Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats

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Rodríguez-Iturbe, Bernardo, Yasmir Quiroz, Mayerly Nava, Lizzette Bonet, Maribel Chávez, Jaime Herrera-Acosta, Richard J. Johnson, and Héctor A. Pons. Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. Am J Physiol Renal Physiol 282: F191–F201, 2002. First published August 30, 2001; 10.1152/ajprenal.00197.2001.—Immunocompetent cells infiltrate the kidney in several models of experimental hypertension. We have previously shown that reduction of this infiltrate results in prevention of salt-sensitive hypertension induced by short-term angiotensin II infusion and nitric oxide inhibition (Quiroz Y, Pons H, Gordon KI, Rincón J, Chávez M, Parra G, Herrera-Acosta J, Gómez-Garre D, Largo R, Egido J, Johnson RJ, and Rodríguez-Iturbe B. Am J Physiol Renal Physiol 281: F38–F47, 2001; Rodríguez-Iturbe B, Pons H, Quiroz Y, Gordon K, Rincón J, Chávez M, Parra G, Herrera-Acosta J, Gómez-Garre D, Largo R, Egido J, and Johnson RJ. Kidney Int 59: 2222–2232, 2001). We therefore studied whether hypertension could be controlled in genetically hypertensive rats (spontaneously hypertensive rats [SHR]) by the administration of 20 mg·kg−1·day−1 of the immunosuppressive drug mycophenolate mofetil (MMF group; n = 35). Other SHR received vehicle (n = 35), and Wistar-Kyoto rats (n = 20) were used as controls. MMF or vehicle was given in two separate 4-wk periods, separated by a 3-wk interval. Systemic hypertension was reduced to normal levels in both periods of MMF treatment in association with a reduction in lymphocyte, macrophage, and angiotensin II-positive cells infiltrating the kidney. Oxidative stress was also reduced by MMF, as indicated by a reduction in urinary malondialdehyde (MDA), renal MDA content, and superoxide-positive cells, and was highly correlated with blood pressure levels. We conclude that the renal immune infiltrate plays a major role in the hypertension in SHR.

immunocompetent donors (4, 39). Conversely, other investigations (53) have reported the partial transfer of hypertension by lymphoid cells.

Despite the evidence for a role of the immune system in the pathogenesis of hypertension, little attention has been given to renal infiltration of immunocompetent cells that is known to be present (1, 20). We have hypothesized that the immune cell infiltration in the kidney may be critical for the development of some forms of hypertension (24, 56). Factors related to the kidney are unquestionably important because cross-transplantation studies with genetically hypertensive and normotensive rats have shown that “hypertension travels with the kidney” (reviewed in Ref. 54). Interestingly, hypertension increases with age in SHR, and so does renal injury and inflammation (12, 30). Furthermore, the possibility that lymphocytes and macrophages infiltrating the kidney could play a role in the genesis or maintenance of hypertension in the SHR is suggested by recent studies by our group of the acquired salt-sensitive hypertension that occurs after

SEVERAL LINES OF EVIDENCE point to the participation of the immune system in the pathogenesis of genetic forms of murine hypertension and, to a lesser extent, essential human hypertension (reviewed in Refs. 13, 18, 26, 33). Most of the evidence has been obtained in the Okamoto strain of spontaneously hypertensive rats (SHR) and includes demonstrations of immunological dysfunction as well as improved hypertension as a result of treatment modalities directed to the immune system. Immunological abnormalities include reduction in subpopulations of T lymphocytes (15), suppressed delayed hypersensitivity (66), depressed mitogen-induced proliferative responses (43, 47), immunogenic arterial wall epitopes (40), increased autoantibody levels in plasma (11), and impaired leukocyte-endothelial cell interactions (64). Treatment modalities directed to the immune system that are known to improve hypertension include cyclophosphamide therapy (6, 27), administration of anti-thymocyte serum (7), and neonatal thymectomy (28) and thymic implants from normotensive donors (4, 39). Conversely, other investigations (53) have reported the partial transfer of hypertension by lymphoid cells.

