Mycophenolate Mofetil Attenuates Renal Ischemia/Reperfusion Injury

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Abstract. Immunosuppressive agents may have an impact on ischemia/reperfusion (I/R) injury. The immunosuppressant mycophenolate mofetil (MMF) presents properties that can attenuate such injury. This study investigated the effects of MMF on renal I/R injury. Male Wistar rats received MMF (20 mg/kg per d) or vehicle by gavage beginning 2 d before ischemia and maintained during the entire study. Ischemic injury was induced by bilateral renal arteries occlusion for 60 min. Control rats received MMF and underwent sham operation. At days 1, 2, and 14, post-ischemia renal function was assessed and kidneys were removed for histologic and immunohistochemical studies. MMF given to nonischemic rats did not alter renal function. There was no functional protection at 24 h post-ischemia with MMF. At 2 d, post-ischemia rats pretreated with MMF presented higher inulin clearance compared with untreated rats (0.42 ± 0.04 versus 0.15 ± 0.02 ml/min per 100 g; P < 0.001) and attenuated renal blood flow decrease (5.23 ± 0.28 versus 3.24 ± 0.37 ml/min; P < 0.01). The immunostaining for intercellular adhesion molecule-1 (ICAM-1) was less intense in rats pretreated with MMF. These rats also presented an earlier decreased infiltrating macrophages/lymphocytes and cell proliferation at day 1 post-ischemia. The functional and immunohistochemical analyses performed at day 14 post-ischemia returned to values similar to controls in both groups of rats. To determine whether mycophenolic acid (MPA) could induce cytoprotection, the effects of MPA on normoxic and hypoxic/reoxygenated (H/R) isolated tubule suspensions were also investigated. MPA was not deleterious to normoxic tubules and it was not protective against H/R tubules. In conclusion, pretreatment with MMF attenuates I/R injury in rats and does not limit the recovery from ischemia. The protective effect of MMF by reducing inflammation precedes the hemodynamic changes and tubular injury.

Renal ischemia/reperfusion (I/R) injury is a common cause of early allograft dysfunction in renal transplanted patients and represents an additional risk factor for the late renal allograft failure (1,2). I/R injury triggers an inflammatory response that causes tissue damage (3,4). In addition, acute renal failure caused by ischemia is associated with high mortality and morbidity in patients with native kidneys (5).

The use of immunosuppressant calcineurin inhibitors, such as cyclosporin A (CsA) and tacrolimus (FK 506), has been associated with limiting adverse events, especially nephrotoxicity (6). However, mycophenolate mofetil (MMF) and non–calcineurin inhibitor drugs, such as sirolimus, present fewer adverse effects with limited nephrotoxicity. Therefore, these latter immunosuppressive drugs could affect the inflammatory response, attenuating renal I/R injury in renal transplantation patients.

MMF, the morpholinoethyl ester of mycophenolic acid (MPA), is a powerful immunosuppressant that is currently used in organ transplantation and under evaluation in immune-mediated inflammatory diseases (7,8). MPA depletes guanosine triphosphate pools in lymphocytes and monocytes and suppresses the de novo synthesis of purines, exerting a selective and reversible antiproliferative activity on these cells. In addition, MPA inhibits production of cell-surface adhesion molecules, which is critical for the recruitment of leukocytes to sites of inflammation (9,10). Several studies have demonstrated that blockade of leukocytes and adhesion molecules in postischemic renal injury is protective against ischemia (25–27).

Therefore, considering that immunosuppressive therapy may have an impact on I/R injury, we studied the effects of MMF in a rat model of ischemic acute renal failure.

Materials and Methods

Experimental Model

Male Wistar rats weighing 180 to 250 g were used for all experiments. Under ether anesthesia, an abdominal incision was made and the renal vessels on both sides were identified. Renal arteries were
bilateral occlusion with a microvascular clamp for 60 min. Clamps were removed, and blood flow to the kidneys was reestablished. The surgical incision was sutured, and rats were allowed to recover with free access to food and water.

