Mycophenolate mofetil prevents salt-sensitive hypertension resulting from nitric oxide synthesis inhibition

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Quiroz, Yasmin, Héctor Pons, Katherine L. Gordon, Jaimar Rincón, Maribel Chávez, Gustavo Parra, Jaime Herrera-Acosta, Dulcenombre Gómez-Garre, Raquel Largo, Jesús Egido, Richard J. Johnson, and Bernardo Rodríguez-Iturbe. Mycophenolate mofetil prevents salt-sensitive hypertension resulting from nitric oxide synthesis inhibition. Am J Physiol Renal Physiol 281: F38–F47, 2001.—Recent studies have suggested that subtle microvascular and tubulointerstitial injury in the kidney can cause salt-sensitive hypertension. To test this hypothesis, we determined whether the mild renal disease induced by transient blockade of nitric oxide (NO) synthesis would result in salt-sensitive hypertension and whether prevention of the renal injury by coadministration of the immunosuppressive agent mycophenolate mofetil (MMF) would block the development of salt sensitivity. Nω-nitro-L-arginine-methyl ester (L-NAME; 70 mg/100 ml in the drinking water) was administered for 3 wk to rats with or without MMF (30 mg·kg−1·day−1 by gastric gavage), followed by a 1-wk “washout” period in which the MMF was continued, which was followed in turn by placement on a high-salt (4% NaCl) diet for an additional 4 wk. Renal histology was examined at 3 and 8 wk, and blood pressure was measured serially. L-NAME treatment resulted in acute hypertension and the development of mild renal injury. During the washout period, blood pressure returned to normal, only to return to the hypertensive range on exposure of the animals to a high-salt diet. MMF treatment prevented the development of hypertension in response to a high-salt diet. This correlated with the ability of MMF to inhibit specific aspects of the renal injury, including the development of segmental glomerulosclerosis, the infiltration of T cells and ANG II-positive cells, and the thickening of afferent arterioles.

lymphocytes; nitric oxide inhibition; angiotensin II-positive cells

Tubulointerstitial injury and microvascular damage were commonly present in many of the clinical and experimental conditions associated with salt-sensitive hypertension and postulated that these changes could result in a malfunctioning pressure-natriuresis mechanism and the induction of a salt-driven elevation of arterial blood pressure. Subsequent experimental studies demonstrated that subtle microvascular and tubulointerstitial injury could be induced in the kidney by short-term administration of ANG II (24), catecholamines (15), or cyclosporin (18) and that this could result in salt-sensitive hypertension that would manifest despite removal of the original vasoconstrictive agent.

The observation that the administration of a vasoconstrictive agent can result in subtle renal injury and persistent salt-sensitive hypertension suggests that a similar process might be induced by blocking key vasodilator systems, such as the nitric oxide (NO) system. Baylis et al. (3) and Ribeiro et al. (30) originally reported on the effects of short-term inhibition of NO synthesis by L-arginine analogs. During the period of NO blockade, rats develop hypertension that involves the sympathetic nervous system (35), endothelin (39), and the renin-angiotensin system (11, 17, 19, 25). Similar to the ANG II (24) and catecholamine (15) models, NO blockade is also associated with the development of focal renal injury, characterized by mild glomerulosclerosis, interstitial fibrosis, arterial and arteriolar lesions, and cellular infiltration in the tubulointerstitial areas of the kidney (3, 4, 30, 39).

Given the remarkable similarity in the renal histological findings observed with NO blockade to those associated with other models of salt-sensitive hypertension (15, 18, 24), we hypothesized that transient blockade of the NO system would also lead to the

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development of sodium-sensitive hypertension. Furthermore, we hypothesized that measures that could reduce the renal injury induced by NO blockade might prevent or ameliorate the later development of hypertension.

