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.

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 process, 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 categorized 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, thio-ester 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. 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, 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 (diagram 1) [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-transplantation 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 clinicopathological 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 (approximately 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 or by immunohistochemical techniques in formalin-fixed and paraffin-embedded specimens (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 (Diagram 2). 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), 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 [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]. Less than 5% of our C4d-positive cases present with so-called acute pure humoral rejection: acute tubular injury, abundant inflammatory cells including polymorphonuclear leukocytes in dilated 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 extensive thrombosis of large arteries is uncommon. ‘Acute pure humoral rejection episodes’ 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 that 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 to the development of chronic rejection currently is only incompletely understood.

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 micro 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 micro 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 favorable, 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 (Table 1) [31].

Treatment (Diagram 3)

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 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 tubulointerstitial 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 with signs of concurrent cellular rejection. 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].

**Future perspective**

A major change of our philosophy explaining renal allograft dysfunction currently is occurring. C4d has ‘magically’ enabled us to detect humoral mediated alloresponses 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 alloantibodies 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.

Diagram 1

![Diagram 1](image-url)
Diagram 2: Histological changes and the detection of C4d along peritubular capillaries

In renal allograft biopsies, C4d can be detected in association with different histologic 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”.
**Diagram 3:** Clinical implications of C4d positive renal allograft biopsies

<table>
<thead>
<tr>
<th>Biopsy Findings</th>
<th>REJECTION</th>
<th>OTHER CHANGES*</th>
<th>NORMAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular</strong></td>
<td><strong>Humoral</strong>**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4d + HLA-DR in tubules +</td>
<td>C4d + HLA-DR in tubules -</td>
<td>C4d + HLA-DR in tubules -</td>
<td>C4d + HLA-DR in tubules -</td>
</tr>
<tr>
<td>S-Cr high</td>
<td>S-Cr low***</td>
<td>S-Cr high</td>
<td>S-Cr low***</td>
</tr>
</tbody>
</table>

**Aggressive therapy**

ATG/OKT3 – or - tacrolimus/mycophenolat-mofetil rescue and/or plasmapheresis – or – immunoabsorption – and/or – IVIG

**No anti rejection therapy**

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 hemorrhage 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, smoldering 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.
<table>
<thead>
<tr>
<th></th>
<th>Graft Dysfunction</th>
<th>Antibodies (donor specific)</th>
<th>C4d</th>
<th>Morphological Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Form I</strong></td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Form II</strong>*</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Form III</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Form IV</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* These cases potentially represent subclinical pure humoral rejection or accommodation

Δ Most of these cases likely represent subclinical rejection, often humoral and cellular

** 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
Appendix: Detection of C4d along peritubular capillaries and MHC-class II (HLA-DR) in tubules

1) 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].

2) 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 degrees C (dilution 1:50) and subsequent avidin/biotin histochemical staining procedures.

3) The upregulation of MHC class II (HLA-DR) 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).

References

32. Montgomery R, Zachary AA, Racusen LC et al. 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 2000; 70: 887–894
Chronic Rejection and Chronic Allograft Nephropathy: Current Concepts

Robert B. Colvin, M.D.

DEFINITION

The term “chronic allograft nephropathy” (CAN) entered primetime in 1993 as a category in the Banff working classification that was to include “at least four entities that at present cannot always be distinguished by biopsy (chronic rejection, chronic cyclosporine toxicity, hypertensive vascular disease and chronic infection and/or reflux) [1]” The rationale was that “because it is often impossible to define the precise cause or causes of chronic allograft damage, the term “chronic/sclerosing allograft nephropathy” is preferable to “chronic rejection,” which implies allogeneic mechanisms of injury, unless there are specific features to incriminate such a rejection process” (underline added) [2]. A rejection process was suggested according to the Banff classification by intimal fibrosis with inflammatory cells and duplication of the glomerular basement membrane (GBM)[2]. CAN was not intended to replace specific diagnostic categories, if these entities could be identified.

Unfortunately, “CAN” is often misused as a generic term for chronic renal allograft dysfunction and fibrosis, or as a synonym for “chronic rejection.” If indiscriminately applied to all causes of renal allograft dysfunction with fibrosis, the term inhibits accurate diagnosis and appropriate therapy and therefore has little value, other than to hide our ignorance. In my view and according to the original Banff conception, CAN should be restricted to the minority of biopsies that are truly nonspecific in their pathology (i.e., CAN, not otherwise specified, or CANNOS for short). This review is based in part on a recent Perspectives article in the New England Journal of Medicine [3], a previous review [4] and an article in press on surrogate markers [5].

IMPORTANCE

The rate of long-term graft loss changed little over the last decade, despite dramatic improvements in short term graft survival. Most late graft loss, other than patient death, has been attributed to CAN. Despite (or perhaps because of) the ambiguous use of the term, CAN publications have increased exponentially from 1 in 1993 [1] to 93 in 2003 (as of 12/13/03: PubMed search).

SPECIFIC DIAGNOSES AND CRITERIA

The pathologist should provide answers to three clinical questions in renal allografts: 1) the cause of graft dysfunction, 2) the current activity of the process and 3) the degree of irreversible damage that has already occurred [5]. Answers to these questions are feasible with current pathologic techniques and should be enhanced by
molecular probes [6]. Clearly, protocol biopsies are critically important, not only for elucidating pathogenetic mechanisms before the process becomes nonspecific, but to guide early interventional therapy in patients who appear to be doing well, but actually have subclinical active graft injury.

How often can the cause of chronic damage be defined? Most of the diseases in the table below have distinctive features recognized by renal pathologists using modern techniques, at least in the early stages [4]. The criteria for active T cell and antibody mediated rejection are well established [7].

### Diagnosis Affects Treatment of CAN

<table>
<thead>
<tr>
<th>DISEASE</th>
<th>PUTATIVE THERAPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Immunologic Rejection</td>
<td>↑ immunosuppression</td>
</tr>
<tr>
<td>T cell mediated</td>
<td>↑ immunosuppression</td>
</tr>
<tr>
<td>Antibody mediated</td>
<td></td>
</tr>
<tr>
<td>2. Drug toxicity</td>
<td>↓ immunosuppression</td>
</tr>
<tr>
<td>Calcineurin inhibitors</td>
<td>Varies with disease</td>
</tr>
<tr>
<td>3. Recurrent disease</td>
<td></td>
</tr>
<tr>
<td>4. Infection</td>
<td>↓ immunosuppression</td>
</tr>
<tr>
<td>Polyomavirus</td>
<td>Management of hypertension</td>
</tr>
<tr>
<td>5. Hypertension</td>
<td></td>
</tr>
<tr>
<td>6. Mechanical</td>
<td>Surgical</td>
</tr>
<tr>
<td>Obstruction/reflux</td>
<td>Angioplasty</td>
</tr>
<tr>
<td>Renal artery stenosis</td>
<td>None, if process inactive</td>
</tr>
<tr>
<td>7. Scarring from past episodes of ischemia</td>
<td></td>
</tr>
<tr>
<td>or acute rejection</td>
<td></td>
</tr>
<tr>
<td>8. Donor disease</td>
<td>?</td>
</tr>
<tr>
<td>Senescence</td>
<td></td>
</tr>
<tr>
<td>Hypertensive nephrosclerosis</td>
<td>?</td>
</tr>
<tr>
<td>9. Chronic allograft nephropathy, NOS</td>
<td>?Requires identification of pathogenesis</td>
</tr>
<tr>
<td>Unclassified tubular atrophy</td>
<td></td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td></td>
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<tr>
<td>Glomerular sclerosis</td>
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</table>