Before surgery, rats were assigned into four groups. Group 1 (Isc+M) received 20 mg/kg MMF (Roche Laboratories, Nutley, NJ) once daily dissolved in a mixture of olive oil and 5% DMSO, and Group 2 (Isc+V) received vehicle. All treatments started 2 d before surgery and were maintained until renal function studies were conducted or until the kidneys removed. Group 3 (M) underwent sham operation and received MMF on the same schedule, and Group 4 (C) underwent sham operation and received no treatment. All experimental procedures were performed according to our institutional guidelines. Doses of MMF were obtained from another study (11).

**Renal Function Studies**

Renal function was assessed by measuring GFR, renal blood flow (RBF), and fractional excretion of sodium, potassium, and water (FE\textsubscript{Na}, FE\textsubscript{K}, and FE\textsubscript{H2O}, respectively). GFR was measured by inulin clearance (C\textsubscript{ICG}).

In the first set of experiments, clearance studies were conducted 24 h after ischemia in Isc+V and Isc+M groups (n = 6) and 2 d after surgery in all groups of rats: C (n = 5); M (n = 5); Isc+V (n = 9); Isc+M (n = 9). Rats were anesthetized with sodium pentobarbital (50 mg/kg body wt, intraperitoneally) and placed on a temperature-regulated surgical table. A tracheotomy was performed, and jugular veins were cannulated with PE60 catheter for infusion of inulin and fluids. Inulin (100 mg/kg body wt) was injected as a loading dose, followed by continuous infusion (0.27 mg/min). A PE50 catheter was placed in the left femoral artery, and mean arterial pressure (MAP) was recorded. Urinary bladder was cannulated with PE240 for collection of urine. After 30-min stabilization, three urine samples (30-min intervals) were collected. Blood samples were obtained at the beginning and end of collection periods.

To determine the effects of MMF administered at the time of ischemia, an additional group of rats (n = 6) received 20 mg/kg MMF by gavage on the day of ischemia and underwent clearance studies 48 h post-ischemia.

In a second set of experiments, we evaluated recovery from ischemic renal failure in rats from group Isc+M (n = 6) and Isc+V (n = 6). On the 2\textsuperscript{nd}, 4\textsuperscript{th}, 6\textsuperscript{th}, 8\textsuperscript{th}, 10\textsuperscript{th}, and 14\textsuperscript{th} days after renal ischemia, a tail blood sample was obtained for determination of blood urea nitrogen (BUN). Clearance studies were performed on the 14\textsuperscript{th} day.

At the end of the experiment, a perivascular transonic ultrasonic flow sensor (T-106; Transonic System Inc., Ithaca, NY) with the probe placed around the left renal artery was used to monitor renal blood flow (RBF) in all groups.

**Biochemical Analyses**

Plasma and urine inulin concentrations were determined by anthrone method, and ions were measured by flame photometry (Instrumental Laboratory Inc.). Urinary volume was measured gravimetrically. Values obtained for the three clearance periods were averaged for each rat. Blood urea nitrogen (BUN) level was measured by a modified Crocker method. Renal vascular resistance (RVR) was estimated by the expression RVR = MAP/RBF, where MAP and RBF represent mean arterial pressure and renal blood flow, respectively.

**Antibodies**

The following antibodies were used: (1) a monoclonal IgG\textsubscript{1} antibody to rat intercellular adhesion molecule-1 (ICAM-1; CD54, clone 1A29; Serotec, Oxford, UK); (2) a monoclonal IgG\textsubscript{1} antibody to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (ED\textsubscript{3}; Bioproducts for Science, Indianapolis, IN); (3) a monoclonal antibody to proliferating cellular nuclear antigen (PCNA; Sigma, St. Louis, MO); (4) a monoclonal IgG\textsubscript{1} antibody to rat T lymphocytes (CD3; clone W3/13HLK; Harlan Sera-Lab., Long- borough, England).

**Immunohistochemical Analyses**

Kidney tissue was harvested at 1, 2, and 14 d after ischemia in Isc+V group (n = 5 to 7 at each time point) and Isc+M group (n = 6 to 8 at each time point). In the control group (n = 5), kidneys were removed at surgery without renal arteries clamping. Representative portions of the kidneys were snap-frozen in liquid nitrogen or fixed by Dubosque Brasil solution, post-fixed in buffered 10% formaldehyde solution, and embedded in paraffin.