To test this latter hypothesis, we utilized the immunosuppressive agent mycophenolate mofetil (MMF). MMF reversibly inhibits the enzyme inosine monophosphate dehydrogenase that regulates de novo purine synthesis and hence strongly inhibits lymphocyte proliferation (1). Our group and others have found that MMF potently suppresses the infiltration of immune cells in the remnant kidney (8, 29, 33) and in the aortic wall of cholesterol-fed rabbits (34). MMF may also have effects on nonhematopoietic cells and has been found to inhibit the expression of adhesion molecules on endothelial cells (13), reduce the proliferation of renal mesangial cells (14), and decrease collagen production by fibroblasts (2). Infiltration of mononuclear cells and expression of adhesion molecules are prominent features in the kidneys after NO inhibition (7, 23, 36). The infiltration of lymphocytes and macrophages is likely to be involved in the mediation of renal injury, but they are also rich sources of cytokines and vasoactive compounds that might modify the physiological mechanisms of sodium excretion. We therefore hypothesized that MMF might act to reduce the infiltration of immunocompetent cells and reduce the glomerular and tubulointerstitial injury, thereby preventing the development of salt sensitivity.

MATERIALS AND METHODS

Experimental Design

Experiments were performed in male Sprague-Dawley rats (Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela) with an initial weight of 300–350 g. The rats were divided into three groups: the L-NAME group (n = 20), the L-NAME+MMF group (n = 20), and a control group (n = 10). Animals were kept in group cages or individual metabolic cages as required, and animal care followed approved institutional protocols. All animals (L-NAME group, L-NAME+MMF group, and control group) received a regular diet with normal salt content (Ratarina, Protinal, Valencia, Venezuela) during the 3 wk of NO synthesis inhibition and the 1 wk of the washout phase. Afterward, they were placed on a high-salt (4% NaCl) diet (Harlan Teklad, Madison, WI) for the remaining 4 wk of the experiment.

Blood pressure, weight, and serum samples were taken weekly. Serum creatinine and urinary protein determinations were done by autoanalyzer methodology (Express Plus, Ciba Corning, Oberlin, OH). Five to ten rats from each experimental group were killed at the end of the period of NO synthesis inhibition and at the end of the experiment by aortic desanguination under ether anesthesia. Kidneys were used for histological and immunohistological studies.

Blood Pressure Determinations

Tail-cuff plethysmography (IITC, Life Scientific Instruments, Woodland Hills, CA) was used to determine systolic and mean arterial pressure weekly (mean value of 3–4 separate measurements/rat). Before the experiments were begun, animals were conditioned three to four times to the procedure.

Histological Studies

As described in previous communications (24), the excised kidneys were divided into two portions. One was included in tissue-freezing medium, snap-frozen in dry ice and acetone, and stored at −70°C. The other was cut in coronal sections that were fixed in methyl carnoys and then embedded in Paraplast Plus (Monoject, Sherwood Medical Scientific Division, St. Louis, MO). Four-micrometer sections of paraffin-embedded tissues were stained with periodic acid-Schiff reagent and trichrome stains and used for evaluation of glomerular and tubulointerstitial histological changes. Frozen sections were used for immunofluorescence studies.

All analyses were performed blinded. Glomerulosclerosis was defined as the increment in periodic acid-Schiff-stained material or green areas (Gomori’s trichrome stain) associated with loss of cellular elements, collapse of capillary lumens, and amorphous hyaline material with or without adhesions to the Bowman’s capsule. Glomerulosclerosis was graded by a semiquantitative index score described by Raij et al. (28). A minimum of 22 glomeruli was evaluated in each biopsy (range 22–56), and they were graded from 0 to 4+ as follows: grade 0 = normal (no sclerosis); grade 1+ = <25% involvement of the glomerular tuft; grade 2+ = 25–50% involvement of the glomerular tuft; grade 3+ = 50–75% involvement of the glomerular tuft; and grade 4+ = 75–100% involvement of the glomerular tuft. The injury score was obtained by multiplying the degree of damage by the percentage of glomeruli with the same degree of damage. The final tissue injury score was the addition of these values (33): [(1 × n of glomeruli with 1+) + (2 × n of glomeruli with 2+) + (3 × n of glomeruli with 3+) + (4 × n of glomeruli with 4+)] × 100/total n of glomeruli examined. In this scoring system, the maximum possible score of a biopsy in which all glomeruli had >75% sclerosis would be 400 = (4 × n glomeruli/n glomeruli) × 100.

