Original Article

Tacrolimus combined with mycophenolate mofetil can effectively reverse C4d-positive steroid-resistant acute rejection in Chinese renal allograft recipients

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Abstract

Background. Tacrolimus (TAC) combined with mycophenolate mofetil (MMF) has been suggested to play a critical role in the reversal of C4d-positive acute humoral rejection (AHR) in renal transplantation, but the efficacy of using only TAC–MMF without immunoadsorption or plasmapheresis has not been investigated. On the other hand, Chinese recipients of renal grafts usually need lower doses of immunosuppressants, and their optimal treatment for acute humoral rejection has not been established.

Methods. Since 1999, we have used TAC–MMF to treat steroid-resistant acute rejection (AR). C4d staining was retrospectively performed in 32 patients with steroid-resistant AR, and the treatments of 19 patients with C4d-positive steroid-resistant AR were investigated.

Results. Thirteen of 19 patients received TAC–MMF treatment only; 11 episodes of rejection in them were reversed (7 completely, 4 partially) and only 2 recipients lost their graft. Another 6 patients received immunoadsorption also. One of them failed to be converted to TAC-MMF and lost her graft. Four of 5 patients treated with immunoadsorption and TAC–MMF recovered (3 completely, 1 partially), but 3 of them had severe pneumonia, a complication rate statistically higher than in patients treated with only TAC–MMF (P < 0.05). AR occurring during the first two weeks after transplantation had a statistically better outcome than that occurring later (P = 0.003).

Conclusion. Our study suggests that the combination of TAC and MMF is a potentially safe and economic treatment for most Chinese renal allograft recipients with C4d-positive steroid-resistant AR, especially for rejections developing within the first two weeks after transplantation.

Keywords: kidney transplantation; acute rejection; C4d; tacrolimus; mycophenolate mofetil

Introduction

Alloantibody-mediated acute rejection (acute humoral rejection, [AHR]) is a major cause of renal allograft loss, despite the use of currently available aggressive therapies [1]. The diagnosis of AHR usually is based on serologic evidence of circulating antibodies to donor human leucocyte antigen (HLA) or to other antidonor endothelial antigens [2]. Unfortunately, antidonor antibodies, especially HLA-II-reactive antibodies, are difficult to detect by conventional methods, while very low levels of them can be associated with the occurrence of severe post-transplant AHR [3,4]. As C4d staining has been found to be 95% sensitive and 96% specific for antidonor antibodies [5], and since the accumulation of neutrophils in peritubular capillaries (PTC) is clearly suggestive of AHR [2], it is suggested that an early post-transplant renal allograft biopsy that shows diffuse C4d-positive staining associated with morphological features suggestive of acute antibody-mediated rejection may be diagnosed as AHR, even in the absence of detectable serum anti-HLA antibodies [6]. C4d can to some degree be regarded as a ‘magic’ in situ marker of antibody-induced injury [7]. Capillary C4d deposition in PTCs is also an independent prognostic factor for renal allograft outcomes: an allograft with C4d deposition usually has a significantly worse outcome than a graft without evidence of C4d [8]. The combined use of immunoadsorption or plasmapheresis with mycophenolate mofetil (MMF),
Anti-HLA antibody. >10% in flow-PRA class I or II were considered positive for study, and divided into two parts: one for formalin fixation from each needle biopsy of a renal allograft for morphologic after the onset of rejection. Two tissue cores were obtained Renal allograft pathology and C4d staining according to the guidelines provided by One Lambda, Inc., PRA screening test was performed pre-transplantation a panel of cells from 50 phenotyped donors; since 2000, flow-antibodies (PRA) reactivity was assessed before 2000 using Eurotransplant Organization. Pre-transplant panel reactivity microcytotoxicity technique according to the protocol of the performed on the subject patients using the standard of severe rejection; (2) resistance to steroid bolus therapy; further analysis. The diagnostic criteria were: (1) evidence frozen biopsy material was stained for C4d, and 19 patients selected for further retrospective investigation. Stored within 5 days after the last dose of methylprednisolone), as creatinine levels not returning to within 20% of baseline 32 of them, whose rejections were steroid-resistant (defined diagnosed to have ARs, based on renal allograft biopsies; of eastern origin usually need lower doses of immunosuppressants than Caucasian recipients, so the combined use of TAC and MMF – without the costly immunoabsorption or plasmapheresis – may be successful in some such Chinese patients. On the other hand, most of the studies were performed in western countries. The optimal treatment of C4d-positive AHR in graft recipients of eastern origin is still undefined. Therefore, to determine the efficacy of various treatments we retrospectively studied 32 Chinese renal allograft recipients with steroid-resistant AR treated in our institution, of whom 19 were diagnosed as C4d-positive AR.