The PCNA and ED1 immunostaining were processed in 4-\mu m paraffinized sections. After deparaffinization, endogenous peroxidase activity was blocked with 0.3% H\textsubscript{2}O\textsubscript{2} in water for 10 min at room temperature. Sections were then subjected to microwave irradiation in citrate buffer to enhance antigen retrieval (12) and incubated with a 1/1000 anti-PCNA antibody or with a 1/2500 anti-ED1 antibody for 60 min at room temperature before incubation with a biotinylated mouse anti-rat IgG for 30 min at room temperature. The reaction product was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The color reaction was developed with 3,3-diaminobenzidine (Sigma), and sections were counterstained with methyl green.

The ICAM-1 and CD3 immunostaining was processed in frozen acetone-fixed 4-\mu m-thick cryostat and deparaffinized sections, respectively. Sections were preadsorbed with avidin-biotin blocking reagents (Blocking Kit, Vector Laboratories). Nonspecific binding sites were blocked by a 60-min incubation with a 10% horse serum. Sections were then incubated overnight at 4°C with a 1/400 anti-ICAM antibody or with a 1/50 anti-CD3 antibody before incubation with a biotinylated mouse anti-rat IgG for 30 min at room temperature. The reaction product was detected with a streptavidin-alkaline phosphatase complex (Vector Laboratories). The color reaction was developed with a fast-red dye solution containing levamisole, an inhibitor of endogenous alkaline phosphatase activity, and counterstained with hematoxylin.

For all sections, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody of normal rabbit or goat IgG. Evaluation of all slides was performed by an observer who was unaware of the origin of the slides. The sections were examined using light microscopy at a magnification of ×400.

The number of PCNA−, ED1−, and CD3−positive cells in each section was calculated by counting the number of positive cells in 30 sequential fields of renal cortex and 20 sequential fields of renal medulla and expressed as mean ± SEM of cells per field.

ICAM-1 expression was calculated by scoring 20 sequential fields of renal cortex using a semiquantitative scale. Each score primarily reflects changes in extent rather than intensity of staining and depends on the percentage of the tubulointerstitial area showing positive staining: 0, 0 to 5% stained; I, >5 to 25%; II, >25 to 50%; III, >50 to 75%; IV, >75%. Data were expressed as mean score ± SE per field.

**Renal Morphology**

Renal morphology was evaluated at 2 and 14 d post-ischemia in pretreated Isc+V and Isc+M rats (n = 6 to 8 in each time point).
Paraffin sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) using standard procedures. Tubulointerstitial injury was defined as tubular necrosis, tubular dilatation and/or atrophy, inflammatory cell infiltrate, or cellular edema. Injury was graded (13) on a scale of 0 to 4 as follows: normal kidney (0), small focal areas of damage (0.5), involvement of <10% of the cortex or outer medulla (1), involvement of 10 to 25% of the cortex or outer medulla (2), involvement of 25 to 75% of the cortex or outer medulla (3), extensive damage involving more than 75% of cortex or outer medulla (4).