### Diagnostic Changes in Renal Allografts

**Chronic Rejection.** Many studies clearly point to immunological mediation of a substantial fraction of chronic allograft damage. For example, 12-28% of protocol biopsies one year or more after transplantation have acute cellular rejection, a finding that predicted the later progression of interstitial fibrosis and tubular atrophy and graft failure [8]. Living related HLA-identical grafts have no CAN in the first 5 years, and about half the frequency of CAN that haplo-identical living related grafts after 5 years [9]. Another study showed that among 10 HLA-identical recipients, none had histologic evidence of CAN in protocol biopsies at 2 years, compared with 50% of
those who received cadaveric kidneys [10]. Circulating antibody to donor HLA antigens is highly associated with later graft loss [11, 12].

“Chronic rejection” refers specifically to graft injury due to immunologic reaction to donor antigens. The typical features attributed to repeated or persistent attack on graft target cells are arterial intimal fibrosis, glomerular mesangial expansion and GBM thickening, tubular atrophy and interstitial fibrosis, in varied degrees and combinations. The vascular and glomerular lesions are the most distinctive and are prime morphologic criteria of chronic rejection.

**Chronic Allograft Arteriopathy (CAA).** The arteries show pronounced fibrous intimal thickening with myointimal cells, collagen fibrils, focal calcification, a variable infiltrate of T cells (often subendothelial), and lipid filled, foamy macrophages disposed characteristically against the external elastica, which is duplicated and disrupted. The adventitia also often has an infiltrate of mononuclear cells, sometimes invading and destroying the outer media. The mononuclear infiltrate and the foamy macrophages (if present) distinguish this lesion from hypertensive intimal thickening. Hyaline change in the outer media and isolated myocyte degeneration are not features of rejection, in contrast to CsA toxicity. Experimental models have indicated that the lesions are due to a response to alloantigens, either class I, II or non-MHC antigens are sufficient. Based on experimental studies antibody is believed to be important in the progression to intimal fibrosis [13], although other immunologic reactivity, including T cells NK cells may contribute [14].

Those findings that I regard as specific for rejection (vs. hypertensive, aging and diabetic changes) are a mononuclear infiltrate in the intima or media, intimal foam cells against the internal elastica, a moth-eaten and thin media due to loss of smooth muscle cells and (less specifically) a prominent perivascular infiltrate. Marked duplication of the internal elastica, a normal or thickened media and relative sparing of the larger arteries (arcuate and larger) are more typical of hypertension [15]. The arterioles are relatively spared in chronic rejection, compared with chronic CsA toxicity, thrombotic microangiopathy/hemolytic uremic syndrome and systemic sclerosis. These processes do not cause a mononuclear infiltrate in the vessels. However, the healing phase of hemolytic uremic syndrome and systemic sclerosis may leave intimal fibrosis that resembles chronic rejection

**Chronic Allograft Glomerulopathy (CAG).** The glomeruli have an increase in mesangial cells and matrix and thickening and duplication of the GBM, with various degrees of scarring and adhesions. Foot process effacement, focal mesangial cell interposition, and mesangiolyisis may be present [16]. Endothelial cell "dedifferentiation" is often evident, as manifested by a loss of the normal fenestrations [17, 18]. Immunofluorescence shows segmental or granular deposits of
immunoglobulin (typically IgM and IgG, rarely IgA), C3 and sometimes fibrin in the capillary wall and in the mesangium. This lesion has been shown to derive from acute allograft glomerulopathy in a few cases [19, 20]. Intraglomerular leukocytes express ICOS and CXCR3, markers of activated effector T cells [21]. An increase in glomerular ecto-AMPase activity and a decrease in ecto-ATPase activity have been demonstrated in glomeruli in chronic allograft injury, a response typical of ischemia [22]. Atubular glomeruli account for up to about 60% of the glomeruli in CAN [23] and in one study were about 18% of the glomeruli in chronically injured allografts. They can be identified in serial sections, but not in single sections [24]. The pathogenesis is probably destruction of the proximal tubule near their origin [23].

The glomerular features are not specific for chronic graft rejection, but are typical. The most distinctive features in my opinion are the loss of endothelial fenestrations and duplication of the GBM. The other diseases with similar light and electron microscopic glomerular features also are characterized by endothelial injury (thrombotic microangiopathy, scleroderma, and eclampsia). Extensive crescents, diffuse granular or linear deposits of IgG, or subepithelial deposits are unusual and suggest recurrent or de novo glomerulonephritis as does IgA deposition [25].

**Peritubular Capillary Basement Membrane Duplication.** By EM a chronic lesion in the peritubular capillaries has been observed consisting of splitting and multi-layered duplication of the basement membrane, analogous to and correlated with the chronic glomerular changes [26] and with C4d deposition in PTC [27]. Thus, the common theme in chronic rejection is endothelial damage at the level of the arteries, glomeruli and peritubular capillaries. The microvascular endothelium remains of donor origin and expresses donor antigen even after 25 years and thus remains a potential target of an alloresponse indefinitely [28]. Decreased PTC density (capillaries/μm2) has been observed in chronic rejection [29].

**Interstitial fibrosis and tubular atrophy (IFTA).** This is the least specific of the findings and accompanies or follows almost any significant injury to the kidney. However, in the setting of an allograft and in the absence of any indicators of specific cause (e.g. glomerulopathy, arteriopathy or hyalinosis), this lesion behaves like chronic rejection, and its presence correlates with the same parameters as chronic rejection with arteriopathy [30]. Late acute rejection episodes (after 3 months post-transplantation) were the strongest risk factor for interstitial fibrosis with or without arteriopathy (OR 14.7). The association of interstitial fibrosis with young recipient age, panel reactive antibodies at the time of transplantation and late acute rejection suggest an immune pathogenesis, consistent with chronic rejection. Tubular atrophy is also associated with C4d in PTC[27]. A history of rejection, calcineurin inhibitory toxicity or CMV infection were the leading predictors of fibrosis and tubular atrophy in protocol biopsies at 2 years [31].
Chronic Humoral Rejection (CHR). C4d, a fragment of the complement component C4, is a reliable marker of antibody mediated rejection [32]. C4d in peritubular capillaries is highly associated with circulating antibody to donor HLA antigens, duplication of the glomerular and peritubular capillary basement membranes, preceding GBM changes [27, 32]. Studies from Vienna indicate that about 34% of biopsies taken after one year have C4d. Furthermore the presence of C4d predicts the later development of CAG. Elution of grafts with CAN usually detects antibodies to donor HLA class I or II antigens (71%), even though a minority (32%) had antibody detectable in the circulation [33]. Criteria for the diagnosis of chronic humoral rejection were defined by an NIH consensus conference [34].