Tubulointerstitial injury was classified according to the extension of damaged areas in tubules (tubular cellularity, basement membrane thickening, dilatation, sloughing, atrophy) and in the interstitium (cellular infiltration, fibrosis, widening) with respect to the areas with normal architecture,
following a previously described scale (27): 0 = no changes present; grade 1 = <10%; grade 2 = 10–25%; grade 3 = 25–50%; grade 4 = 50–75%; grade 5 = 75–100%. For each biopsy, the entire cortical and juxtedudillary regions were evaluated.

Cellular counts in glomeruli were expressed as positive cells with a given staining per glomerular cross section and in the tubulointerstitium as positive cells per square millimeter. Computer-assisted image-analysis software (Optimas, version 6.2, Media Cybernetics, Silver Spring, MD) was used to study osteopontin, which is a marker of tubulointerstitial injury. The digitalized images were acquired with a Leica DMRB microscope fitted with a microimage iii08 low-light video camera with a Flashpoint video framegrabber (Bartels & Stout, Bellevue, WA) as described before (24).

Afferent arteriolar thickness was measured in individual arterioles by using computerized analysis of images acquired with a Zeiss Axioscope fitted with a Kodak DC 120 digital megapixel camera. Afferent arterioles were identified by their location adjacent to the vascular pole of the glomerular tuft, the presence of an elastic lamina, and by having fewer endothelial cells than the efferent arteriole. Afferent arteriolar wall thickness was quantified in individual arterioles by measuring the width of arteriole wall (exclusive of the endothelium), using the mean of the thinnest and thickest segments. At least 7–12 arterioles were examined per biopsy.

**Immunohistology**

As described previously (24, 26, 32), indirect immunoperoxidase methodology was used to identify osteopontin, macrophages (ED-1-positive cells), proliferating cells (PCNA-positive cells), and angiotensin AT1 and AT2 receptors (AT1- and AT2-positive cells). Indirect immunofluorescence was used to identify lymphocytes (CD5-positive cells), interleukin-2 (IL-2) receptor expression (CD25-positive cells), and ANG II-producing cells (ANG II-positive cells).

Double immunostaining was done to define the percent of CD5 (lymphocytes)- and ED-1 (macrophages)-positive cells that stained positive for ANG II (ANG II-positive cells). The technique has been described previously in detail (32) and includes, first, incubation with Mabs anti-CD5 or anti-ED-1; second, incubation with rhodamine-conjugated affinity-purified F(ab’2) IgG with minimal cross-reactivity to rat serum proteins; third, incubation with anti-ANG II antibody; and, finally, incubation with fluorescein-conjugated (dichlorotriazinyl aminofluorescein affinity-purified donkey anti-rabbit IgG antibody with minimal cross-reactivity to rat serum proteins. The following negative controls were carried out: 1) staining using a nonpertinent monoclonal antibody instead of the first primary antibody (anti-CD5 or anti-ED-1); 2) staining in which the second primary antibody (rabbit anti-human ANG II) was omitted; and 3) staining omitting either the rhodamine-conjugated rat anti-mouse IgG or the dichlorotriazinyl aminofluorescein affinity-purified donkey anti-rabbit IgG.

**Antisera**

Antisera used in this study included anti-osteopontin (OP 199, gift of C. Giachelli, Univ. of Washington), ED-1 (monoclonal antibody to macrophages, Harlan Bioproducts, Indianapolis, IN), anti-CD5 (clone MRCOX19, Biosource, Camarillo, CA), anti-CD25 (Accurate Chemical and Scientific, Westbury, NY), anti-AT1 and AT2 (goat anti-rat AT1 and AT2 antibodies, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-ANG II (rabbit anti-ANG II-human IgG, Peninsula Laboratories). Secondary rat anti-mouse and donkey anti-rabbit antibodies were purchased from Accurate Chemical and Scientific.