Proximal Tubules Isolation Method
Male Wistar rats (150 to 250 g) were anesthetized with sodium pentobarbital (50 mg/kg body wt, intraperitoneally) and underwent laparotomy. The aorta was cannulated with polyethylene tubing (PE 100), and the kidneys were perfused with 60 ml of cold heparinized (4000 U) oxygenated solution (solution A) containing: 112 mM NaCl, 25 mM NaHCO3, 5 mM KCl, 1.6 mM CaCl2, 2.0 mM NaH2PO4, 1.2 mM MgSO4, 5 mM glucose, 2.5 mM HEPES, 10 mM mannitol, 1 mM glutamine, 1 mM sodium butyrate, and 1 mM sodium lactate, buffered to pH 7.4. Subsequently, kidneys were perfused with 30 ml of solution A containing 15 mg of collagenase (type V, Sigma) and 15 mg of hyaluronidase (Sigma). After perfusion, kidneys were placed in ice-cold solution A containing 40 mg of collagenase and 10 mg of hyaluronidase. Tissue digestion was performed at 37°C and superfused continuously with 95% O2/5% CO2 in a shaking water bath. Digestion was halted at 15, 20, 25, and 30 min to remove the suspended digested tubules. These segments were poured off, washed in solution A and placed in a solution containing 1 g of fatty acid-free albumin (Sigma). Meanwhile, larger particles were returned to water bath for further digestion. At 30 min of digestion, all tissue was washed free of collagenase and placed in the albumin-supplemented solution A for 10 min. After filtering through a coarse wire mesh, tissue was washed free of albumin three times with solution A and suspended in 60 ml of oxygenated cold 45% Percoll layered onto 5 ml of 100% Percoll. Tissue was centrifuged at 1500 x g for 10 min in a refrigerated centrifuge (Hitachi, Japan). The lowest band, which was composed primarily (95%) of proximal tubules, was removed and refrigerated ultracentrifuge (Hitachi, Japan). The lowest band, which was removed and refrigerated ultracentrifuge (Hitachi, Japan). The lowest band, which was removed and refrigerated ultracentrifuge (Hitachi, Japan).

LDH Release Determination
One milliliter of proximal tubules suspension was centrifuged for 10 s in a refrigerated centrifuge at 1500 x g. The pellet was lysed with Triton X-100, 1.5% (vol/vol). LDH activity was measured in the supernatant and pellet according to Bergmeyer (14). LDH release was converted to percentage by dividing supernatant activity by total activity.

Statistical Analyses
All values were reported as mean ± SEM. Data were analyzed by ANOVA with the Student-Newman-Keuls multiple comparison post-test or by unpaired t test when appropriate. P < 0.05 was considered statistically significant.

Results
BUN determinations before bilateral renal clamping and on subsequent 14 d in MMF-treated and untreated rats are shown in Figure 1. BUN levels increased after ischemia in both groups, peaked at 48 h, and decreased toward basal levels at day 14. The values of BUN at day 2 and day 4 were lower in rats pretreated with MMF.

No differences were observed in RBF between Isc+V (4.54 ± 0.24 ml/min) and Isc+M (3.57 ± 0.11 ml/min) at 24 h post-ischemia. CIN (ml/min per 100 g body wt) and FEPO4 were 0.076 ± 0.018 and 16.1 ± 3.0% in Isc+V group, respectively. These values were not different from Isc+M group (0.057 ± 0.030 and 11.2 ± 2.2%). These data indicate that earlier, at 24 h post-ischemia, no protection on renal hemodynamic and tubular function was observed with MMF pretreatment.

Renal function studies performed 48 h after ischemia are summarized in Table 1. There were no significant differences in renal function of nonischemic MMF-treated rats (M) versus control (C) rats.

Hypoxia/Reoxygenation (H/R) Protocol
Isolated tubules were divided into an experimental group and a time control group. The pO2 in time control group was kept throughout the experiment in the 300 to 400 mmHg range. The experimental group was made hypoxic for 15 min (pO2 averaged 20 to 40 mmHg) by gassing with 95% N2/5% CO2 for 5 min. After hypoxia, tubules were reoxygenated by gassing with 95% O2/5% CO2 for 5 min. The pO2 after reoxygenation returned to the 300 to 400 mmHg range. Flasks were capped and kept oxygenated for 45 min.

Samples were obtained at baseline, after 15 min of hypoxia (H-15), and after 45 min of reoxygenation (R-45). Samples from time control groups (C) were obtained at the same time points. Cell injury was assessed by lactate dehydrogenase (LDH) release.

Figure 1. Urea nitrogen levels in serum (BUN) over 14 d after 60-min bilateral renal clamping. Rats pretreated with mycophenolate mofetil (MMF; *) presented a significant decrease in BUN values at days 2 and 4 after ischemia compared with untreated rats (■). Values at each point represent mean ± SEM. *P < 0.001; #P < 0.01.
Urine volume was higher in Isc, significant increase in renal vascular resistence (RVR). This decrease in renal perfusion was associated with a decrease in GFR, FE Na, FE K, and FE H2O, fractional excretion of sodium, potassium, and water, respectively. C, control (n = 5); M, MMF-treated rats (n = 5); Isc + V, ischemic vehicle-pretreated rats (n = 9); Isc + M, ischemic MMF-pretreated rats (n = 9).

