The histological development of acute antibody-mediated rejection in HLA antibody-incompatible renal transplantation

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Abstract

Background. The aim of this study was to examine the development of acute antibody-mediated rejection in HLA antibody-incompatible renal transplantation in relation to the Banff 07 histological classification.

Methods. Renal biopsies were scored using the Banff 07 diagnostic criteria, and paraffin-embedded sections were stained with the pan-leucocyte marker CD45.

Results. Thirty-six patients had 72 renal biopsies. In biopsies performed 30 min after graft reperfusion, the mean number of CD45+ cells per glomerulus was higher than in control grafts (P<0.04) and was associated with the donor-specific antibody (DSA) level at transplantation measured by microbeads (P<0.01), and eight out of nine patients with greater than five CD45+ cells per glomerulus had early post-transplant rejection or oliguria, compared to 11 out of 20 with less than five cells per glomerulus (P<0.01). In the first 10 days post-transplant, although peritubular capillary (PTC) leucocyte margination grade 3 and C4d deposition were specific for rejection, their sensitivities were low. PTC C4d staining was only seen in two out of 11 biopsies taken in the first 5 days after transplant, even in the presence of rejection, but was present in the majority of later biopsies with rejection. In biopsies stained for CD3, CD68 and CD20, it was notable that CD20+ cells were not seen during acute rejection, the infiltrates comprising CD3+ and CD68+ leucocytes.

Conclusions. Glomerular margination of leucocytes occurred early after transplantation and was associated with DSA level and early graft dysfunction. The Banff 07 PTC margination scoring system was easy to apply, especially when CD45 staining was used, and PTC margination grade 3 was always associated with clinical rejection.

Keywords: antibody-incompatible transplantation; CD20; complement C4d; HLA antibodies; renal allograft histology

Introduction

Previous reports of the histological appearances of acute antibody-mediated renal allograft rejection (AMR) have been reviewed in detail [1,2]. The 1997 (updated 2003) and 2007 Banff classifications of renal allograft pathology define criteria for histological diagnosis [3,4]. The key feature of acute AMR is margination of neutrophils and mononuclear leucocytes into glomeruli and peritubular capillaries (PTC). There is also diffuse and circumferential staining for the complement component C4d on PTC. These appearances contrast with those seen in acute T cell-mediated rejection where there is tubulitis associated with a cellular infiltrate largely made up of cells staining for CD68 or CD3 [5].

Grafts that have failed from hyperacute rejection have cortical necrosis and widespread thromboses in the microvasculature. A 1968 study found that the earliest feature predictive of hyperacute rejection was neutrophil margination into glomeruli, four or more neutrophils per sectioned glomerulus in a biopsy taken after reperfusion [6].
The important histological findings of severe acute antibody-mediated rejection were described by Halloran and colleagues in 1990 [7]. Biopsies in the first 5 days showed predominantly glomerular changes with neutrophil infiltrates and some staining for complement C3. Electron microscopy showed swelling or denudation of glomerular endothelial cells. In contrast, changes in the rest of the renal cortex were mild with acute tubular necrosis and mild cellular infiltrates. Also, in the cyclosporin era, three of nine patients who were transplanted immediately after protein A immunoadsorption to remove donor-specific antibodies (DSA) and underwent post-perfusion renal biopsy had focal glomerular thrombosis [8].

Histological data from the Johns Hopkins University have been reported in several studies [9–12]. Changes compatible with antibody injury were seen on post-perfusion renal biopsies, and additionally, two cases also had C4d staining in PTC that was not seen in pre-implantation biopsies, but without glomerular changes [10]. In another study of patients receiving HLA antibody-incompatible living donor transplants, strongly positive staining for C4d in PTC was seen in 17% of protocol biopsies and 47% of those performed for graft dysfunction, although over 20% of biopsies that were negative for C4d in PTC had some element of cellular margination in glomeruli [11].

More recently, microarray analysis of renal allograft biopsies showed similar disturbances in the selected microarray sets in AMR and T cell-mediated rejection, suggestive of significant T cell involvement in AMR [13].