Draft Proposal for Chronic Humoral Rejection [34]

1. Clinical evidence of chronic graft dysfunction
2. Histologic evidence of chronic injury: need 3 of 4
   • Arterial intimal fibrosis
   • Duplication of glomerular basement membrane
   • Interstitial fibrosis / tubular atrophy
   • Laminated PTC basement membrane
3. Evidence for Ab action/deposition in tissue (C4d in PTC)
4. Serologic evidence of anti-HLA antibody

Four elements should be present (Table): graft dysfunction, histologic evidence of chronic injury, immunopathologic evidence of antibody action (e.g., C4d) and evidence of antibody reactive to the donor in the circulation. Chronic humoral rejection may arise through a series of stages as diagrammed below. Further studies should identify the significance and appropriate therapy for each of these stages.

Hypothesized Stages of Chronic Humoral Rejection

<table>
<thead>
<tr>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Graft dysfunction (clinical chronic rejection)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Graft injury (pathology in graft biopsy)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>C4d detectable in graft microvasculature</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>De novo antibodies detectable in circulation</td>
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</tr>
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</table>

Transplant

Antibodies first produced

Time (not to scale)

Graft loss
**Chronic Cellular Rejection.** This category probably exists, but has not been defined in the Banff system. A practical marker equivalent to C4d, that indicates current T cell activity, has not been identified. In analogy to CHR, one would require 1) graft dysfunction, 2) chronic structural change (probably the same ones as for CHR), 3) presence of T cells in sites of chronic damage, i.e., the arterial intima, glomeruli, tubules, and interstitium and 4) demonstrable T cell reactivity to donor. In studies of late protocol biopsies concomitant acute cellular rejection often accompanies chronic lesions (e.g., in one series 37% at 6-12 months and 12-20% thereafter) [35] and predicts the later occurrence of chronic injury[8, 10, 35, 36]. Late acute cellular rejection (after 4 months) was a stronger predictor of CAN than rejection in the first 3 months [8]. Furthermore, the presence of tubulitis in CAN probably indicates ongoing activity, since about 41% of patients respond to increased immunosuppression [37].

The simplest definition of chronic cellular rejection would be presence of acute cellular rejection according to standard criteria and the presence of chronic lesions (interstitial fibrosis, chronic allograft arteriopathy or glomerulopathy). However, this would presume that the chronic and acute lesions were related. Certainly, numerous studies have shown a strong correlation between acute cellular rejection in protocol biopsies and the later development of chronic injury [35, 38].

**Chronic Calcineurin Inhibitor Toxicity.** Chronic calcineurin inhibitor toxicity characteristically causes nodular hyaline deposition in the periphery of arterioles; in contrast, diabetes, hypertension and senescence have subendothelial hyaline (Fig 4) [39]. Later biopsies show progressive scarring of arterioles, intimal fibrosis and segmental glomerular obsolescence. Focal segmental glomerulosclerosis is correlated with arteriolar hyalinosis, suggesting a relationship with cyclosporin toxicity. FSGS was associated with increased rate of graft loss independent of the degree of interstitial fibrosis [40]. Hyalinosis has a better prognosis than allograft arteriopathy and is not associated with episodes of acute rejection [41]. The features that favor chronic rejection over chronic CsA toxicity are duplication of the GBM and marked intimal fibrosis of the small arteries [39]. Numerous plasma cells in the infiltrate are also more typical of chronic rejection (plasma cells were an average of 21% of the infiltrate vs. 3% in CsA toxicity) [42]. Late protocol biopsies in diabetics has shown evidence of a high frequency (50-100%) of chronic calcineurin inhibitor toxicity [35].

**Polyomavirus Infection.** Polyomavirus infection can be demonstrated immunohistochemically in the active phase [43]. Late biopsies may reveal only tubular destruction and interstitial fibrosis, without viral antigens. Residual virus may be detected by PCR [44, 45].

**Recurrent Disease.** Recurrent disease is a small, but significant, cause of late allograft failure, estimated to be 1-5% of recipients [46]. Obviously, the diagnosis of
recurrence requires accurate classification of the original disease and lesions that differ from chronic allograft glomerulopathy. The frequency and clinical significance of recurrence varies with the disease [46-48]. Recurrent glomerular disease is usually recognizable by the same features that permitted the original diagnosis [4].

**Obstruction/Reflux.** The features of chronic obstruction include dilated collecting ducts, rupture of tubules with leakage of PAS+ Tamm-Horsfall protein into the interstitium with associated inflammation and dilated lymphatics. Interstitial fibrosis and tubular atrophy with a modest mononuclear infiltrate is common. Among patients with “borderline” scores for rejection, 12% had evidence of obstruction [49].

**Hypertensive Arteriosclerosis/Benign Nephrosclerosis (Donor Disease).** Donor disease is most definitively recognized by implantation biopsies or other biopsies early in the course (< 1 mo). The features that favor rejection over hypertension or calcineurin inhibitor toxicity are given above.

**Senescence (Donor Disease).** Halloran has proposed that senescence of the donor kidney contributes to an ineffective reparative response to injury and hence the consequences of ischemia, rejection and drug toxicity on the allograft [50]. One measure of senescence in the human is loss of telomere length, which has been demonstrated in allografts [51]. New markers have been proposed to identify senescence, namely p21 (WAF1/CIP1) cyclin-dependent kinase (CDK) inhibitor gene [51], p16 and p27 cyclin-dependent kinase inhibitor genes[52] and senescence-associated beta-galactosidase [53]. In one study the markers had a linear correlation with age in normal kidneys’ this relationship was then applied to CAN in grafts, which had biological age values about 15 years older than their chronological age [52]. Senescence as judged by FISH telomere length or beta-galactosidase correlated with the severity of the chronic allograft damage and donor age, but not with prior acute rejection or ATN [51].

**Renal artery stenosis.** The pathology of pure renal artery stenosis is distinctive from the other conditions above and include diffuse tubular atrophy without fibrosis or vascular disease, small unscarred glomeruli and no interstitial infiltrate.