**Statistical Analysis**

Comparison between groups was done by one-step ANOVA with Tukey-Kramer posttests. Serial changes were evaluated with repeated measures ANOVA and Tukey-Kramer posttests. Two-tailed \( P \) values are used throughout, and \( P < 0.05 \) was considered statistically significant. A commercially available statistical program (Instat, GraphPad, San Diego, CA) was used for the statistical calculations.

**RESULTS**

**Effects of MMF Treatment on Blood Pressure, Weight, and Renal Function**

Figure 1 shows that systolic blood pressure rose progressively during the 3 wk of L-NAME administration. At the end of the NO synthesis inhibition period, blood pressure reached 194 ± 24 mmHg in the L-NAME group and 182 ± 13 mmHg in the L-NAME+MMF group (\( P = \text{NS} \)). During the 1-wk washout period, blood pressure decreased similarly in both experimental groups and returned to the upper limits of the 95% confidence interval of the arterial pressure found in the control group (Fig. 1). During the subsequent high-salt-diet period (weeks 4–8), systolic blood pressure rose to levels (mean) of 170 mmHg in the rats untreated with MMF whereas it remained in the normal range in the MMF-treated animals.

The serial weight changes are shown in Fig. 2. MMF-treated and untreated rats had similar weight changes during the experiment. These changes included a re-

![Fig. 1. Systolic arterial pressure changes during and after nitric oxide synthesis inhibition. Experimental groups received vehicle (●) or mycophenolate mofetil (MMF; □) during the first 4 wk [3 wk of \( N^n \)-nitro-L-arginine-methyl ester (L-NAME) administration and 1 wk of washout] before the start of a high-salt diet (last 4 wk of the experiment). A high-salt diet was given to examine the development of salt-sensitive hypertension. Values are means ± SD. Dotted lines, 95% confidence interval (CI) of systolic arterial pressure in control rats. \( * P < 0.05 \) and \( ** P < 0.001 \) vs. control. \( * P < 0.001 \) vs. control and MMF. \( b P < 0.001 \) vs. control and \( P < 0.01 \) vs. MMF. \( c P < 0.001 \) vs. control and \( P < 0.05 \) vs. MMF.](http://ajprenal.physiology.org/DownloadedFrom)
suggested that MMF may have altered the renal injury induced by NO blockade. Studies therefore focused on the effects of MMF on the various renal compartments both at 3 (at the end of the L-NAME period) and at 8 wk (at the end of the high-salt diet).

Glomerular changes. Some segmental glomerulosclerosis was found in 61% of the glomeruli in the L-NAME group and in 37% of the glomeruli in the rats of the L-NAME+MMF group at 3 wk. However, the degree of glomerulosclerosis was mild, and only rare glomeruli showed involvement of >50% of the glomerular tuft (grade 3 sclerosis index score). MMF treatment significantly reduced the glomerulosclerosis index in L-NAME treated rats at 3 wk from 74.5 ± 17.7 in the L-NAME group to 44.6 ± 18.0 in the L-NAME+MMF group (P < 0.05) (Table 1). During the period of the high-salt diet (8-wk studies), the experimental groups did not have statistically significant changes in their sclerosis index scores from the values observed at the end of the NO blockade (3-wk studies) (Table 1).

Tubulointerstitial injury. L-NAME-treated rats developed mild to moderate tubulointerstitial injury, and this was not reduced by MMF treatment. Tubulointerstitial scores at the end of the L-NAME administration were comparable in the L-NAME group and in the L-NAME+MMF group. Similarly, osteopontin scores were similar in the L-NAME (0.69 ± 0.3%) and L-NAME+MMF groups (0.67 ± 0.5%) at 3 wk. In contrast, during the post-L-NAME period the tubulointerstitial injury in the normotensive MMF-treated group was significantly less compared with the hypertensive MMF-untreated group (Table 1).