Materials and methods

Patients

We reviewed the records of 442 cadaveric renal allograft recipients who had been transplanted between April 1999 and October 2003 in Jinling Hospital, Nanjing University School of Medicine. Of them, 112 patients had been diagnosed to have ARs, based on renal allograft biopsies; 32 of them, whose rejections were steroid-resistant (defined as creatinine levels not returning to within 20% of baseline within 5 days after the last dose of methylprednisolone), were selected for further retrospective investigation. Stored frozen biopsy material was stained for C4d, and 19 patients meeting the criteria of C4d-positive AHR were selected for further analysis. The diagnostic criteria were: (1) evidence of severe rejection; (2) resistance to steroid bolus therapy; (3) C4d deposition in PTC and (4) typical pathologic features (granulocyte infiltration in glomeruli and PTCs).

Pre-transplantation cytotoxic crossmatching had been performed on the subject patients using the standard microcytotoxicity technique according to the protocol of the Eurotransplant Organization. Pre-transplant panel reactivity antibodies (PRA) reactivity was assessed before 2000 using a panel of cells from 50 phenotyped donors; since 2001, flow-PRA screening test was performed pre-transplantation according to the guidelines provided by One Lambda, Inc., as described by Böhmig et al. [12]. Sera with reactivities >10% in flow-PRA class I or II were considered positive for anti-HLA antibody.

Renal allograft pathology and C4d staining

The diagnostic biopsies had been performed 3.9±5.0 days after the onset of rejection. Two tissue cores were obtained from each needle biopsy of a renal allograft for morphologic study, and divided into two parts: one for formalin fixation and one for quick-freezing. Haematoxylin and eosin, periodic acid-Schiff, methenamin silver and Masson stains were routinely used on formalin-fixed tissue. Fresh-frozen tissue were analysed by immunofluorescence microscopy (IF) after exposure to a conventional panel of antibodies against IgG, IgM, IgA, C3, C4 and C1q. The residual biopsy tissues were stored for future use. The frozen tissue samples of 32 patients who had steroid-resistant AR were retrieved, and C4d staining was performed using an indirect immunofluorescence technique, as described previously [13]. Positive C4d staining was defined as bright linear staining along capillary basement membranes, involving more than half of the sampled capillaries – according to the 2001 Banff Meeting criteria [2]. All biopsies met the Banff criteria for adequacy: at least 10 glomeruli and 2 arterial sections were available in any one slide. In that cohort of 32 patients, 19 had positive C4d staining (Figure 1A). These 19 were selected for further analysis in this study. The other 13 patients were defined as C4d-negative controls.

Initial immunosuppression

Nine of the 19 recipients had received a primary immunosuppression consisting of cyclosporine A (CsA) plus MMF plus steroids. The other 10 had been treated with TAC plus MMF plus steroids; and among them, 4 had also received a two-dose CD25-mAb (Zenapax, Roche) induction. The initial dose of MMF used was 1.5 g/d, and the doses of TAC and CsA had been adjusted to trough levels (TAC, 6–12 ng/ml during the first half-year and 5–10 ng/ml during the second half-year; CsA, 180–250 ng/ml during the first half-year and 150–200 ng/ml during the second half-year). A standard tapering corticosteroid regimen had been used, consisting of an intravenous bolus of methylprednisolone 500 mg on days 0 to 2, followed by oral prednisolone 80 mg/day on day 3, dose was tapered by 10 mg increments per day down to 20 mg/day, and then tapered more slowly to 5 mg/d thereafter.