Untreated ischemic rats presented a significant decrease in the number of inulin clearance (ml/min per 100 g body wt) compared with control rats (0.15 ± 0.02 versus 0.85 ± 0.07; P < 0.001). Pre treatment of ischemic rats with MMF resulted in a significant protection compared with Isc + V rats (0.42 ± 0.04 versus 0.15 ± 0.02; P < 0.001). RBF (ml/min) measurements were significantly lower in Isc + V group compared with control (3.214 ± 0.37 versus 6.98 ± 0.15; P < 0.001) and Isc + M rats (5.2 ± 0.3; P < 0.01 versus Isc + V). This decrease in renal perfusion was associated with a significant increase in renal vascular resistance (RVR). Urine volume was higher in Isc + V rats (0.027 ± 0.003; P < 0.01 versus all groups). The FE Na was markedly elevated in Isc + V rats (5.6 ± 1.0 versus 0.5 ± 0.1; P < 0.001), and pretreatment with MMF reduced FE Na (2.1 ± 0.6; P < 0.1 versus Isc + V), indicating an additional tubular protection. FE K and FE H2O followed a similar pattern.

Renal function results obtained at 14 d after ischemia (16 d of treatment) are presented in Table 2. All functional parameters returned to baseline in both groups, indicating that MMF did not influence recovery from ischemia.

When MMF was administered at the time of ischemia, no protection of renal function was observed 48 h after ischemia. (C IN, 0.19 ± 0.04 ml/min per 100 g; RBF, 4.9 ± 0.26 ml/min; d FE Na, 2.0 ± 0.2%).

Immunohistochemical Analyses

The graphical representation of the expression of ICAM-1 24 h after 60 min of bilateral renal artery occlusion is depicted in Figure 2. Control kidneys (Figure 3A) did not stain for ICAM-1. Untreated rats (Figure 3B) showed an increased staining for ICAM-1 on endothelium and tubular cells. This staining was significantly decreased in the kidneys of animals treated with MMF (Figure 3C), reaching values not statistically different from control. Our results indicate that pretreatment with MMF downregulates the expression of ICAM-1 in ischemic kidneys.

The number of interstitial cells demonstrating ED-1 staining for macrophages/monocytes were significantly increased at 24 h and 48 h after ischemia in untreated rats (Figure 4C). The pretreatment with MMF induced a significant reduction of ED1-positive interstitial cells (Figure 4E). In control kidneys, we noticed only a few ED1-positive cells (Figure 4A). At day 14 post-ischemia, the number of ED1-positive cells was not different from control in both groups. Figure 5 shows the number of ED1-positive cells in cortex and renal medulla at the time points studied.

Cells demonstrating staining for PCNA were minimal in control kidney (Figure 4B). At 48 h post-ischemia we noticed a marked increase in PCNA-positive cells. The majority of staining was found in tubular cells (Figure 4D). Rats pretreated with MMF presented a significant decrease in PCNA-positive cells (Figure 4F). As seen in Figure 6, PCNA staining was detected in renal cortex and medulla as early as 24 h after ischemia, peaked at 48 h, and presented a minor increase (although significant) by day 14 post-ischemia. The pretreatment with MMF resulted in a significant decrease of PCNA-positive cells in any time point.