Cellular infiltration. Macrophage (ED-1-positive cells) infiltration in the interstitium was mild and was not different between experimental groups at 3 wk (L-NAME group, 36 ± 17; L-NAME+MMF group, 49 ± 25) or at the end of the subsequent high-salt-diet phase at 8 wk (L-NAME group, 13 ± 5.5; L-NAME+MMF group, 7.7 ± 5.80). However, the number of macrophages at 8 wk was significantly less than the corresponding values at 3 wk (P < 0.02).

An accumulation of lymphocytes (CD5-positive cells) was also present in the interstitium of L-NAME-treated rats (Fig. 3). The number of lymphocytes was sixfold greater than that observed in the control group. In contrast to the lack of effect of MMF on inhibiting the macrophage accumulation, MMF treatment re-

**Histological Findings**

The observation that blood pressure was reduced during the high-salt phase in the MMF-treated rats was also present in the interstitium of L-NAME-treated rats (Fig. 3). The number of lymphocytes was sixfold greater than that observed in the control group. In contrast to the lack of effect of MMF on inhibiting the macrophage accumulation, MMF treatment re-

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**Table 1. Histological findings**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3 wk</th>
<th>8 wk</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>L-NAME</td>
<td>L-NAME + MMF</td>
<td>L-NAME</td>
</tr>
<tr>
<td>Glomerulosclerosis</td>
<td>0</td>
<td>74.6 ± 17.6</td>
<td>44.6 ± 18.0*</td>
</tr>
<tr>
<td>score index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulointerstitial</td>
<td>&lt;1</td>
<td>1.3 ± 0.7</td>
<td>1.6 ± 1.0</td>
</tr>
<tr>
<td>score (0–5)</td>
<td></td>
<td></td>
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<tr>
<td>Afferent arteriolar</td>
<td>2.52 ± 0.32</td>
<td>3.30 ± 0.73</td>
<td>2.48 ± 0.32*</td>
</tr>
<tr>
<td>thickness, μm</td>
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Values are means ± SD. L-NAME, N\(^\circ\)-nitro-L-arginine methyl ester; MMF, mycophenolate mofetil. Significant level vs. L-NAME, *P < 0.05.
Reduced the lymphocyte infiltration by 60% (Fig. 3). At the end of the high-salt period (8 wk), the infiltration of lymphocytes had decreased in the L-NAME group to values comparable to those in the L-NAME + MMF group, which remained at stable levels.

Activated lymphocytes [IL-2 receptor (CD25)-positive cells/mm²] were also detected in rats with NO synthesis inhibition, and their numbers were also markedly reduced by MMF treatment (L-NAME group, 8.4 ± 2.8; L-NAME + MMF group, 1.2 ± 2.7; P < 0.001, Fig. 4). After 4 wk of a high-salt diet, the accumulation of activated lymphocytes was still more prominent in the hypertensive L-NAME group than in the normotensive L-NAME + MMF group (Fig. 4).

Representative photomicrographs of histological changes and cellular infiltration are shown (see Fig. 7).

Expression of ANG II-positive cells. A marked infiltration of interstitial cells expressing ANG II was observed in L-NAME-administered rats (Fig. 5; see also Fig. 8), and the infiltration of ANG II-positive cells became more intense (P < 0.01) during the subsequent period of the high-salt diet. MMF treatment reduced the number of ANG II-positive interstitial cells by 50% compared with the L-NAME group both at the end of the NO synthesis blockade and at the end of the salt-loading period (Fig. 5). ANG II-positive cells were also found rarely in glomeruli. In individual rats, the number never exceeded 2 positive cells/glomerular cross section, and the differences in the L-NAME and the L-NAME + MMF groups were not statistically significant.