The aims of this study were to examine the interrelationships between DSA, cell infiltrations and other histological findings after HLA antibody-incompatible transplantation. Our hypothesis was that novel information could be gained from looking at the histological development of antibody-mediated rejection, especially as DSA measurement by the new method of microbead analysis allowed a more precise correlation between histology and potentially pathogenic antibodies to be made.

Materials and methods

Patients

Patients sensitized to HLA antigens were selected for the programme if they had current reactivity with donor-specific HLA antigens measured by complement-dependent cytotoxic (CDC) cross-match, flow cytometric (FC) cross-match or microbead assay. Pre-transplant, patients were treated with double-filtration plasmapheresis with the aim being to achieve a negative FC cross-match at the time of surgery. Patients who received ABO-incompatible grafts with no HLA antibody incompatibility were excluded. Five post-perfusion renal biopsies taken from antibody-compatible living and deceased donor transplants were also stained with CD45. Not every antibody-incompatible patient had a post-perfusion biopsy; the main reason for omitting it was concern about haemostasis.

Immunosuppression consisted of mycophenolate mofetil 1000 mg bd started 10 days before transplant with dose reduced if white cell count fell below 4.0 × 10^9/L. Tacrolimus was started 4 days before transplant at a dose of 0.15 mg/kg/day in divided doses with a target trough level of 10–15 μg/L in the first month. Prednisolone 20mg od was started at the time of surgery, and methylprednisolone 500 mg was given as a single intravenous dose during the transplant operation. Two doses of basiliximab 20 mg were given at days 0 and 4.

Post-transplant serum samples for antibody analysis were taken daily for the first 2 weeks, and then three times a week for the next 2 weeks.

AMR was diagnosed on renal biopsy by Banff criteria or by a combination of a decline in renal function, DSA and a biopsy showing the cellular changes of acute antibody-mediated rejection (peritubular capillaritis and/or glomerulitis), but negative for C4d (i.e. a biopsy ‘suspicous’ for AMR by Banff criteria). Severe rejection was defined as patients needing dialysis during the rejection episode. Rejection was treated with 3 days’ high-dose methylprednisolone, additionally for non-responders or severe rejection daily plasmapheresis and rituximab early in the series; subsequently, muromonab CD3 (OKT3) or anti-thymocyte globulin ATG (Genzyme) with little or no plasmapheresis.

Cross-matching and measurement of HLA antibody levels

All patients had HLA antibody levels measured with CDC cross-matching [anti-human globulin (AHG) enhancement was not used], FC cross-matching and microbeads [One Lambda Inc. (Canoga Park, CA, USA), analysed on the Luminex platform (XMap 200)] as described in detail previously [14]. This was performed at initial evaluation, prior to plasmapheresis and on the afternoon of the day prior to transplantation after the course of plasmapheresis had been administered. Monitoring after the transplant was primarily with Luminex on a daily basis for the first 2 weeks, three times a week for 2 weeks and then according to clinic attendance.

Biopsies

Renal biopsies were taken approximately 30 min post-reperfusion in the operating theatre or to investigate sub-optimal or declining renal function. Biopsies were fixed in 10% neutral buffered formalin and routinely processed into paraffin wax for standard light microscopy.

Immunohistochemistry on de-waxed sections was undertaken using the following antibodies: C4d (Biomedica Gruppe C4dpAb, Cat. No. BI-RC4D) and C4d5 (DAKO CD45, Cat. No. M0701 or Vision Biosystems CD45 X16/99, Cat. No. PA0042). A subset of specimens was also stained for CD3 (Vision Biosystems CD3, Cat. No. NCL-L-CD3-565), CD68 (DAKO CD68, Cat. No. M0814 or Vision Biosystems CD68 514H12, Cat. No. PA0273) and C2D (DAKO CD20, Cat. No. M0755). Sections were stained using Biogenex Polymer Detection System or Leica Microsystems Bond Max Immunostainer with Bond Polymer Refine Detection System.

Slides were reviewed using CD45 immunostaining and scored according to the Banff criteria for cellular (T cell-mediated) rejection and the Banff 07 for PTC margination (grades PTC0–PTC3) and also for C4d staining (C4d0–C4d3). Additionally, the numbers of cells in all glomeruli in the sample and in at least three high-power fields (hpf) of cortical kidney were counted. Biopsies with four or more CD45+ cells per glomerulus were examined with haematoxylin and eosin (H&E) stain for an independent PTC margination score and to count glomerular neutrophils. Since neutrophils stained positive with the CD45 antibody used in this study, there was no point in looking for neutrophils in biopsies with few CD45+ cells, and the selection cut-off value of 4 CD45+ cells per glomerulus was chosen arbitrarily.