The immunohistochemical analysis also showed a significant increase in infiltrating lymphocytes at 24 h and 48 h post-ischemia (Figure 7B) compared with controls (Figure 7A). The lymphocyte infiltration in the MMF-pretreated rats

| Table 1. Renal function and hemodynamic parameters 48 h after ischemia

<table>
<thead>
<tr>
<th>Groups</th>
<th>GFR ml/min per 100 g</th>
<th>MAP mmHg</th>
<th>RBF ml/min</th>
<th>RVR ml/min per mmHg</th>
<th>V ml/min</th>
<th>FE Na %</th>
<th>FE K %</th>
<th>FE H2O %</th>
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<tbody>
<tr>
<td>C</td>
<td>0.85 ± 0.07</td>
<td>117 ± 3</td>
<td>6.98 ± 0.15</td>
<td>16.2 ± 0.2</td>
<td>0.012 ± 0.001</td>
<td>0.5 ± 0.1</td>
<td>18.9 ± 3.8</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>M</td>
<td>0.80 ± 0.09</td>
<td>116 ± 2</td>
<td>6.82 ± 0.26</td>
<td>17.1 ± 0.9</td>
<td>0.008 ± 0.001</td>
<td>0.5 ± 0.1</td>
<td>26.7 ± 2.4</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Isc + V</td>
<td>0.15 ± 0.02</td>
<td>108 ± 2</td>
<td>3.24 ± 0.37</td>
<td>36.3 ± 3.6</td>
<td>0.027 ± 0.003</td>
<td>5.6 ± 1.0</td>
<td>139.3 ± 22.4</td>
<td>11.3 ± 1.6</td>
</tr>
<tr>
<td>Isc + M</td>
<td>0.42 ± 0.04</td>
<td>110 ± 3</td>
<td>5.23 ± 0.28</td>
<td>21.4 ± 1.4</td>
<td>0.017 ± 0.004</td>
<td>2.1 ± 0.6</td>
<td>26.2 ± 4.4</td>
<td>2.0 ± 0.4</td>
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* Data are expressed as mean ± SEM. GFR, glomerular filtration rate; MAP, mean arterial pressure; RBF, renal blood flow; RVR, renal vascular resistance; V, urine volume; FE Na, FE K, and FE H2O, fractional excretion of sodium, potassium, and water, respectively. C, control (n = 5); M, MMF-treated rats (n = 5); Isc + V, ischemic vehicle-pretreated rats (n = 9); Isc + M, ischemic MMF-pretreated rats (n = 9).

| Table 2. Renal function and hemodynamic parameters 14 d after ischemia

<table>
<thead>
<tr>
<th>Groups</th>
<th>GFR ml/min per 100 g</th>
<th>MAP mmHg</th>
<th>RBF ml/min</th>
<th>RVR ml/min per mmHg</th>
<th>V ml/min</th>
<th>FE Na %</th>
<th>FE K %</th>
<th>FE H2O %</th>
</tr>
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<tbody>
<tr>
<td>Isc + V</td>
<td>0.93 ± 0.07</td>
<td>109 ± 3</td>
<td>6.37 ± 0.51</td>
<td>17.6 ± 1.0</td>
<td>0.005 ± 0.001</td>
<td>0.4 ± 0.1</td>
<td>22.5 ± 4.4</td>
<td>0.54 ± 0.1</td>
</tr>
<tr>
<td>Isc + M</td>
<td>0.87 ± 0.08</td>
<td>105 ± 3</td>
<td>6.10 ± 0.27</td>
<td>18.2 ± 1.1</td>
<td>0.010 ± 0.002</td>
<td>0.6 ± 0.2</td>
<td>21.3 ± 3.2</td>
<td>1.02 ± 0.2</td>
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</table>

* Data are expressed as mean ± SEM. Isc + V, ischemic vehicle-pretreated rats (n = 6); Isc + M, ischemic MMF-pretreated rats (n = 6).
was also markedly diminished (Figure 7C). At 14 d after ischemia, the number of these cells returned to values not different from the control group (Figure 8).

Renal Morphology

Light microscopy studies showed tubular necrosis, tubular dilatation, inflammatory cell infiltration, and cellular edema in the tubulointerstitial compartment of the renal cortex and outer medulla from animals sacrificed 48 h after renal ischemia. These lesions were less intense in rats treated with MMF (P < 0.05 versus untreated rats). We also observed tubular dilatation and/or atrophy and inflammatory cell infiltrate in the cortex and medulla from the animals sacrificed 14 d after renal ischemia, but these alterations were mild in most of them, and no difference was observed between the two groups of rats (Figure 9).