Treatment of AR

Once an episode of rejection occurred, bolus corticosteroid therapy (methylprednisolone 500 mg/d for 3 days) was used as the initial treatment. As TAC and MMF had shown effect in the treatment of steroid-resistance AR [11], all the patients had been administered TAC–MMF (MMF, 1.5 g/d; TAC trough levels maintained between 8 and 15 ng/ml). As to patients who received TAC plus MMF plus steroids as primary immunosuppression, their dose of TAC was adjusted to maintain a trough level between 8 and 15 ng/ml. Between August 2002 and March 2003, immunoadsorption was used in our unit to treat steroid-resistant rejection, and 6 episodes of rejection occur during this period were treated also with immunoadsorption. Immunoabsorption therapy was performed with the Citem 10 Immunoadsorption System and a staphylococcal protein A column (Fresenius Haemo Care, Inc., Redmond, WA). Plasma was separated with a traditional plasma separator, passed through the column for adsorption, and then re-infused. Continuous veno-venous haemofiltration, other than regular dialysis, was performed when necessary.

We use ‘completely reversed’, ‘partially reversed’ and ‘not reversed’ in this report to indicate the effect of therapy. Completely reversed means that graft function recovered to a normal range (serum creatinine <110 µmol/l in our
department) within 1 month after the initiation of therapy. Partially reversed means that the graft’s function did not recover to the normal range within 1 month, but that there was a stable function without need for dialysis. Not reversed means that the recipient lost the graft due to rejection and required dialysis again. All patients were followed continuously for more than 1 year after the rejection episodes, including the patients who lost their grafts.

Statistical methods
Descriptive statistical values are expressed as mean±SD. Between-group differences in frequencies of clinical characteristics were determined using the Fisher exact test. The analyses were done using the Stata 6.0 software (Stata Corporation, College Station, TX, USA). A P-value of 0.05 or less was considered significant.

Results
Baseline characteristics
C4d staining was positive in 19 recipients of kidney allografts (Table 1), 9 males and 10 females. The average age of the males was 33.2±10.4 years (range 20–54); in females it was 41.3±8.6 years (range 29–54). No patient had ever received a renal allograft before except for one, a 54-year-old male from a minority group in China. He was the only male older than 40 in this cohort. All transplantations were ABO compatible. For social reasons, HLA matching had not been performed in the 19 recipients. Pre-transplantation sensitization data (Table 1) showed that 8 of the 19 patients had been sensitized pre-transplantation.

Clinical manifestations
Thirteen episodes of rejection occurred in the first two weeks after transplantation and 6 happened later. All grafts functioned initially (as reflected in a decrease of serum creatinine) before the AHR occurred. Fever, decreased urine volume, and swift allograft dysfunction were observed in the 19 recipients. Delayed graft function (defined as a need for renal replacement therapy within the first week after transplantation) occurred in 4 recipients. The 14 patients who needed haemodialysis had been subjected to continuous venovenous haemofiltration. Ultrasound examinations revealed an enlarged renal allograft and increased vessel resistance index in each recipient. An immediate (within 4 days) renal biopsy was performed in 18 of the 19 recipients; the other recipient was treated for AR which was confirmed by biopsy 24 days later (Table 2).