Double staining (see Fig. 8) documented that many of the ANG II-positive cells were CD5-positive lymphocytes. However, the proportion of CD5-positive lymphocytes that were ANG II positive was similar in the L-NAME group (24 ± 6%; L-NAME + MMF group, 22 ± 7%) at the end of NO synthesis inhibition and also at the end of the high-salt-diet period (L-NAME group, 38 ± 6%; L-NAME + MMF group, 39 ± 6%). The differences between the 3- and 8-wk findings were not statistically significant.

ANG II receptors. AT1 receptors were found in glomeruli, proximal and distal tubules, and renal vasculature (see Fig. 8). As shown in Fig. 6, at the end of NO blockade the expression of AT1 receptors in tubular cells (AT1-positive cells/100 tubulointerstitial cells)
was increased in the L-NAME group (35 ± 3.5) with respect to values in control animals (19 ± 3.5, \( P < 0.001 \)), but it was not increased in the L-NAME+MMF group (22 ± 8.4). The number of AT\(_1\) receptor-positive cells during the period of the high-salt diet was also increased significantly in both experimental groups with respect to the values at the end of NO synthesis inhibition (Fig. 6); however, the difference between the L-NAME group and the L-NAME+MMF group was no longer significant.

AT\(_2\) receptors were scarce and limited to vascular regions and occasional proximal tubular cells (<3 positive cells/100 tubular cells).

**Microvascular changes.** Afferent arteriolar thickness was increased with L-NAME administration, and MMF treatment prevented this abnormality (Table 1, Fig. 7). The differences between the L-NAME and L-NAME+MMF groups were observed at the end of 3 wk (\( P = 0.052 \)) and persisted during the subsequent high-salt-diet period (\( P < 0.05 \) at 8 wk).

**DISCUSSION**

We have been interested in the role of subtle renal injury in the development of salt-sensitive hypertension. In this study, we utilized the model of chronic NO blockade to induce the renal injury to determine whether we could induce a salt-sensitive state. Given previous reports that MMF can reduce injury in “nonimmune” models of renal injury such as the remnant kidney model (9, 29, 33), we further tested the hypothesis that MMF treatment during the period of NO blockade could prevent the subsequent salt sensitivity.

Rats were treated with L-NAME (a NO synthesis inhibitor) for 3 wk, and during this time they developed the well-characterized arterial hypertension that has been shown to be largely dependent on the renin angiotensin, endothelin, and sympathetic nervous systems (11, 17, 19, 25, 35, 39 and reviewed in 41). Although it is known that the coadministration of a high-salt diet during the L-NAME administration can induce much more severe hypertension and renal injury (9, 17), in this study a normal-salt diet was used as only mild and focal renal injury was desired. Indeed, histological studies performed at the end of the L-NAME administration showed characteristic thickening of the afferent arterioles (4) and only mild tubulointerstitial injury. Although segmental sclerosing changes were present in the majority of glomeruli, only rarely was glomerulosclerosis severe. Serum creatinine was also within normal limits, and there was no significant proteinuria. The observation that proteinuria was not elevated has been observed previously by others (19), although clearly in some studies proteinuria may have been present (39).

After the L-NAME period, blood pressure returned to the normal range. However, the subsequent placement of these rats on a high-salt diet resulted in the first new finding of the study, which was the demonstration of a rapid rise in blood pressure in response to a high-salt diet. Control rats that had never been exposed to L-NAME did not show the increased blood pressure response to the high-salt diet. The observation that hypertension developed in the rats that had been previously exposed to L-NAME suggests that the kidney had been altered by the L-NAME treatment. Indeed, the microvascular and tubulointerstitial changes induced by L-NAME are similar to what is observed with transient exposure to ANG II (24) or catecholamines (15) that also result in a salt-sensitive state.