Statistical analysis

Statistical analysis of comparison between groups was performed using Student’s t-test on SPSS for Windows, version 12.0.

Results

Patients

During the study period, 36 patients had 72 adequate renal biopsies. Another 11 patients were transplanted, but did not have renal biopsies, and are not included. Two patients had HLA antibody and ABO incompatibility. There was one inadequate biopsy; this was repeated and the sample was adequate. Characteristics of the patient population are shown in Table 1.
Post-perfusion biopsies

There were 29 post-perfusion biopsies from antibody-incompatible and five from non-sensitized patients receiving transplant with no current anti-donor antibody reactivity (termed ‘antibody-compatible’). The mean numbers of glomerular CD45+, interstitial CD45+ and tubular CD45+ cells per hpf were 3.9 (range 0.8–12.3), 10.6 (range 1.6–34.0) and 0.8 (range 0–4.3) for the antibody-incompatible patients and 1.2 (range 0–1.7), 14.1 (range 3.3–27.7) and 0.8 (range 0–2) for the antibody-compatible transplants, respectively (P<0.04 for glomerular cells, P=ns for others).

The number of CD45+ cells per glomerulus and the DSA level measured by microbead on the final pre-transplant cross-match were correlated (P<0.01) (Figure 1). Glomerular CD45+ cells and the relative mean fluorescence (RMF) of the flow cytometric cross-match on final pre-transplant cross-match is also shown in Figure 1. This shows that some infiltration occurred with low levels of DSA measured by this technique (the cut-off for reporting a positive cross-match in our laboratory is 2.5 for regrafts and 4.0 for first grafts). The RMF values and the extent of glomerular infiltration were not significantly correlated.

Of the nine patients with five or more CD45+ cells per glomerulus, eight had either post-transplant oliguria (mean urine output days 2–4 <50 ml/h) or a rejection episode, compared with 11 out of 20 patients with less than five CD45+ cells per glomerulus (P<0.001). One case with a low DSA level at transplant and a low glomerular CD45+ infiltration count developed a rapid decline in renal function with marked rise in DSA levels at day 10. Anti-rejection treatment was started without biopsy, she made a good recovery and a diagnosis of rejection was made on clinical grounds. If the diagnosis of rejection was incorrect, this did not affect the conclusion from these data, as she was in the low DSA/low glomerular cell count group.

No post-perfusion biopsy showed peritubular capillaritis, i.e. PTC score of 0 in all cases, and there was no tubulitis.

One biopsy showed weak focal staining for C4d in PTC, the others were negative. Ten biopsies had at least focal glomerular staining for C4d. Four of the five biopsies in antibody-compatible transplants also showed some mesangial staining for C4d. Examples of glomeruli showing cellular infiltration are shown in Figure 2.

Table 1. Study population

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
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<tr>
<td>Number</td>
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</tr>
<tr>
<td>Age in years, mean (range)</td>
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<td>Duration of ERF in years, mean (range)</td>
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<tr>
<td>Living donor/deceased donor</td>
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<tr>
<td>Pre-treatment DSA level</td>
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<tr>
<td>CDC positive/FC positive/microbead positive</td>
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</tr>
<tr>
<td>CDC negative/FC positive/microbead positive</td>
<td>15</td>
</tr>
<tr>
<td>CDC negative/FC negative/microbead positive</td>
<td>9</td>
</tr>
<tr>
<td>Had plasmapheresis pre-transplant</td>
<td>29</td>
</tr>
<tr>
<td>Time biopsy</td>
<td></td>
</tr>
<tr>
<td>Post-perfusion</td>
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</tr>
<tr>
<td>Days</td>
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<td>41–90</td>
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<tr>
<td>&gt;90</td>
<td>6</td>
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</table>

Fig. 1. Mean number of CD45+ cells per glomerulus on renal biopsies taken 30 min after reperfusion for 29 HLA antibody-incompatible and five control patients; cases were ranked according to DSA level at pre-transplant cross-match, as measured by microbead (upper panel) and by flow cytometric cross-match (lower panel) (MFI, mean fluorescence intensity in microbead assay; RMF, relative mean fluorescence).