Pathology findings
In diagnostic biopsies, all patients had neutrophil and mononuclear cell infiltration in PTCs (Figure 1C), and glomerulitis (Figure 1B) was found in 84.2% of the biopsies. Tubulitis was observed in...
63.2% patients and interstitial haemorrhage in 42.1%. Endo-arteritis (Figure 1D) was found in all but one case (94.7%). Three patients (15.8%) had arterial intimal necrosis and 2 (10.5%) had arterial thrombosis. According to the Banff 97 diagnostic categories for AR, 11 met criteria for grade IIA, 4 for IIB, 3 for III and 1 for IB. C4d staining of frozen slides (Figure 1A) was positive in all 19 patients, who were thus diagnosed as having C4d-positive steroid-resistant AR.

Treatments and clinical outcomes

Anti-rejection therapy consisting of boluses of steroid and TAC–MMF had been given to 13 patients, their rejection episodes occurring between August 2002 and March 2003; the other 6 patients had received in addition two sessions of immunoadsorption beside TAC–MMF. Unfortunately, one of them had reverted to CsA from TAC because of severe gastrointestinal side effects. None of the 19 patients had an immediate response to steroid treatment. Of the 13 patients who received only TAC–MMF, 7 (53.8%) had complete reversal and 4 (30.8%) were partially reversed; in only 2 (15.4%) did the treatment have no effect. During the 31.5±16.3 months of follow-up, the renal allograft function in 4 of the 7 completely reversed patients stayed normal; 3 developed chronic allograft dysfunction, but none of them returned to dialysis. One of the partially reversed patients returned to dialysis 13 months after the initial rejection; the other 3 continued on TAC–MMF, and have had stable graft function up to now. Of the 6 patients treated also with immunoadsorption, one who had not successfully been converted to TAC lost her graft; 4 of the other 5 patients experienced recovery (3 completely reversed, 1 partially reversed), and 1 returned to dialysis (Table 2); circulating antiendothelial cell antibodies were 1:80 positive in this patient when AR occurred. All the three completely reversed patients had normal graft function for greater than one year of follow-up. In the completely reversed patients; it took 18.3±8.7 days (16.4±7.9 days in the TAC–MMF only group and 23.8±9.7 days in the immunoadsorption group) for the treatment to begin taking effect.

With regard to the 19 patients, 3 of the 10 females lost their grafts; but only 1 of the 9 males lost his graft. Of the 13 rejection episodes occurring during the first two weeks after transplantation, 10 (83.3%) were completely reversed – 1 patient (8.3%) returned to continuous dialysis. Of the 6 rejection episodes occurring later, 3 (50%) caused graft’s loss and 3 (50%) were partially reversed. None of the episodes occurring in this cohort after the second post-transplantation week were completely reversed (Table 3) ($P=0.003$ vs episodes occurring during the first 14 days). We did not find any difference in the efficiency of TAC–MMF treatment for C4d-positive and -negative steroid-resistant rejections (Table 4).

Infectious complications

Pre-transplantation donor and recipient cytomegalovirus serological status was D+/R+ in all 19 patients. No severe infectious complications were observed in patients receiving TAC–MMF, except for 1 case of urinary tract infection happening one month after the rejection episode. Of the patients who had been treated with immunoadsorption, 3 of the 4 completely reversed patients experienced severe cytomegalovirus pneumonia during the two months following the rejection episode. Of the 6 patients treated also with immunoadsorption, one who had not successfully been converted to TAC lost her graft; 4 of the other 5 patients experienced recovery (3 completely reversed, 1 partially reversed), and 1 returned to dialysis (Table 2);
Table 2. Clinical features of Chinese patients with acute humoral rejection

<table>
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<tr>
<th>Case</th>
<th>Onset of AR (days)</th>
<th>Diagnostic biopsy (days)</th>
<th>Trough SCr before rejection</th>
<th>SCr at diagnosis (μmol/l)</th>
<th>Highest level of SCr (μmol/l)</th>
<th>IA (sessions)</th>
<th>TAC/MMF</th>
<th>Reversal of rejection</th>
<th>Time to effect</th>
<th>Infectious complications</th>
<th>Current Scr (months)</th>
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*Measured by days after transplantation.
*Days after onset of rejection.
*This patient did not successfully converted to tacrolimus.
episodes, all accompanied with acute respiratory distress syndrome. As to the 2 patients who lost renal allografts, most immunosuppressants were withdrawn in them and no infectious episodes were observed. The incidence of severe pneumonia in the patients who received immunoadsorption was significantly higher than in the patients treated simply with TAC–MMF ($P = 0.021$) (Table 5).