**Chronic allograft nephropathy, not otherwise specified.** In our experience, a majority of biopsies taken for late graft dysfunction can be assigned to a specific diagnosis. In one series only 37% had non-specific tubulo-interstitial fibrosis and tubular atrophy [32]. This residual group is rightfully defined as “CAN” by Banff and should be the subject of research to define its pathogenesis.
Specific Diagnoses Late Renal Allografts with Slowly Progressive Dysfunction (n=155) [32]

- Chronic humoral rejection 12%
- De novo glomerular disease 13%
- Recurrent glomerular disease 16%
- Calcineurin inhibitor toxicity 14%
- Chronic rejection, C4d-
- Interstitial fibrosis with tubular atrophy 37%*
- 100%

*This category alone meets the definition of chronic allograft nephropathy, not otherwise specified.

ASSESSMENT OF ACTIVITY

Among the established measures of immunologic activity are those related to injury mediated by T cells (tubulitis, interstitial inflammation, infiltration of arterial intima) and antibody (C4d deposition in PTC). Other assessments are likely to be useful in the future. Many of these have shown promise in research studies, such as include markers of cell death (TUNEL), cell proliferation (Ki67, PCNA), IFNγ action (HLA-DR) [54], matrix synthesis (mRNA for collagen types, matrix related proteases and their inhibitors) [55], cytokines (transforming growth factor β, TGFβ). These molecular markers and others to come will enhance the pathologists ability to detect ongoing activity and determine which pathways are most appropriate for therapeutic intervention.

The complexity of interpretation can be illustrated by the extensive literature on TGFβ. TGFβ expression in grafts has been associated with chronic allograft arteriopathy [56] and glomerulopathy [57], and the severity of interstitial fibrosis [58, 59]. Subsequent loss of renal function is more severe in patients with increased TGFβ, according to one study [60], but another finds that TGFβ expression in acute rejection predicts less CAN later [55]. TGFβ is increased more in patients on cyclosporin compared with tacrolimus [36]. Angiotensin II receptor protein and mRNA is increased in “CAN” [61] and angiotensin II receptor antagonists such as losartan inhibit expression of TGFβ and may slow the progress of fibrosis [62]. One must also consider that some forms of TGFβ are inactive or are precursors and that it is rendered inactive by antagonists that bind it, such as decorin [63].

MEASUREMENT OF DAMAGE

The features of CAN can be scored by “eyeball” estimation or by morphometric techniques. Either can use special stains to accentuate the measured variable. The Banff system uses 4 grades of cortical fibrosis (0, <6%; 1, 6-25%; 2, 26-50%; 3, >50%). The reproducibility of these grades is poor, according to a recently published
international study (kappa score of 0.295) and did not improve with practice or by supplying photomicrographs [64]. Other Banff parameters were similarly poorly reproducible (hyalinosis, intimal thickening grade, glomerulitis, extent of infiltrate tubulitis (kappas 0.195-375). Reproducibility improved substantially for intimal fibrosis, intimal arteritis and hyalinosis when microphotographs were used, indicating that the problem for these parameters was identifying the lesion in the glass slides. A small group of pathologists who had worked together had better kappas (0.53-0.65) evaluating protocol biopsies for interstitial fibrosis, but allograft glomerulopathy scoring was still not reproducible [65]. The solution would appear to be to improve the definitions of the lesions, the procedure for evaluation, and/or the knowledge of the evaluators.

Two sources of error are important: 1) sampling and 2) interpretation. It has been estimated that 25% of biopsies are over- or under-scored for fibrosis by sampling [66]. Sampling is particularly a problem in evaluating vascular lesions, since typically, few arteries are included. Errors due to interpretation can be minimized by using morphometric analysis, although the threshold for positive staining (aka, “segmentation), remains subjective [67]. Most morphometry studies have focused on interstitial fibrosis, which is amenable to morphometry and may be less subject to sampling error than vascular lesions. However, because a measurement is more reproducible, it is not necessarily more relevant to progression.

Morphometric Analysis of Fibrosis. The markers used include antibodies to collagen (I or III [68]) and special stains (Masson/Mallory trichrome [69], Sirius red [70]) and picrosirius red [71]). Digital images and computerized data analysis are used to calculate cortical interstitial volume fraction ($V_{IF}$). Since interobserver segmentation is variable, a computer program has been developed which sets the segments automatically for Sirius red staining of interstitial and mesangial areas [67].

Some examples show the power of morphometry. $V_{IF}$ in 6 month protocol biopsies, quantitated with Sirius Red-stained protocol biopsies, was highly correlated with time to graft failure [70]; similar data was found for $V_{IF}$ measured by IHC for collagen III [68]. Measurement of $V_{IF}$ in 12 month biopsies using picrosirius red has demonstrated increased fibrosis in patients on cyclosporin (vs. tacrolimus) [72]. In children, a picrosirius red $V_{IF}$ above 10% predicts a decline in GFR at 2 years [71]. The type of collagen deposited may have diagnostic value. Collagen III accumulates in chronic rejection, CAN and cyclosporin toxicity, but collagen I accumulates to a lesser degree in cyclosporin toxicity [55]. Collagen I content of the whole cortex can be accurately assessed by biopsy samples [73]. It is important to recognize that fibrosis can be reversible after the stimulus is removed, as has been demonstrated in animals [63].
**Combined Scores.** A composite histologic score, the “chronic allograft damage index (CADI),” consisting of the following six assessments: interstitial fibrosis, tubular atrophy, glomerular mesangial expansion, intimal fibrosis, interstitial infiltrates and glomerular sclerosis (scored from 0-3+ each and added), correlates with graft loss at three years [74]. Of these features, the interstitial fibrosis at six months was the most predictive of graft loss by multivariate analysis [74]. In children, previous acute cellular rejection predicts elevated CADI at 3 years and the latter predicts inferior graft function at 5 years.

Other combined scores have been used. A chronic graft damage score at 6 months, calculated from the degree of vascular intimal hyperplasia, glomerular mesangial changes, focal lymphocytic infiltration, focal and diffuse interstitial fibrosis, and tubular atrophy, is strongly associated graft loss 2-3 years after transplantation [75]. The Banff Chronic Sum (calculated as the sum of cg, gi, ct and cv) was not correlated with morphometric analysis of Sirius red staining, but did correlate with graft failure [70].

However, intimal and interstitial fibrosis are correlated not only with rejection, but also with donor disease, age and ischemia [76]. Thus, whether the pathogenesis of the changes, as well as their severity, are relevant to outcome has not been established. A donor biopsy may prove invaluable in assessing the post-transplant change in such parameters.

When predictive variables are combined arithmetically to give a single score, as in the CADI or the Banff “CAN” grade, the individual predictors should ideally be independent (or nearly so), strongly correlated to outcome, and provide some unique contribution to the assessment of the score [3]. These proposed surrogate markers need to be validated in long-term follow-up to see if changes in the scores correlate with changes in the late outcome.

