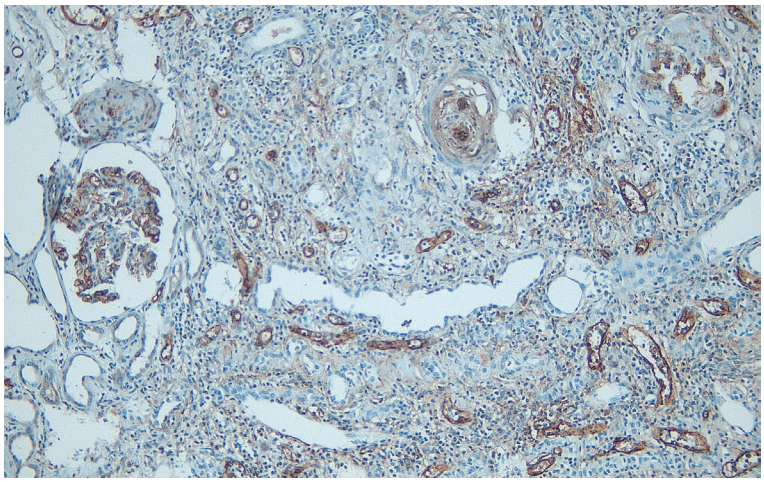

Fig. 1. Example of C4d staining in rejected kidney tissue with Anti-C4d monospecific clonal antibody DB107 manufactured according to our proprietary methodology. The staining shows specific positivity of peritubular and glomerular capillaries in the tissues of the rejected kidneys.

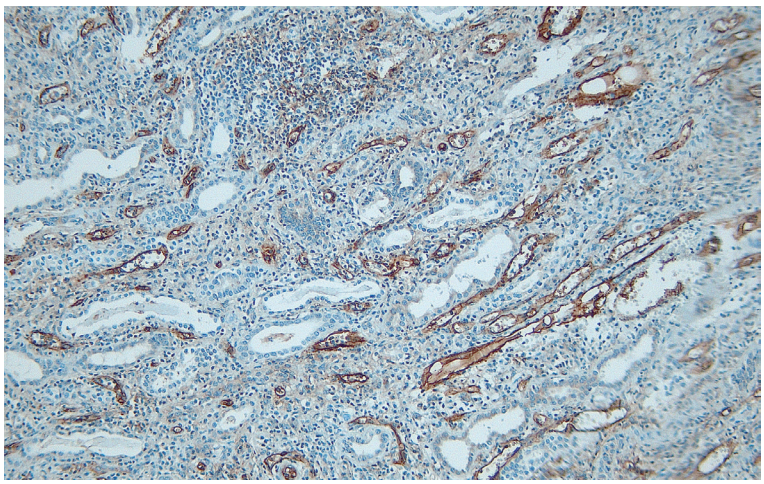

Fig. 2. Kidney rejection after transplantation. Histological section of kidney tissue after biopsy, fixed in formalin and embedded in paraffin, immunohistochemically stained using primary anti-C4d antibody against complement component C4d and secondary antibody labeled with horseradish peroxidase. Brown staining confirms the presence of the C4d component of complement in the peritubular capillaries, positively indicating kidney rejection.

peptide to stimulate its immune system and produce specific antibodies against the injected peptide. After approximately three months, a blood sample is taken from the rabbit in order to obtain hyperimmune serum (antiserum) containing the desired antibody.

Using proprietary antibody clone selection technology ("Epitope-Specific Entropic In-Vitro Antibody Capture System" or "EVAC"), the monospecific immunoglobulin is then separated from the crude anti-peptide polyclonal antiserum based on minimum-entropy criteria against the respective originally designed epitope. This immunoglobulin is a product of one B-cell line. Traditional immunoaffinity purification is not employed because it often results in a heterogeneous mixture of immunoglobulins. The purification process yields a monospecific clonal antibody.

Utilizing rabbits allows for providing an antibody that is fully post-translationally modified, properly glycosylated, and therefore more stable than antibodies produced by traditional cell systems. The use of rabbits to generate the native complete antibody structure avoids the employment of a cellular system, which often results in an incorrectly or incompletely glycosylated antibody.

The produced anti-C4d antibody was tested, confirming its binding to the C4d protein fragment in the peritubular capillaries of the rejected kidney (Figs 1, 2). The antibody can be applied to detect C4d fragment in various laboratory methods used for diagnostic and research purposes.

Conclusions and further perspectives

Evaluation of complement components is essential in the clinical practice of nephrology and renal pathology. A complement-focused approach has influenced the pathological classification of glomerulopathies and provides invaluable mechanistic insights into these disorders (45). To summarize, the consistent pattern of C4d staining in membranous nephropathy and immune complex-mediated membranoproliferative glomerulonephritis can be used as a valuable adjunct tool in establishing the diagnosis, especially when immunofluorescence findings are limited by inadequate sampling. C4d reactivity in other kidney glomerular diseases is variable and as such may not serve as a diagnostic tool in renal biopsy evaluation (46). The clinical significance after kidney transplantation of the category of C4d staining without evidence of rejection remains questionable and requires further investigation (37). The risk of no induction immunosuppression significantly exceeds the risks associated with its administration and is desirable even in patients at low immunological risk. Induction immunosuppression should be tailored individually and thus it differs from patient to patient (47)

Immunohistochemical staining for C4d has revolutionized the field of renal histopathology. Despite being a simple diagnostic test, its utility can be of utmost importance, especially in a resource-poor setting where immunofluorescence and frozen tissue may not be available (26). It should be considered that kidney transplantation may be affected not only by autoimmune reactions, but also by particular congenital or acquired urological diseases such as infections, vascular complications, or complications connected with the reconstruction of the lower urinary tract. The incidence of urological complications is reported to be in the range of 1% to 30% of transplants, representing up to one half of all surgical complications (48–53).

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