Biopsies taken up to day 20, before administration of ATG or OKT3

In contrast to post-perfusion biopsies which showed glomerular infiltration, biopsies in patients with rejection during the first 20 days showed the development of peritubular capillaritis and, later, C4d staining. Between days 1 and 20 post-transplant, 25 biopsies were performed in 20 patients. The indication for renal biopsy was renal dysfunction, protocol biopsies were not performed. No biopsy was normal. The mean number of glomerular cells was 9.2 (range 3.9–22.2), compared with 3.9 (range 0.8–12.3) on post-perfusion biopsies (P<0.001), and for interstitial CD45+ cells, 84.3 (range 24.3–250) per hpf, compared with 10.6 (range 1.6–34.0) per hpf on post-perfusion biopsies (P<0.0001). The majority of interstitial leucocytes were within PTC; the mean numbers of interstitial CD45+ cells in patients treated for rejection was 91.4 (range 38–250), and those within tubular basement membranes was 2.44 (range 0–6). The rate of development of changes during the 20-day period is shown in Table 2; this shows the maximal changes seen on biopsies taken up to each of the indicated time intervals.
The extent of cellular infiltration in these biopsies was not associated with DSA levels on the day of the biopsy. We have previously shown significant absorption of DSA by the graft in this early period, especially of HLA class 1 and HLA DR antibodies [15]. This may have affected the relationship between circulating DSA levels and events occurring in the grafts.

Thirteen patients received ATG or OKT3 treatment and seven had improved renal function with either methylprednisolone or no escalation of immunosuppression. The only features with 100% specificity for proceeding to ATG or OKT3 treatment were a PTC leucocyte margination score of 3 and a PTC C4d score of 3. However, the sensitivity of these indicators was poor, at 44% and 13%, respectively. Likewise, of the eight biopsies that showed a PTC margination score of 2, three were in patients who did not proceed to ATG or OKT3 therapy. Figure 3 shows a patient who showed positive PTC staining for C4d after treatment for rejection had been initiated.

Biopsies after therapy of rejection

These biopsies showed continuing intensity of staining for C4d developing over time with less cellular infiltration than in pre-treatment biopsies, which may be a result of anti-leucocyte treatment. Nine biopsies were performed in six patients because of poor function after the initiation of ATG or OKT3 therapy. This showed continued margination of leucocytes, though to a lesser extent in patients treated with ATG by the first post-transplant day. In all six patients, there was evidence of PTC C4d staining: C4d grade 1 in four and C4d grade 3 in two. In all these cases, the biopsy was performed at least 7 days after transplantation. These cases had DSA present, but so did the majority of patients with good graft function who were not biopsied, as previously described [14]. The numbers of interstitial and tubular CD45+ cells were 48.4 (range 6–110.3) and 1.64 (range 0–6.33), respectively. As illustrated in Figure 3, much of the early CD45+ infiltration was within PTC. When the CD45+ infiltrate was dense, however, it was not possible to measure exactly how many cells were in capillaries and how many were in other interstitial spaces, so the total interstitial count is presented.

### Table 2. The development of histological changes in renal biopsies

<table>
<thead>
<tr>
<th></th>
<th>Glomerular CD45</th>
<th>Cortical CD45</th>
<th>PTC leucocyte margination score</th>
<th>PTC C4d score</th>
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</thead>
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<td>Post-perfusion</td>
<td>12.3</td>
<td>26.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Up to day 2</td>
<td>17.7</td>
<td>77.7</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Up to day 5</td>
<td>17.7</td>
<td>107.3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Up to day 20</td>
<td>22.2</td>
<td>250</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

The maximal change in each category seen on any biopsy by the indicated period is shown. Glomerular CD45, mean number of positive staining cells per glomerulus; cortical CD45, mean number of positive staining cells per high-power field; PTC leucocyte margination score and PTC C4d score as per Banff 07 criteria.