To better understand the pathogenesis of the salt sensitivity, we treated another group of rats with MMF during the L-NAME period. MMF is a potent immunosuppressive agent that primarily acts to reduce T cell proliferation and activation (1); however, it also has some effects on other cell populations (2, 13, 14). MMF was selected because prior observations had shown that it can reduce renal injury in animal models in which macrophage and T cell accumulation occur, such as the remnant kidney model (8, 29, 33). In this study, the MMF was well tolerated. Although MMF can cause diarrhea and weight loss that could affect blood volume and hence blood pressure, no diarrhea was observed in the MMF-treated rats, and there were also similar changes in weights between the MMF+L-NAME and L-NAME groups.

MMF did not protect animals from the acute hypertension induced during L-NAME administration. However, the second new finding in this study was the observation that MMF treatment during the period of NO blockade completely prevented the development of salt-sensitive hypertension in the subsequent high-salt-diet phase. The ability of MMF to prevent the development of salt-sensitive hypertension led us to...
focus on the individual compartments in the kidney to obtain insight into the mechanisms by which salt sensitivity could be induced or prevented.

One potential mechanism by which L-NAME-mediated renal injury could cause hypertension would be by reducing nephron mass. Certainly, there was some segmental glomerulosclerosis after L-NAME treatment, and this was significantly reduced by MMF therapy (Table 1). The mechanism involved may relate to the ability of MMF to block mesangial cell proliferation (14). The only measurement of renal function that was made was serum creatinine, and this is a relatively insensitive measure of the glomerular filtration rate (GFR). However, the fact that serum creatinine remained in the normal range throughout the study and in both groups suggests that any depression in the GFR was not severe. Nevertheless, we cannot exclude the possibility that one mechanism by which MMF may have prevented the development of salt-sensitive hypertension could be better preservation of glomeruli and GFR.

A second mechanism by which MMF may have prevented the development of post-L-NAME salt-sensitive hypertension is via effects on the tubulointerstitium. Interestingly, MMF did not block tubulointerstitial injury (tubular dilation, cast formation, etc.) or tubular osteopontin expression in response to L-NAME. This might be expected, as MMF did not block the acute hypertensive response and therefore may not have blocked the acute vasoconstriction and ischemia associated with inhibition of NO synthesis, which can directly damage tubules and stimulate osteopontin expression (21). MMF also did not block the macrophage infiltration induced by L-NAME. However, consistent with the known effect of MMF to suppress lymphocyte proliferation, there was a profound reduction in T cells

Fig. 7. Microphotographs of a glomerulus showing grade 2+ glomerulosclerosis (Gomori's trichromic stain) in a biopsy from the L-NAME group (A) in contrast to a normal glomerulus in the L-NAME+MMF group (B). Focal tubulointerstitial infiltrate is evident in a biopsy from the L-NAME group (C) and absent in the L-NAME+MMF group (D). T lymphocyte (CD5-positive cells) infiltration in the L-NAME group (E) is almost absent in the L-NAME+MMF group (F). Activated lymphocytes expressing IL-2 receptor-positive cells (G) and afferent arteriole thickening (H) are shown in biopsies from rats in the L-NAME group.
(CD5+ cells) in L-NAME+MMF-treated animals compared with L-NAME alone. MMF also reduced the number of activated lymphocytes (IL-2 receptor-positive cells) in L-NAME-treated rats, and this effect persisted at the end of the high-salt period (Fig. 4).

A potential mechanism linking the reduction in T cells with the prevention of salt-sensitive hypertension was revealed when the tissue was examined for the presence of ANG II with a specific antibody. A new finding was that L-NAME treatment was associated with a significant interstitial infiltration of mononuclear cells that were expressing ANG II and that MMF reduced this number by >50% at both the end of the L-NAME period and at the end of the high-salt-diet period (Fig. 3). When double immunolabeling was performed, it was shown that between 5 and 40% of the ANG II-positive cells were T cells. The observation that T cells may express ANG II is consistent with previous studies documenting that T cells and macrophages can express angiotensin-converting enzyme activity and mRNA (5, 22, 31, 40). It is possible that positive staining for ANG II in the infiltrating lymphocytes represents ANG II binding to receptor sites; however, the staining appeared to be in the cytoplasm (Fig. 8). The observation that T cells were actively expressing this vasoactive mediator suggests potential new roles for these cells in intrarenal hemodynamics and sodium handling.