Proximal Tubule Suspension Studies

LDH release revealed no cytotoxicity of MPA in oxygenated proximal tubules after 60 min of incubation (Figure 10). Acute addition of MPA to H/R tubules also had no effect on LDH release (Figure 11, A and B).

Figure 2. Intercellular adhesion molecule-1 (ICAM-1) expression in renal cortex 24 h after ischemia. Values are expressed as mean ± SEM. *P < 0.001 versus other groups.

Figure 3. Immunostaining for ICAM-1 in renal cortex at 24 h after ischemia from control rats (A), untreated rats with ischemic injury (B), and MMF-treated rats (C). Note that the expression of ICAM-1 is higher in panel B. Magnification, ×400.
Discussion

We investigated the effects of pretreatment with MMF on renal ischemia reperfusion (I/R) injury. It was observed that pretreatment with MMF resulted in functional protection of renal I/R injury and did not limit recovery from ischemia.

Decreased infiltrating macrophages and lymphocytes and reduced expression of ICAM-1 were observed 24 h post-ischemia in MMF-treated animals, and an improvement in renal function was observed only 48 h after ischemia. These findings indicate that the protective effect of MMF by reducing inflammation is seen before there is amelioration of hemodynamic changes and tubular dysfunction.

Control rats treated with MMF did not present any change in GFR, RBF, or tubular function. This observation is in agreement with other studies that demonstrate that MMF is not nephrotoxic (15). In contrast, calcineurin inhibitors, such as cyclosporin (CsA) and tacrolimus (FK506), are associated with nephrotoxicity, which limits their benefits in long-term treatments (6). CsA strikingly decreased GFR and RBF in experi-

Figure 4. Immunostaining for ED-1 (macrophages/monocytes; A, C, and E) and proliferating cell nuclear antigen (PCNA; B, D, and F) in juxtamedullary region from control rats (A and B) and untreated rats with ischemic injury (C and D) or MMF-treated rats (E and F). Note that the number of ED-1– and PCNA–positive cells is higher in C and D, respectively. Magnification, ×280.
mental models of nephrotoxicity. Similar effects were also demonstrated with FK 506 (16,17).

MMF pretreatment in rats submitted to 60 min of bilateral renal artery occlusion was associated with higher GFR and RBF compared with untreated rats. These findings were accompanied by a corresponding tubular protection, as demonstrated by FE_{\text{Na}} and FE_{\text{K}}.

After 14 d, all functional parameters returned to baseline values, demonstrating that MMF-pretreated rats presented similar renal function recovery compared with untreated rats. This is an additional beneficial effect of MMF in contrast with rapamycin, which may limit renal recovery from ischemia injury (18).

The decreased expression of ICAM-1 and reduced macrophages and lymphocytes infiltration presented by MMF-pretreated rats may be explained by the inhibitory action of MMF on adhesion molecule synthesis. MPA depletes guanosine triphosphate pools, inhibiting fucose and mannose transfer to membrane glycoproteins, including adhesion molecules (10). Several studies suggest that MMF can suppress leukocyte attachment and penetration through endothelial cells (19,20). MPA treatment of human monocytes decreased their attachment to endothelial cells (19). Similarly, MMF treatment of CD4 and CD8 cells was shown to suppress binding to P-selectin, VCAM, ICAM-1, and E-selectin (20). In another study, MPA also inhibited the overexpression of ICAM-1 and osteopontin in ischemia/reperfusion injury (21).