**PROTOCOL BIOPSIES/SURROGATE ENDPOINTS**
Protocol biopsies have been used to predict outcome, with the potential benefit of shortening the follow-up time needed to evaluate efficacy in drug trials [3]. For clinical trials, quantitative pathology generally provides more precise data for correlations. Candidates at this time for an early surrogate marker of long-term graft survival rely on protocol biopsies at a time relatively close to transplant, i.e., 3-6 months. Such biopsies are critical, because structural changes in renal parenchyma that are predictive of graft loss occur significantly in advance of evidence of loss of graft function or proteinuria [74, 76, 77]. In this regard, among the strongest published predictors of graft loss at 2-10 years are arterial intimal fibrosis and interstitial fibrosis, detected on biopsy at 3-6 months post-transplant [74, 76, 78, 79]. However, it should be emphasized that the outcome of a graft is not predetermined.
by early events alone, but is also influenced by subsequent events that are not always predictable, such as non-compliance, drug toxicity and infections.

Nankivell et al [35] reported a valuable study of protocol biopsies in diabetics to assess the “natural” history of CAN (defined as chronic interstitial fibrosis and tubular atrophy, independent of other changes). Functional studies underestimated graft pathology: CAN developed in 94% patients by one year and correlated with previous acute ischemia or subclinical rejection. Subclinical acute cellular rejection was a risk factor for progression, arguing that immunologic rejection is a major pathogenetic factor. Acute rejection was rare after the first 3 months, but subclinical rejection was common, detected in about 25% at 1-2 years and in 5-10% thereafter. After one year the grafts showed progressive arteriolar hyalinosis and glomerular sclerosis; only 5.4% had the chronic arterial or glomerular features suggestive of rejection. Calcineurin inhibitor toxicity was diagnosed in a surprising 50-100% of the biopsies from 7-10 years, as judged by hyalinosis, striped fibrosis and tubular microcalcification. However, other causes, such as hypertension, senescence, or recurrent diabetes, may also contribute to these changes. It was not reported whether the more specific lesion of nodular hyalinosis was present or whether other factors such as polyomavirus infection and humoral rejection played a role.

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Renal Allograft Pathology and the Banff Working Classification

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The Johns Hopkins University
School of Medicine

Purposes - Transplant Biopsy

- Diagnose/grade pathology
- Predict response to therapy
- Predict long-term outcome
  “surrogate markers”

Specimen Adequacy (a necessary prerequisite for coding)

- Unsatisfactory: No glomeruli or arteries
- Marginal: 1-9 glomeruli with one artery
- Adequate: 10 or more glomeruli with at least two arteries

Minimum Sampling:

- 7 slides: 3 H&E, 3 PAS or silver stains, and 1 trichrome
Inflammation in the Allograft

- Acute rejection
- Pre-existing
- Ischemia
- Infection
  - bacterial
  - viral
- Drug reaction
- Neoplastic - PTLD
- “Non-specific”
- Acceptance reaction
Allograft Rejection

Acute
- Cell-mediated
- Antibody-mediated
Chronic
- Cell-mediated
  (Antibody-mediated)

Common Features of Acute Rejection

- Inflammatory infiltrates
- Targeting of structural components

**MORPHOLICAL FEATURES COMMON TO ALL SOLID ORGAN ALLOGRAFTS**
Acute Rejection - cell-mediated

The cells:

Morphology - mononuclear lymphocytic

Immunophenotyping

T cells - CD8 > CD4 - αβ, γδ
CD45RO > RA perforin/granzyme/GMP-17- cytotoxic cells
macrophages

Other cells - eosinophils plasma cells

Acute semiquantitative scores

g – glomerulitis
i – interstitial inflammation
t – tubulitis
v – vasculitis

Graded on scale of 0-3+
Glomerulitis – Banff grading

- G1 – mononuclear infiltration +/- endothelial cell swelling involving <25% of glomeruli
- G2 – involving 25-75% of glomeruli
- G3 – involving > 75% of glomeruli
Tubulitis – Banff grading

- T1 – less than 4 intra-epithelial lymphocytes in the most inflamed tubular cross-section
- T2 – 4-10 intra-epithelial lymphocytes
- T3 - > 10 intra-epithelial lymphocytes
Vasculitis – Banff grading

V1 – intimal arteritis with intimal lymphocytes/edema reducing lumen by < 25%
V2 – intimal arteritis reducing lumen by > 25%
V3 – inflammation/fibrinoid necrosis involving media/transmural

Table 1. (cont.)

<table>
<thead>
<tr>
<th>Type (Grade)</th>
<th>Histopathological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>Cases with significant interstitial infiltration (&gt;25% of parenchyma affected) and foci of moderate tubulitis (&gt;4 mononuclear cells/tubular cross section or group of 10 tubular cells)</td>
</tr>
<tr>
<td>IB</td>
<td>Cases with significant interstitial infiltration (&gt;25% of parenchyma affected) and foci of severe tubulitis (&gt; 10 mononuclear cells/tubular cross section or group of 10 tubular cells)</td>
</tr>
<tr>
<td>IIA</td>
<td>Cases with mild to moderate intimal arteritis (v1)</td>
</tr>
<tr>
<td>IIB</td>
<td>Cases with severe intimal arteritis comprising &gt;25% of the luminal area (v2)</td>
</tr>
<tr>
<td>III</td>
<td>Cases with “transmural” arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells (v3 with accompanying lymphocytic inflammation)</td>
</tr>
</tbody>
</table>

2 Key Concepts

- Borderline/Suspicious for acute rejection
- Subclinical Rejection
Acute Rejection
Pathologic Findings Predicting Refractory Rejection
- Intimal arteritis
- Severe acute glomerulitis
- Necrotizing arteritis
- Interstitial hemorrhage
- Numerous eosinophils, plasma cells, monocyte/macrophages
- CD8-rich infiltrate

Specific Vascular Lesions and Prognosis
- Renal biopsies with AR - scored for variety of lesions
- Steroid resistance - correlates: vascular AR, mononuclear cell adherence, fibrinoid necrosis
- 1 yr. Graft survival - correlates: fibrinoid necrosis, activated endothelium, int. hemorrhage

Renal Allograft Rejection with Arteritis – Predictors of Response to Therapy and Survival
- 185 index biopsies with Type 2A or 2B AR
- Initial response to therapy:
  - worse in 2B vs 2A
  - worse in 1,2,3 vs 0, 1
- Graft survival:
  - worse in 2B vs 2A (HR 1.9, p<0.5)
  - (NS when adjusted for initial response)
- Conclusion: distinction between 2A and 2B significant; degree of tubulitis should be specified
Plasma cell-rich rejection
(Charney, et. al., Transplantation 68: 1999)