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ANG II or Nω-nitro-L-arginine methyl ester (L-NAME) exposure. Indeed, the transient administration of ANG II or L-NAME to rats will result in salt-sensitive hypertension that persists after removal of the inciting stimulus, and this is associated with the infiltration of immunocompetent cells in the renal interstitium (49, 57). Many of the mononuclear cells, including some T cells, were shown to express ANG II, and there was also local generation of oxidants (49, 57). These findings appear particularly relevant in light of studies that have shown that the renin-angiotensin system is activated during monocyte/macrophage differentiation (42) and upregulation of the AT1 receptor in macrophages contributes to the increment in peroxide production (68). Treatment with mycophenolate mofetil (MMF) during ANG II or L-NAME administration was able to reduce the T cell infiltration, decrease the number of ANG II-positive cells present in the interstitium, and prevent the generation of oxidants, and these effects were shown to prevent the subsequent development of salt-sensitive hypertension (49, 57).

Because SHR have renal interstitial infiltration of mononuclear cells (12, 30, 32), an activated intrarenal renin-angiotensin system (16, 20, 34, 36, 44), and evidence of oxidative stress (60, 63, 65, 67), we hypothesized that measures that could reduce the renal interstitial inflammation might prevent or treat the hypertension in this rat strain. We therefore treated the SHR with MMF, a selective lymphocyte-suppressive agent that reversibly inhibits the enzyme inosine monophosphate dehydrogenase (3), which regulates the de novo pathway of purine synthesis. MMF treatment reduces proliferation and infiltration of immunocompetent cells in the experimental models of progressive renal failure (19, 52, 58), atherosclerosis (59), and experimentally induced salt-sensitive hypertension (49, 57). We postulated that this drug would also suppress the renal infiltration of lymphocytes and macrophages in the SHR and, therefore, could be used to gain further insight into the role of immunocompetent cells in the maintenance of hypertension in this strain of rats.

MATERIALS AND METHODS

Experimental design. Male SHR from the Okamoto strain and control normotensive Wistar-Kyoto rats (WKY; 25–27 wk of age; Instituto de Investigaciones Científicas, Los Teques, Venezuela) were provided unrestricted access to regular rodent chow (Purina, Proinital, Valencia, Venezuela) and water throughout the experiments and were handled in accordance with institutional guidelines of animal care.

The SHR were randomly divided into two groups: the MMF-treated group (n = 35) and the vehicle-treated group (n = 35). The MMF-treated group received the drug by gastric gavage in daily doses of 20 mg/kg body wt. Because the drug is insoluble in water, it was suspended in 500 µl of water by vigorous agitation immediately before administration, as discussed in previous reports (49, 57–59). The vehicle-treated group received 500 µl of water daily by the same method. Control WKY rats (n = 20) did not receive either MMF or vehicle. In separate experiments, the effect of MMF on blood pressure was tested in an additional group of normotensive WKY rats (n = 12) that received 20 mg/kg of MMF daily by gastric gavage for 4 wk. The initial weight of the rats in the SHR experimental groups did not differ significantly (MMF-treated group, 356.7 ± 45.6 g; vehicle-treated group, 364.5 ± 43.2 g), nor did it differ from the weight of the control group (WKY, 365.3 ± 38.5 g).

Studies were designed to test the effects of intermittent MMF therapy on blood pressure, infiltration of immunocompetent cells, and oxidative stress. Preliminary studies demonstrated that 4 wk of treatment with MMF would cause a significant decrease in the blood pressure in SHR (48) and that this effect would disappear after 3 wk without administration of the drug; therefore, the experimental protocol included three successive periods, extending over a total of 11 wk. Period I lasted for 4 wk (weeks 0–4), during which the SHR received MMF or vehicle; period II lasted for 3 wk (weeks 5–7), during which the rats did not receive any treatment; and period III lasted 4 wk (weeks 8–11), during which the SHR received again either MMF or vehicle in a manner identical to that described for period I. Rats were weighed, and their blood pressure determined weekly. Serum creatinine, serum albumin, serum sodium, white blood cell and lymphocyte counts in peripheral blood, and 24-h urine protein were determined before the experiments were begun (baseline), after 4 wk (end of period I), at 6–7 wk (end of period II), and at the end of the experiment (week 11, end of period III). Biochemical determinations were done by autoanalyzer (Express Plus, Ciba Corning, Oberlin, OH), and white blood cell counts were determined with a hematology autoanalyzer (ADVIA 60, Bayer, Tarrytown, NY).