There is a growing body of literature showing that ICAM-1, leukocyte adhesion molecule CD_{11/CD}_{18}, and leukocyte endothelial adhesion molecules play an important role in renal I/R injury. Ischemic injury triggers release of inflammatory mediators and upregulates adhesion protein expression on endothelial cells and leukocytes. These factors result in rolling, adherence, and extravasation of leukocytes in the renal tissue (22,24). Earlier experimental studies have suggested a protective effect of anti-adhesion therapy on I/R injury (25–27). Administration of monoclonal antibodies against both CD_{11/CD}_{18} and ICAM-1 afforded impressive functional and morphologic protection in rats (25). Haller et al. (26) used ICAM-1 antisense oligodeoxyribonucleotides to inhibit the surface expression of ICAM-1 in a model of rat renal injury and showed inhibition of granulocyte and macrophage infiltration associated with less pathologic and functional damage. Finally, knockout mice lacking the gene for ICAM-1 were also protected from acute ischemic renal injury (27). An increase in renal leukocyte infiltration after I/R injury has been demonstrated in several studies. The presence of these leukocytes has been related to renal parenchymal damage exacerbating I/R injury (22–24). Polymorphonuclear cells (PMN) have long been considered as main mediators of injury in ischemic kidneys, but their actual role remains controversial (28–31). Recently, some studies have demonstrated that mononuclear cells present prominent features in experimental models of ischemic ARF (32–36). An increased number of macrophages (ED1-positive cells) in renal interstitium is demonstrated from day 2 through day 8 post-ischemia (33,34). Similarly, an increased number of T lymphocytes have been identified in experimental model of ischemic renal failure, and these cells can mediate I/R injury (35,36). In this study, we demonstrate an increased infiltration of interstitial macrophages and lymphocytes in untreated rats 48 h post-ischemia, which could be significantly reduced by pre-treatment with MMF. These observations suggest that these infiltrating cells are implicated in I/R injury, which is in agreement with several studies demonstrating the importance of inflammatory pathways in renal I/R injury (22–24).

In parallel with the antiinflammatory effects of MMF protecting against I/R injury, we observed increased RBF in MMF-pretreated rats. Therefore, MMF may also have a hemodynamic beneficial effect on renal ischemia. Further investi-
gations are necessary to elucidate the mechanisms that resulted in this observation.

To determine whether MPA could induce cytoprotection, we investigated the effects of MPA on normoxic and hypoxic/reoxygenated isolated proximal tubules in suspension. It was demonstrated that MMF had no direct effect on isolated tubules, suggesting that MMF protection against I/R is not consequent to direct cytoprotection.

ARF induced by many agents results in increased cell proliferation by damaged tubular cells, presumably a reparative

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**Figure 7.** Immunostaining for CD3 (T lymphocytes) in juxtamedullary region from control rats (A) and untreated rats with ischemic injury (B) or MMF-treated rats (C) at 48h. Note that the number of CD3-positive cells is higher in panel B. Magnification, ×400.

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**Figure 8.** Number of CD3-positive cells/mm² in renal cortex and outer medulla at 24 h, 48 h, and 14 d after ischemia. Values are expressed as mean ± SEM. *P < 0.01 and **P < 0.05 versus Isc+V; *P < 0.01 and ***P < 0.05 versus control. ■, control; □, Isc; □, Isc+V; □, Isc+M.

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**Figure 9.** Tubular necrosis scores in renal cortex (A) and outer medulla (B) at 48 h after ischemia. *P < 0.003 and **P < 0.01 versus Isc+V. ■, Isc+V; □, Isc+M.
event (37). PCNA is an acidic nuclear protein that increases from the late G1 to S phases of the cell cycle; its detection parallels other standard methods for assessing cell proliferation (38). Studies applying PCNA antibodies to renal tissue sections in post-ischemic experimental models of acute renal failure found a large number of PCNA-positive cells in tubules of the renal cortex and outer medullary stripe (39). There is evidence that tubular cell proliferation may be related to the severity of acute tubular necrosis in humans (40). We show an increased tubular proliferation 24 and 48 h post-ischemia in untreated animals, which is significantly reduced in animals pre-treated with MMF. It is likely that this finding reflects milder renal injury in rats pre-treated with MMF, as demonstrated by a lesser degree of tubular necrosis in these animals.

At 14 d post-ischemia, an increased number of macrophages were detected compared with control group. This finding is in agreement with other observations that the number of monocytes and macrophages was increased at 16 d after renal ischemia, which had resolved by day 180, indicating that this infiltration had no implication for later scarring (41).

In summary, pretreatment with MMF protected against I/R injury in rats. MMF protection may have occurred by an earlier antiinflammatory effect and a beneficial hemodynamic effect rather than a direct cytoprotection. Therefore, MMF used as part of an immunosuppressant regimen may also afford protection against renal ischemia.

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