- Plasma-cell rich (>300/hpf) vs “classic” AR
- Comparison of clinical, pathological data
- Greater rate of graft failure in PCAR
- PCAR not correlated with CAN, virus infection

Antibody-mediated rejection
Morphologic clues may lead to diagnosis

- Marginating cells - neutrophils, mononuclear
- Early/severe arteritis/fibrinoid necrosis
- Thromboses
- Hemorrhage/infarction
- Complement deposition

Antibody-mediated rejection

The target

- Endothelium of arteries, capillaries
- AB antigens
- HLA antigens - class I, class II,
- Endothelial antigens
Acute Rejection - antibody mediated

Effectors

Complement
Neutrophils
Platelets
Acute Humoral Rejection - Complement

Collins, et al, JASN 10:2208, 1999

- 16 bx from 10 pts with AHR, positive cross-match
- 14 control biopsies (ACR, toxicity, normal)
- C4d in peritubular capillaries in 16/16 with AHR
- IgM/C3 present in 19/44%
- No C4d in controls
- Graft loss AHR/control - 40%/0%
- C4d specific and sensitive marker for AHR
Ab-mediated Acute Rejection
67 (of 232) patients with early AR + Bx
20 (30%) had widespread PTC C4d
18/20 C4d + had DsAb
1/47 C4d – had DsAb
Morphologic features – C4d+ vs C4d–
PMN in PTC, PMN in glomeruli, PMN tubulitis, severe ATI, fibrinoid necrosis in glomerular arteries
Not different - "v" endarteritis, inflammation, hemorrhage, infarcts
AHR-subclasses - capillary vs arterial
1 year graft failure 27% vs 40%
   (versus 3-7% in ACR)

C4d in Renal Allografts – Implications for Dx, Rx
- 398 biopsies from 265 patients; 125 controls
- C4d+ - 30% (median 38 days post-transplant)
- C4d+ associated with transplant glomerulitis, MHC class II expression, ↑ PRA (> 10%) in 43% (vs 19%)
- C4d+ associated with higher creat at biopsy, more ATG/OKT3 Rx
- No difference in outcome – but C4d+ patients treated more aggressively
- No association with ATG induction, infection, intimal arteritis, other histologic changes, CAN

C4d and Monocyte/Macrophages (Mo)
(Magil & Tincham, Kidney Int, 2003)
- 23 Biopsy with strong diffuse capillary C4d
  ATI +/- cellular rejection
- 29 biopsies with AR, C4d negative
- C4d+ group more glomerular and interstitial monocyte/macrophages (p=0.0023)
- More glomerular and peritubular capillary
  PMN (p=0.003)
- Conclusion: close association between PTC C4d and monocyte/macrophages (and PMN)
Antibody-mediated rejection

("Suspicious" if C4d or circulating anti-HLA or other anti-donor AB negative)

1) ATN-like - “ATN” with circulating anti-HLA AB and C4d+
2) Capillary - Capillaritis with anti-HLA AB and/or C4d+
3) Arterial – transmural inflammation and fibrinoid change with anti-HLA Ab and/or C4d+

Causes of fibrosis in the allograft

- Chronic rejection
- Infection
- Drug toxicity
- Atherosclerosis/hypertensive vascular disease
- Obstruction
- Recurrent disease
- Donor-related

Chronic Allograft Nephropathy – Fibrosis evolving in the renal allograft

Chronic Rejection – Fibrosing/sclerosing changes due to allo-immune reaction to the allograft
Chronic Rejection

The Cells

Monocyte/macrophage - IL2R+
Fibroblast/myofibroblast
T cells - CD4+

Chronic Rejection

The targets

Parenchymal elements
most closely correlated with outcome
“the final common pathway”
Arteries
lesions often not sampled on Bx
Capillary lesions
better sampled

Chronic Allograft Nephropathy

Criteria for true “chronic rejection”

Histological features
Arterial
Capillary
Molecular - C4d?
Intragraft Events Preceding CR

Kidney Int 61:1867, 2002

Inbred swine – unstable tolerance model
Progression group
(1) Persistent T cell infiltration, activation, proliferation
(2) Increased levels of donor-reactive CTL, anti-donor class I IgG
(3) Prominent apoptosis of parenchymal cells
(4) Persistent capillaritis, tubulitis

IMMUNOPATHOLOGICAL FINDINGS
Pathologic Definition

- cg – chronic glomerulopathy (based on extent in most severely affected glomerulus)
- *ci - interstitial fibrosis (% cortex)
- *ct - tubular atrophy (% cortex)
- cv - fibrointimal thickening (based on luminal impingement)
- * Features used to grade severity

Table 1. (cont.)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Histopathological findings</th>
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</thead>
<tbody>
<tr>
<td>I (mild)</td>
<td>Mild interstitial fibrosis and tubular atrophy without (a) or with (b) specific changes suggesting chronic rejection</td>
</tr>
<tr>
<td>II (moderate)</td>
<td>Moderate interstitial fibrosis and tubular atrophy (a) or (b)</td>
</tr>
<tr>
<td>III (severe)</td>
<td>Severe interstitial fibrosis and tubular atrophy and tubular loss (a) or (b)</td>
</tr>
</tbody>
</table>

6. Other Changes not considered to be due to rejection, see Table 14.

Chronic Rejection and C4d
Mauiyedi et al, JASN 12:574, 2001

- 38 specimens with “CR”
- 23/38 C4d + (vs 2% of controls)
- 88% of C4d + had DSA
- 1 yr. graft survival 62% vs 25% (rescue Rx initiated)
- C4d may ID antibody-mediated AR sub-group
Chronic Allograft Changes
New Directions (Banff 2001)

- Identify cases due to chronic alloimmune reaction
  - Morphological features
  - C4d staining
- Determine type, “activity” of sclerosing changes
- May enable future interventions

Draft Schema for Chronic Allograft Changes

1. Progressive Allograft Dysfunction – Biopsy unavailable/uninformative
2. Chronic/fibrosing rejection (Bx Dx)
   - Active - with evidence of immunologic activity (active infiltrates; C4d+)
   - Inactive - characteristic lesions (arterial, capillary) without activity
3. Specific other diseases (Bx Dx)
   - (e.g. chronic CIN toxicity, chronic PPV)
4. Chronic Allograft Nephropathy (Bx Dx)- Fibrosis with no etiologically specific lesions
Kidney Graft Bx - “Other Dx”

- Acute tubular injury - necrosis, oxalate
- CNI toxicity - Acute or chronic
- Bacterial or viral infection
- Acute interstitial nephritis
- Obstruction/reflux
- De novo disease
- Recurrent disease
- PTLD
TMA- Differential Diagnosis in Graft