### Discussion

In this article, we report on 19 Chinese renal allograft recipients in whom C4d-positive steroid-resistant AR was later confirmed. Of them, 13 were treated with the combination of TAC and MMF without immunoadsorption or plasmapheresis. Rejection episodes were controlled in 11 recipients (84.6%, with 7 complete and 4 partial reversals), and 1-year graft survival rate was 84.6%. Our results suggest that combined TAC and MMF treatment is a potentially safe and economical method with acceptable efficacy for most Chinese renal allograft recipients who have C4d-positive steroid-resistant AR.

According to the Banff 2001 criteria [2], the diagnosis of AHR requires serologic evidence of circulating antibodies to donor HLA or to other antidonor endothelial antigens. The antibodies, especially HLA-II-reactive antibodies, are very difficult to detect, so it is suggested that an early post-transplantation biopsy showing diffuse C4d-positive staining associated with suggestive morphological features of acute antibody-mediated rejection can be diagnosed as AHR [6]. Our patients met the criteria used in some publications [4,14]. Nevertheless, C4d deposition in PTCs is also an independent prognostic factor for renal allograft outcomes. Most rejections in this cohort were accompanied with histological signs of acute cellular rejection, a finding reported in some publications [15]. Endoarteritis was found in 94.7% cases of acute cellular rejections, and interstitial haemorrhage and arterial intimal necrosis and arterial thrombosis were not rare in our cohort, most such lesions are associated with a poorer outcome [16].

The combined use of immunoadsorption or plasmapheresis with MMF, TAC and one or more of intravenous immunoglobulins has been reported to effectively reverse C4d-positive AHR in western countries [4,6,9,10]. It seems that removal of existing pathogenetic alloantibodies with immunoadsorption or plasmapheresis is an essential component of treatment for this kind of patients. The use simply of the combination of TAC and MMF in the treatment of AHR had not been studied. Actually, some investigations showed that TAC–MMF played a critical role in the rescue of AHR [9]. It is obvious that immunoadsorption or plasmapheresis can be used to remove existing alloantibodies, but it does not suppress antibody synthesis, and a rebound in circulating donor-specific antibodies (DSA) after plasmapheresis has been documented [17]. It is TAC–MMF that suppresses the production of new DSA. MMF inhibits in vitro
antibody production by B cells and has in vivo been shown to reduce humoral responses in renal transplant recipients [18]. In the pre-cyclosporine era, attempts to treat AHR by removing DSA with plasmapheresis were largely unsuccessful [19]. All these findings suggest that TAC–MMF played a more important role in the rescue of AHR than immunoadsorption and plasmapheresis. Finally, as C4d-positive AR is frequently accompanied by signs of T-cell mediated rejection, and since MMF and, especially, TAC are drugs approved for the treatment of severe steroid-resistant cellular rejection, both antibody mediated rejection and the accompanying T-cell activity can be controlled by TAC–MMF.

Significant differences exist between races in eastern and western countries; and renal allograft recipients of eastern origin usually need lower doses of immunosuppressants than recipients of western origin. Therefore, we reasoned that the combined use of MMF and TAC might effectively control the ongoing alloantibody mediated rejections in Chinese patients. It is very interesting that the treatment needs 18.1±9.0 days to take effect: this is coincident with the half-life of IgG, the major immunoglobulin in humans.