An interesting observation was that the number of ANG II-positive cells increased during the high-salt-diet period in both experimental groups ($P < 0.01$, Fig. 5) and reached values in the 8-wk studies that are similar to (L-NAME+MMF group) or higher (L-NAME group) than the number of infiltrating CD5-positive cells at the corresponding time (Fig. 3). This confirms that cells other than lymphocytes were also staining positive for ANG II during the high-salt period. Importantly, MMF therapy was associated with a reduction by 50% in the number of ANG II-positive cells at the end of the high-salt-diet period.

We also examined the renal distribution AT1 and AT2 angiotensin receptor in the L-NAME model. AT1 receptor expression was increased during the period of NO synthesis inhibition in the L-NAME group but not in the MMF-treated rats (Fig. 6 and Fig. 8, E and F). This observation is in agreement with the finding that type 1 ANG II receptors are stimulated by increased ANG II activity in this experimental model (20) and that they play an important role in the inflammatory cardiovascular changes (38) and fibrosis resulting from transforming growth factor-β induction (37). The reduction in ANG II-positive cells associated with MMF treatment (Fig. 5) is likely responsible for the finding of lower expression of AT1 receptors in the MMF-treated group (Fig. 6). In the subsequent period of the high-salt diet, AT1 receptor expression increased in both experi-
mental groups, and although the rats in the L-NAME group had a higher expression of AT_1 receptors, the differences between the experimental groups were not statistically significant (Fig. 6). As suggested by Harrison-Bernard et al. (12), failure to downregulate AT_1 receptor expression may help explain why chronic elevation of ANG II induces sustained hypertension. In these experiments, sustained salt-sensitive hypertension in the L-NAME group was associated with increased ANG II-positive cells and increased AT_1 receptor expression in tubulointerstitial regions of the kidney.

An additional mechanism that may account for the induction of salt sensitivity was the development of an arteriolopathy in L-NAME-treated rats (Table 1). This lesion, which has been observed in previous studies by other groups (4), is characterized by thickening of the smooth muscle cell layer due to the presence of more smooth muscle cells and matrix (“hypertrophic vascular remodeling”). Importantly, MMF treatment was associated with a significant reduction in the arteriolar thickening. Theoretically, a reduction in the arteriolopathy in the MMF-treated rats may improve renal blood flow in the post-L-NAME period and thereby help prevent continued renal ischemia. Indeed, recent studies in the cyclosporin A model have found a close relationship between the presence of afferent arteriolar-atheriolyopathy and the development of salt-sensitive hypertension (18), and Goldblatt himself (10) postulated a key role for afferent arteriolar disease in the pathogenesis of salt sensitivity.

Finally, it is of interest that the mild tubulointerstitial injury that resulted from L-NAME administration increased afterward in the rats with salt-sensitive hypertension, in contrast to the normotensive rats that had been treated with MMF during the L-NAME period (Table 1). One possibility is that the continued tubulointerstitial injury represents hypertension-related injury; a second possibility is that the continued presence of arteriolopathy and infiltration of ANG II-positive cells led to continued ischemia and local activation of fibroblasts and cytokines.

In summary, rats treated for 3 wk with an inhibitor of NO synthesis develop subtle glomerular, arteriolar, and tubulointerstitial renal damage that predisposes the animals to salt-sensitive hypertension. Although MMF treatment does not block the acute hypertensive response to NO synthesis inhibition, MMF prevents the subsequent salt-sensitive hypertensive response. The mechanism by which MMF protects appears to involve a reduction in renal injury, as manifested by less arteriolar thickening, less glomerulosclerosis, and less infiltration in the interstitium of T cells and ANG II-positive cells. Thus MMF may be helpful in the prevention of renal injury and the development of hypertension in conditions in which NO synthesis is impaired.

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