- Drugs - calcineurin inhibitors, OKT3, ? Sirolimus
- Antibody-mediated rejection
- Recurrent Disease
- Infection-associated - eg HCV
- De novo
- ENDOTHELIAL INJURY

Osmotic Injury

Recurrence of primary glomerulopathies

<table>
<thead>
<tr>
<th>Recurrence Rate</th>
<th>Graft Loss</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSGS 20 to 30%</td>
<td>30 to 40%</td>
<td>High-risk group—malignant course, recurrence in a prior allograft</td>
</tr>
<tr>
<td>Membranous GN</td>
<td>3 to 7%</td>
<td>Rare</td>
</tr>
<tr>
<td>Type I MPGN</td>
<td>20 to 30%</td>
<td>30 to 40%</td>
</tr>
<tr>
<td>Type I MPGN</td>
<td>&gt;80%</td>
<td>10 to 20%</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>&lt;50%</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Anti-GBM nephritis</td>
<td>&lt;12%</td>
<td>Rare</td>
</tr>
</tbody>
</table>
Recurrence Rate | Graft Loss | Comments
--- | --- | ---
Henoch-Schönlein purpura | 10 to 15% | Loss
Lupus nephritis | <1% | None
HIV | 10 to 20% | Loss
Diabetic nephropathy | 50% | Loss
Amyloidosis | 50% | Loss
Wegener's granulomatosis | Reported | Loss
Essentially mixed cryoglobulinemia | 75% | Loss

Mesangial IgG deposits seen in ~50%, graft loss with tubular epithelial involvement.
Recurrences successfully treated with steroids or plasmapheresis/chlorambucil.
Baseline living-related transplant and CA.
Simultaneous renal transplants/transplantation may prevent diabetic renal disease.
Overall high mortality and morbidity due to infection, sepsis, atherosclerosis, etc.
Recurrences successfully treated with cyclophosphamide and steroids.
Recurrence may occur despite citrulline and angiotensin-1 blockade before transplantation.
Allograft glomerular lesions

- Glomerulitis/chronic transplant glomerulopathy
- Anti-GBM disease in recipients with hereditary nephritis
- Membranous glomerulopathy - transplant antigens
- Diabetic glomerulopathy due to immunosuppressive therapy

“Acceptance” Reactions

Recognizable in experimental models
T-lymphocytes and monocyte/macrophages
no vasculitis, may be tubulitis
less T cell activation, apoptosis than rejection
down interferon, up IL-10 compared to rejection
Cytotoxic cells present

NOT BEING RECOGNIZED CLINICALLY
Chronic allograft dysfunction

Predictors
- Clinical - Delayed graft function
  Acute rejection, especially late
  CsA/FK level/toxicity
- Pathological - AR - type, severity
  Nature of infiltrates
  Chronic /inflammatory changes - may be very early

Chronic Allograft Damage Index

- Bx at 2 years
- Inflammatory and sclerosing features
- Correlation with function 4 years later

Clinical and Pathologic Factors

- Prospective clinicopathologic data
- Protocol biopsies at 1, 2, 3, 6, and 12 mos
- End point - 24-month creatinine
- Independent correlative variates - chronic
  Bx score, late AR, CsA level, DGF
Baseline Glomerular Size and Late Allograft Function

- 100 baseline biopsies
- GPA - glomerular planar area
  - by point-counting
  - by computerized morphometry
- Correlation with creatinine at 6, 12, 24, 36 and 48 months

Donor Biopsies - Predictive Features

- Glomerulosclerosis
  - Gabur, et al, Transplantation, 1995
  - Pokorna, et al, Transplantation, 2000
  - Randhawa, et al, Transplantation, 2000
- Arteriosclerosis
  - Karpinski, et al, Transplantation, 1999
  - Pokorna, et al, NDT, 2000
- Hyaline arteriolar change/Arteriolosclerosis
- Fibrosis
  - Savov, et al, NDT, 1993
  - Randhawa, et al, Transplantation, 2000

Donor Biopsy and Outcome


- 78 donor biopsies - multivariate analysis
- Graded gs, ci, ct, cv, ah
- Any degree of ci-O.R. 2.6 for worse outcome at 6 months (p=0.002), but NS when corrected for donor age
- GS incremental grade - O.R. 2.3 for worse outcome at 12 months (p=0.05)
- Controlling for AR, high PRA did not obviate these factors
- Histologic parameters independently predictive
Acute Rejection - cell mediated

The molecules

Activation - TK, CD45, transcription factors
Costimulatory - B7-1/B7-2, LFA, CAMS
Targeting - HLA, CAMS
Cytokines - IL-2, IFN
Cytotoxins - enzymes, Fas-fas ligand, TNF

Demonstrable by immunohistology, immunoblotting PCR

Chronic Rejection

The Molecules

Fibrogenic Factors
TGF, FGF, PDGF
Macrophage-associated
RANTES, MCP-1, IL-6
Cytokines
IFN, TNF
Molecular Tools for Rejection Diagnosis

- Hold great promise
- Closer to utility
- Will likely not completely replace morphology
  type of rejection important
  concurrent processes may be missed
Gene Array Analysis of Renal Allograft Biopsies

Acute rejection is an endpoint of complex interplay between a multitude of immune and non-immune host and graft factors. In renal transplant biopsies, a lymphocytic infiltrate is most often due to acute rejection (AR); however, there is significant overlap with other processes such as infection, drug reaction, obstruction/reflux and lymphoproliferative disorders. Banff criteria for allograft rejection have been very helpful in the identification and grading of the severity of acute rejection.

In recent years, several studies have established the clinical significance of Banff classification in predicting outcome. All these studies have shown that vascular rejection (Banff II, III) is indeed associated with adverse prognosis. Although severity of tubulitis did not have an impact on outcome, a subset of patients with Banff I AR did experience graft failure. Moreover, many cases of “borderline” rejection do in fact represent rejection. It cannot be overemphasized that a kidney biopsy cannot be interpreted in isolation without clinical correlation. In addition to these issues, interobserver concordance among pathologists is less than desirable.

Thus, while Banff criteria have proven to be very useful, there is a need to improve risk stratification based on both biopsies and non-invasive methods. Recently, Sarwal et al. investigated the gene expression patterns in 67 renal allograft biopsies diagnosed with AR and related disorders, in an attempt to identify molecularly distinct subgroups of acute rejection, which have differences in clinical course and outcome.

Gene Array Analysis of 67 Renal Allograft Biopsies:

67 allograft biopsy samples were obtained from 50 pediatric patients. While 52 were obtained during allograft (acute or chronic) dysfunction, 7 were from patients with stable graft function; 8 other biopsies were obtained at the time of engraftment.