Rats were killed by aortic desanguination under ether anesthesia at the end of period I (week 4), at the end of period II (week 7), and at the end of period III (week 11). At each time point, kidneys from 7–12 rats from each experimental group were processed for light microscopy and immunohistochemistry.

Blood pressure determinations. Systolic blood pressure (SBP) and mean arterial pressure were measured by tail-cuff plethysmography (ITC, Life Scientific Instruments, Woodland Hills, CA) as described previously (49, 57). Before the experiments were started, rats were conditioned to the procedure three to four times. The value recorded for each week represented the mean of three to four determinations.

Histological studies. At the end of each experimental period, the rats were killed and their kidneys were excised and divided into two parts. As described in previous communications (35, 57), one part was cut in coronal sections, fixed in methyl Carnoy’s, and embedded in Paraplast Plus (Monoject, Sherwood Medical Scientific Division, St. Louis, MO). Four-micrometer sections were sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and trichrome stains and used for evaluation of light microscopic findings. The other part of the excised kidney was embedded in tissue-freezing medium, snap-frozen in dry ice and acetone, and stored at −70°C. Frozen tissue was used for immunohistochemical studies. All studies were done blinded. In each biopsy, the entire cortical and juxtamedullary regions were evaluated.

Gomorrosclerosis was defined as PAS-stained material or green areas (Gomori’s trichrome stain) and was associated with loss of cellular elements, collapse of capillary lumen, and presence of amorphous material with or without adhesion to Bowman’s capsule. It was graded by a semiquantitative index score described originally by Raij et al. (50) and detailed in a previous communication (49). The scale consists of the following grades: 0 = normal, no sclerosis; 1+ = <25% involvement of the glomerular tuft; 2+ = 25–50% involvement.
ment of the glomerular tuft; 3+ = 50–75% involvement of the glomerular tuft; and 4+ = 75–100% involvement of the glomerular tuft. The final tissue injury score was obtained by the formula [(1 × n of glomeruli with grade 1+) + (2 × n glomeruli with 2+) + (3 × n glomeruli with 3+) + (4 × n glomeruli with 4+)] × 100/total n of glomeruli examined, where n is number.

Tubulointerstitial injury was classified according to the extension (%) of the damaged areas in the tubules (dilatation, sloughing, disruption of basement membrane, cellularity) and in the interstitium (cellular infiltration, widening, scle-

osis) as described before (46): 0 = no changes present; 1+ = <10%; 2+ = 10–25%; 3+ = 25–50%; 4+ = 50–75%; and 5+ = 75–100%.

Afferent and efferent arterioles were identified because of their location near the vascular pole of the glomerular tuft. Afferent arterioles were identified by the presence of elastic lamina. Afferent and efferent arteriolar wall thickness were defined as the mean value obtained from measuring the wall width (exclusive of endothelium) at the thickest and thinnest section of the arteriolar measurements were done using comput-

erized analysis of images acquired with a Zeiss Axioscope fitted with a Kodak DC 120 megapixel camera, as described in previous communications (17). Eight to twelve arterioles were evaluated in each biopsy.

Immunohistology. Indirect immunofluorescence was used to identify lymphocytes (CD5-positive cells), activated lymphocytes [interleukin (IL)-2 receptor-expressing cells], macrophages (ED1-positive cells), and ANG II-positive cells as described before (35, 57). Cellular counts in the glomeruli are given as positive-staining cells per glomerular cross section (gcs). Cellular infiltration in the tubulointerstitial areas are given as positive cells per square millimeter.

Double-staining methodology was used to determine whether lymphocytes and macrophages expressed ANG II. The technique has been described before in detail (55) and includes an initial incubation with monoclonal antibody anti-

CD5 or anti-ED1; a second incubation with rhodamine-con-

jugated, affinity-purified F(ab')2 anti-mouse IgG; incubation with rabbit anti-ANG II antibody; and, finally, incubation with fluorescein-conjugated, affinity-purified donkey anti-

rabbit IgG antibody.