Nevertheless, immunoadsorption and plasmapheresis often cause severe infections – especially cytomegalovirus pneumonia, which is associated with a high mortality in Chinese recipients [20]. Many centres routinely use gancyclovir to prevent cytomegalovirus pneumonia when giving immunoadsorption treatment. As to our study cohort, no pneumonia was observed in the group treated with TAC–MMF, but 2 of the 3 reversed patients (66.7%) who received immunoadsorption developed cytomegalovirus pneumonia (P < 0.05). The study presented here suggests that TAC–MMF rescue is safer than immunoadsorption for most Chinese patients with C4d-positive steroid-resistant AR. Our former study found that circulating antiendothelial cell antibodies may be associated with poor graft outcomes [13], this might account for the loss of grafts in two patients who received immunoadsorption.

Our investigation revealed that late-onset C4d-positive AHR might be associated with a worse outcome. Significantly most of the episodes of C4d-positive rejections occurring in the first two weeks were completely reversed (P = 0.003), and only one (7.7%) graft was lost; but 3 (50%) of the 6 patients whose AR developed 2 weeks or more after transplantation lost their grafts. In the 4 patients who lost their grafts, 3 (75%) were females. This suggested that C4d-positive AHR that occurs 2 weeks or more after the operation might need more aggressive therapies, besides the combined use of MMF and TAC, especially in females. As the sample size of our study is very small, it is difficult to find any association between the Banff grades and patients’ outcomes.

In the study, 19 out of 442 grafted patients developed C4d-positive steroid-resistant AR, a rather low incidence. Actually, C4d-positive AHR is not always steroid-resistant [15], and in our cohort the staining for C4d was performed in tissue stored for a long time. Accordingly we should have more cases of C4d-positive AR responding to TAC–MMF treatment. So, we think that the possible selection bias in this retrospective study should not affect the conclusion derived from this investigation.

Nickeleit et al. [15] have reported that an antilymphocytic preparation can effectively reverse C4d-positive rejection with pronounced allograft dysfunction – immunoadsorption or plasmapheresis was not used in their cohort. We have presented another proof that AHR may have a favourable outcome without immunoadsorption or plasmapheresis treatment. Our study suggests that the use of only TAC–MMF may have an efficacy in Chinese recipients similar to that of using TAC–MMF combined with immunoadsorption, but with fewer severe infectious complications. This is not a prospective controlled study, which keeps us from drawing a stronger conclusion; but, our investigation provides the rationale for further investigations and a new safe and economic choice for treating patients from eastern countries – especially developing countries, where most patients cannot afford the costly immunoadsorption or plasmapheresis treatment. Further prospective research should be performed to confirm the efficacy of this treatment. Also, whether this protocol is suitable for the treatment of the so-called ‘pure humoral rejection’ needs to be studied.

**Conflict of interest statement.** None declared.

**References**


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**Table 5. Complications in patients receiving different treatments**

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<tr>
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<th>TAC–MMF</th>
<th>IA ± TAC–MMF</th>
<th>P-value</th>
</tr>
</thead>
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<td>Morbidity of entire cohort</td>
<td>0/13 (0%)</td>
<td>3/6 (50.0%)</td>
<td>0.021</td>
</tr>
<tr>
<td>Morbidity of rejection-reversed patients</td>
<td>0/11 (0%)</td>
<td>3/4 (75.0%)</td>
<td>0.009</td>
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<tr>
<td>Patients who received temporary dialysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morbidity of the entire cohort</td>
<td>9/13 (69.2%)</td>
<td>6/6 (100%)</td>
<td>0.255</td>
</tr>
<tr>
<td>Morbidity of rejection-reversed patients</td>
<td>7/11 (63.6%)</td>
<td>4/4 (100%)</td>
<td>0.275</td>
</tr>
</tbody>
</table>

TAC: tacrolimus; MMF: mycophenolate mofetil; IA: immunoadsorption.


Received for publication: 26.4.05
Accepted in revised form: 12.8.05