Biopsy diagnosis on blinded study of 67 samples

<table>
<thead>
<tr>
<th>Pathological Diagnosis</th>
<th># Biopsies</th>
<th>Gene Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Rejection (AR)</td>
<td>20* (borderline- IIA Banff)</td>
<td>A (11), B (4), C (5)</td>
</tr>
<tr>
<td>Drug Toxicity (DT)</td>
<td>15#</td>
<td>B</td>
</tr>
<tr>
<td>Chronic allograft Nephropathy (CAN)</td>
<td>16</td>
<td>C</td>
</tr>
<tr>
<td>Normal</td>
<td>15**</td>
<td>D</td>
</tr>
<tr>
<td>Others (ATN, infection, reflux)</td>
<td>Seen along with AR or DT</td>
<td>B, C</td>
</tr>
</tbody>
</table>

*gene array analysis was done in duplicate on one biopsy
# includes 5 patients treated prior to biopsy, who no longer had definite features of AR
** includes biopsies at engraftment and during stable graft function

Gene Array Analysis: Total RNA isolated from frozen biopsy tissue was used on microarrays containing 28,032 DNA spots, representing approximately 12,440 human
genes. The data from all 67 samples were used for unsupervised clustering. Supervised clustering was also performed, excluding the 5 patients who were treated prior to biopsy.

The gene expression patterns of the biopsy samples loosely fell into four clusters (A-D), which for the most part corresponded to the pathological category. The AR cases were predominantly in cluster A, but were also seen in clusters B and C, interspersed with DT and CAN. These were designated as AR-I, AR-II and AR-III respectively.

<table>
<thead>
<tr>
<th>AR Clusters</th>
<th>Selected upregulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR-I (A)</td>
<td>HLA, T-cell receptor, INFγ regulated genes, STAT cluster, Ig chains, CD20</td>
</tr>
<tr>
<td>AR-II (B)</td>
<td>TGFβ, VGEF, FGF, complement activation, lymphocyte activation, NFAT, apoptosis</td>
</tr>
<tr>
<td>AR-III (C)</td>
<td>Cyclin B, Cyclin A2, CC chemokine receptor 5</td>
</tr>
</tbody>
</table>

In addition to overexpression of T cell related genes, a B cell signature was also seen in AR-I, when compared other gene clusters. AR-II upregulated genes shared features with AR-I, but there was overlap with drug toxicity and infection. The striking feature of AR-III was expression of genes involved in cellular proliferation and cell cycling.

*Graft Function Recovery (time to return to pre-txp sCr) in various AR groups:*
Patients in the AR-I group had significantly poorer functional recovery of graft. Four of five study patients with steroid resistant AR (requiring antibody therapy) belonged to AR-I group.

**Correlation with Banff Grading:**

<table>
<thead>
<tr>
<th>Cluster (n)</th>
<th>Banff IB</th>
<th>Banff IIA</th>
<th>Graft loss/Incomplete functional recovery</th>
<th>Median f/u in months</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR-I (11)</td>
<td>3</td>
<td>2</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>AR-II (4)*</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>AR-III (5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
</tbody>
</table>

*none of the 5 additional “clinical rejection” cases treated prior to biopsy had adverse outcome

**Immunohistochemistry on histologically proven AR samples (n=20):**

CD4, CD8: Overall, there were more CD8+ than CD4+ lymphocytes in all AR groups.

CD20: Large aggregates of B-lymphocytes were present in less than half of AR cases, mainly in areas of atrophy and glomerulosclerosis. Scattered B cells interspersed within preserved cortex were seen in all cases.

EBV (in situ hybridization), SV40: Negative

C4d: Positive in 5 cases (ARI-2; ARII-2; ARIII-1) (Immunofluorescence for IgG, IgM and C3 was negative)

PCNA: Performed on all cases of Cluster C. There was increased expression of PCNA (proliferating cell nuclear antigen) in ARIII cases compared to CAN.

Calculation of positive cell density was performed on CD4, CD8, CD20 and PCNA stained slides. CD20+ cluster density was defined as > 275 cells/hpf

**CD20 cluster density predicts graft loss in cases of Acute Rejection**

![CD20 cluster density predicts graft loss in cases of Acute Rejection](image-url)
9 cases CD20 +: AR-I: 7/11; AR-II: 1/4; AR-III:1/5

To confirm these results, CD20 staining was done on a separate set of 31 biopsies with acute rejection, retrieved from Stanford pathology files. This confirmed that CD20+ staining was associated with poor graft outcome (p=0.11) and correlated strongly with resistance to steroid therapy (p<0.001)

Discussion

Gene array analysis of renal allograft biopsies identified three molecularly distinct subgroups of acute rejection, which had significantly different prognoses in terms of graft outcome. There were “intra-cluster” variations as well, reflecting the molecular heterogeneity of acute rejection. Although a T cell signature was present in all groups, there seemed to be differences in the phenotype such as activated T cells in AR-I and relatively quiescent T cells in AR-III. AR-II samples showed significant overlap with those of “clinical rejection”, infections and drug toxicity, underscoring the pathologic diagnostic difficulties and biological similarities of these entities.

AR-III clustered along with a subset of chronic allograft nephropathy (CAN) cases, further reiterating the likely quiescent nature of the rejection. However, AR-III seems to be different from just CAN, in that there is more proliferative/regenerative activity, presumably as part of the healing process.

One distinctive feature of the AR-I group that is associated with worse prognosis is the B cell signature. On immunohistochemical stains, CD20+ (defined as >275 cell/hpf) cases were significantly associated with steroid resistance and eventual graft failure. Although additional studies are required to further characterize the phenotype of these B-lymphocytes, this finding underscores the emerging importance of humoral rejection in transplant patients. C4d staining in our study did not correlate with either CD20 staining or graft outcome. However, our sample size is limited and further studies are required to recognize the relative importance and interactions of C4d and various B cell markers.

Recently, several other studies using gene arrays in kidney transplant patients have attempted to improve our understanding of rejection. Akalin et al used oligonucleotide arrays to generate gene expression profiles of seven renal allograft biopsies with acute vascular rejection (Banff IIa or IIb). They identified several upregulated genes that are associated with immune activation and inflammation. Interestingly, cytotoxic T cell effector molecules were not upregulated and this may be due to the small sample size. No prominent B cell signature was identified either. One other study analyzed gene expression in 13 transplant nephrectomies with chronic rejection compared to that of normal kidneys and end-stage polycystic kidneys. Two distinct subsets of chronically rejected transplants were identified; they were clinically
and histologically indistinguishable. This study further emphasizes the complexity and multifactorial causation of chronic allograft nephropathy/chronic rejection.

Gene array studies provide valuable insights into the pathobiology of rejection and will subsequently help us develop rapid diagnostic tools and prognostic indicators. Quantitative PCR analysis of peripheral blood and urine has been shown to be helpful in diagnosis of allograft rejection \(^{17-19}\). We can hope that simpler and less expensive techniques, which can reliably diagnose rejection, will evolve over time.
References