Antiserum. Antiserum used in this study included anti-CD5 (clone MRCOX19, Biosource, Camarillo, CA); anti-ED1 (monoclonal antibody to macrophages, Harlan Bioproducts, Indianapolis, IN); anti-CD25 (monoclonal antibody to IL-2 receptor, Accurate Chemical and Scientific, Westbury, NY); and anti-ANG II (rabbit anti-ANG II-human IgG, Peninsula Laboratories). Secondary rat anti-mouse and donkey anti-

rabbit antibodies with minimal cross-reactivity to rat serum proteins were obtained from Accurate Chemical and Scientific.

Urinary and renal malondialdehyde determination. Thio-

barbituric reactive substances were determined by the method of Ohkawa et al. (41) as described previously (57, 58) in 24-h urine samples obtained at baseline and at the end of the first treatment period (period I: 4 wk) in 14 rats from the MMP-treated group, 10 rats from the vehicle-treated group, and 7 control WKY rats. Briefly, the method consists of reacting a 400-μl sample with a mixture of 200 μl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid, and 600 μl of doubly distilled water. The mixture was heated at 95°C for 60 min, and 1 ml of water and 5 ml of n-butanol-pyridine were added. The mixture was centrifuged at 2,000 g for 15 min, and the absorbance of the upper organic layer was read at 532 nm (Shimatzu Spectrophotometer model UV211008, Kyoto, Japan). Malonaldehyde (MDA) bis-dimethyl acetal was used as an external standard. Results are expressed as nanomoles MDA in 24-h urine.

Renal MDA content was determined also by the method of Okawa et al. (41) in 400 μl of supernatant of homogenized kidney slices prepared as described below. Results are expressed as nanomoles MDA per milligram homogenate protein.

Renal tissue content of glutathione and renal catalase ac-
tivity. Studies in renal tissue were done in 1 of the 2 kidneys obtained at the time of death at the end of the second period of treatment (period III; at 11 wk) from 8 rats in the MMAF-
treated group, 7 rats in the vehicle-treated group, and 10 control WKY rats. Kidney slices for MDA and GSH determinations were placed in a cold mixture of 100 mM KCl and 0.003M EDTA, homogenized, and centrifuged at 600 g for 15 min. Supernatants of this preparation were used for MDA and GSH determinations, as described previously (38).

Renal GSH was measured by a modification of the method of Beutler et al. (9) in supernatants of the homogenized and centrifuged kidney slices prepared as indicated above. One milliliter of supernatant was added to 1.5 ml metaphosphoric acid and, after removal of particulate debris by centrifugation, 500 μl of supernatant were reacted with 2 ml of 0.2 M phosphate buffer and 0.25 ml 0.04% 5,5’-dithio-bis-2-nitrobenzoic acid. Absorbance was read at 410 nm. Glutathione con-

tent was expressed as nanomoles GSH per milligram homog-

enate protein. Gluthatione (Sigma, St. Louis, MO) was used as an external standard.

Renal catalase activity was determined following the method of Aebi (2) as reported before (38). Kidney slices were transferred to tubes containing 50 mM potassium phosphate, pH 7.0, homogenized, and centrifuged at 600 g for 10 min. Twenty-five microliters of the supernatant were added to 725 μl of a mixture of 7.7 mM H2O2 in 10 mM phosphate buffer, pH 7.0. The change in absorbance was read at 240 nm. The rate constant of a first-order reaction (κ) was used: κ = (1/Δt) × ln (A1/A2), where Δt is a measured interval (30 s), and A1 and A2 are the absorbance at the initial and final measurement times, respectively.

Superoxide-positive cells. Superoxide production in renal cells was studied in cryostat sections of the kidneys obtained at the end of both treatment periods (periods I and III) by the cytochemical method of Briggs et al. (10) with minor modifications, as reported in previous studies (38). Slides were incubated for 60 min at 37°C in a solution containing 50 ml 0.05 M Tris-HCl buffer, 1 ml dianimobenzidine stock solution (5 g dianimobenzidine-132 ml 0.05 M Tris buffer, pH 7.6), 250 μl 8% NiCl2, 32.5 μl 10% NaNO3, and 50 μl 0.5 M MnCl2. Sections were fixed with 10% formalin and counter-

stained with 1% methyl green. Results were expressed as O2- -positive cells per square millimeter.