Fig. 2. Glomeruli from biopsies taken 30 min after graft reperfusion stained with PAS and for CD45+ cells with the immunoperoxidase method: (A) antibody-compatible transplant showing no CD45+ cells in the glomerulus; (B) and (C) antibody-incompatible transplants with CD45+ cells in the glomeruli.
Later biopsies

These biopsies showed some cases with marked tubular leucocyte infiltration, not marked on earlier biopsies, and some cases of transplant glomerulopathy. Seven patients had eight biopsies after day 30; in addition, four patients had biopsies after transfer back to their original referral centres. Six of these biopsies were performed in the first year post-transplant, the other five at time points up to 41 months. Five biopsies showed changes compatible with acute antibody-mediated rejection, and blood tests showed persistent or resynthesized DSA. Three of these patients had previously experienced early rejection; other clinical events in the month before biopsy were CMV infection [1], surgery for lymphocele [1] and non-adherence with medication [1]. Renal function responded to high-dose steroids in four cases, one patient was treated with OKT3.

One patient developed a thrombotic microangiopathy, and the graft failed just after day 90. There was a considerable degree of glomerular infiltration by CD45+ cells as well as the thrombotic process, but C4d staining was persistently negative while DSA persisted at high levels.

The mean numbers of interstitial and tubular CD45+ cells were 81.2 (range 11–207) and 4.69 (range 0–25.7), respectively, with two patients having marked tubulitis in contrast to earlier biopsies.

Four patients had appearances of transplant glomerulopathy, two of whom also had acute AMR with PTC margination. Two of these had prior rejection in the early post-transplant period. Two of these patients had subsequent improvement in transplant function after vigorous blood pressure treatment; two others have restarted dialysis at 32 and 59 months after transplantation respectively.

Leucocyte subsets and tubular infiltration

Twenty-eight biopsies were stained for CD20, CD68 and/or CD3 leucocyte subsets. These showed similar distributions of cells by proportion. In biopsies taken on days 1–20 in

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Fig. 3. Early development of antibody-mediated rejection post-transplant with negative C4d staining in PTC: (A) renal biopsy on day 2 post-transplant stained for CD45, showing glomerular and peritubular infiltration (this patient had poor renal function and was treated for rejection); (B) C4d staining on day 2 renal biopsy, minor glomerular mesangial staining; (C) renal biopsy on the same patient taken at day 12 post-transplant, after ATG treatment, showing persistent peritubular capillaritis with CD45+ cells; (D) biopsy on day 12 stained for C4d.
patients who had not received ATG or OKT3, the mean percentage of glomerular cells that were CD20+ was 0.2% (range 0–0.8%), CD68+ 67.2% (range 54.7–85.7%) and CD3+ 32.6% (range 14.3–45.6%). The mean percentage of cortical cells that were CD20+ was 1.8% (range 0–5.8%), CD68+ 50.2% (range 28.9–68.7%) and CD3+ 48% (range 30.3–65.3%).

Glomerular neutrophils and reproducibility of PTC margination scores

Thirty-one biopsies with greater than four CD45+ cells per glomerulus were examined for glomerular neutrophils and an independent PTC margination score. Eight of these biopsies were post-perfusion, the mean number of CD45+ glomerular cells was 7.5 (range 4.9–12.3) and the mean number of neutrophils was 3.7 (range 1.1–7). Twenty-three biopsies were taken at other times after transplantation, the mean number of CD45+ glomerular cells was 9.7 (range 4.6–22.2) and the mean number of neutrophils was 3.9 (range 0.9–9). Examination of morphology on CD45-stained sections showed that neutrophils stained positive with CD45. Comparison of the PTC margination scores obtained independently by two observers, one using H&E staining and the other using CD45 staining, showed that 46% of the scores were in complete agreement; in 43%, the difference in score was one grade; in all but one case, the CD45 score being higher than the light microscopy score; in 11% of cases, the scores differed by two grades; and in no cases did the score differ by three grades.

Discussion

The extent of glomerular margination 30 min after graft reperfusion was associated with DSA levels at transplant as measured by microbead analysis. A mean of five or more CD45+ cells per glomerulus was associated with significant of rejection or early graft dysfunction. There was also an association between the extent of early glomerular CD45+ margination and DSA levels. However, this was not absolute (Figure 1), and further work examining the immunoglobulin subclasses of DSA and their affinity for antigen is indicated. It is also possible that early oliguria and ischaemia could have contributed to some of the cellular changes, though the high early dysfunction rate in our patients was likely ultimately to have been due to DSA. A larger control group including antibody-compatible living donor transplants with delayed function would be desirable, but the delayed function rate after living donor transplantation is low and it has not been the policy in our unit to perform post-perfusion biopsies in all transplants.

These findings in post-perfusion biopsies add to the previous recognition of early glomerular infiltration by defining an association with DSA levels and clinical outcomes [6,8,10]. The Banff 07 criteria differ from the Banff 97 criteria in that glomerular inflammation is now a criterion for diagnosis of antibody-mediated rejection, and this study reinforces that view [3,4].

Very few CD20+ cells were found in the biopsies, and there were roughly equal proportions of CD68+ and CD3+ cells. This finding is compatible with both the failure of anti-CD20 therapy to control established acute rejection and the finding by microarray studies that T cells rather than B cells are found within kidneys undergoing acute antibody-mediated rejection [13,16]. Likewise, it is also compatible with the previous observation [7] and our own experience (92% graft survival and 100% patient survival) that the T cell-specific drug muromonab CD3 (OKT3) was effective in the treatment of established antibody-mediated rejection. This of course does not rule out a critical role for B cells, particularly CD20− B cells, in the overall process of DSA production. On average, neutrophils made up about 50% of the glomerular-infiltrating leucocytes, but the neutrophil count in individual patients did not add to the clinical associations obtained from CD45 staining.

Interobserver comparison of the PTC margination grade was performed, with one observer using H&E sections and the other CD45-stained sections. There was good agreement between observers, albeit with a tendency for the CD45-graded PTC score to be one level higher than the H&E-graded score. This comparison compares very well with the interobserver variation previously reported for PTC scoring in which the kappa score was only 0.43, graded as moderate agreement [17].

The main apparent discrepancy between the Banff criteria and our findings was the lack of PTC staining for C4d in the first few days after transplantation, even in some biopsies with PTC grade 3 cellular margination, circulating DSA and a clinical picture of antibody-mediated rejection. C4d-negative AMR has been observed clinically [18], and in a murine model of AMR, C4d staining was focal at day 5 after transplantation, progressing to diffuse by day 7 [19]. The sensitivity of the staining method for C4d may also be important as we used the immunoperoxidase method on paraffin-embedded tissue, which is less sensitive than immunofluorescence. However, we used the Banff 07 criteria, which take account of the reduced sensitivity of immunoperoxidase, and our method was sensitive enough to detect the low levels of constitutive C4d production in glomerular mesangial cells in many biopsies. Further work is indicated to examine C4d staining in biopsies during the early phase of onset of antibody-mediated rejection, especially studies using immunofluorescence staining for C4d. The development of staining for C4d on PTC appeared to be associated with new-onset staining for C4d on glomerular capillary loops, and this also requires further investigation.

Immunophenotyping studies of glomerular- and PTC-infiltrating leucocytes have previously been performed, examining the differences between infiltrates in biopsies that were positive or negative for C4d. These studies show T cells present in both circumstances, but with increased proportions of monocytes in C4d-positive biopsies [20,21]. The relationships between antibody deposition, complement and cellular infiltration would appear to be complex and remain to be fully elucidated. Studies of cellular infiltration and complement staining according
to the class or subclass of DSA, its affinity with antigen, binding of complement types including the MBL pathway and complement regulatory molecules are all likely to be important.

Conclusion

In summary, this study further illuminates the development of acute antibody-mediated rejection in HLA antibody-incompatible transplantation. Glomerular margination, principally by CD68+ cells including neutrophils, commenced within minutes of reperfusion of the graft. Significant glomerular infiltration was even seen in some patients with low DSA levels, for example, those who were flow cytometric cross-match-negative at the time of transplantation. PTC margination of leucocytes, both CD68+ and CD3+, occurred within the first 24 h after transplantation, but the extent of margination increased progressively over the first week post-transplant.

Conflict of interest statement. None declared.

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