Statistical analysis. Comparisons between groups were done with one-step ANOVA and Tukey-Kramer posttests. Serial determinations were evaluated with repeated mea-

sures ANOVA. Correlations were examined with Pearson’s linear correlation coefficient. Throughout the paper values are expressed as means ± SD, and two-tailed P values of <0.05 are considered significant. A commercially available statistical package (Instat, GraphPad, San Diego, CA) was used for statistical calculations.

RESULTS

Blood pressure and general data. Administration of 20 mg/kg body wt MMF to normotensive WKY rats for 4 wk had no effect on blood pressure (baseline SBP = 131.6 ± 12.2 mmHg, 4-wk SBP = 130.4 ± 8.0 mmHg).
The serial blood pressure determinations in the SHR, vehicle-treated, and MMF-treated rats are shown in Fig. 1. In the vehicle-treated SHR, hypertension gradually became more severe during the 11 wk of the study. Four weeks of MMF treatment induced a progressive fall of SBP to mean levels of 147.8 ± 16.8 mmHg at the end of period I. After MMF was stopped, SBP increased rapidly in this group of rats and, at week 7, reached hypertensive levels that were equal to the ones existing in the SHR that had never been exposed to MMF (Fig. 1). At that point, MMF was restarted and again the SBP began to decrease over the following 4 wk in a manner similar to that observed in the first period of MMF treatment (Fig. 1).

There were no significant differences in the weight of the vehicle-treated SHR and the weight of MMF-treated SHR in any of the weekly determinations. At the end of the experiment (period III), the vehicle-treated SHR group weighed 381 ± 27.2 g and the MMF-treated SHR group weighed 370.3 ± 37.4 g.

Table 1 shows that the biochemical and hematologic parameters tested were essentially unchanged during the experiment and similar in the MMF-treated and vehicle-treated rats.

**Light histology.** Histological and immunohistological microphotographs are shown in Figs. 2 (A–F) and 3 (A–F). Renal histology was largely preserved during the experiment. Glomerular and tubulointerstitial scores were not significantly different in the vehicle-treated and MMF-treated SHR (Table 2). Mesangial expansion was occasionally observed, but glomerular sclerosis was infrequent and, when present, it was restricted to 10–15% of the glomerular tuft (Fig. 2A). Tubulointerstitial findings were also scarce and consisted of widening of the interstitial areas, mild tubular dilatation, and damage to tubular cells. Areas of intense cellular infiltration, such as those shown in Fig. 2B, were exceptional.

**Immunocompetent cells.** The renal infiltration with T lymphocytes (CD5-positive cells) was analyzed separately in glomeruli and tubulointerstitial areas. In glomeruli, there were very few lymphocytes in the biopsies in periods I, II, and III (0.3–1.2 CD5-positive cells/gcs) with no significant differences in the experimental groups (vehicle group = 0.8 ± 0.75, MMF group = 0.5 ± 0.29). The findings in tubulointerstitial areas are demonstrated in Fig. 4, which shows that interstitial CD5-positive cells increased progressively in the vehicle-treated SHR during the 11 wk of the experiment. MMF treatment induced a reversible reduction in lymphocyte infiltration. In the initial 4 wk of treatment (period I), lymphocyte infiltration was reduced to less than half, but it increased to levels comparable to those in untreated SHR after 3 wk without the drug (Fig. 4). After reinitiation of MMF (period III), the infiltration of CD5-positive cells was reduced to

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**Table 1. Biochemical data in vehicle-treated and MMF-treated SHR rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (n = 35)</th>
<th>4 Wk (n = 35)</th>
<th>6–7 Wk (n = 32)</th>
<th>11 Wk (n = 9)</th>
</tr>
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<tr>
<td>S&lt;sub&gt;Cr&lt;/sub&gt;, mg/dl</td>
<td>0.5 ± 0.09</td>
<td>0.4 ± 0.22</td>
<td>0.5 ± 0.04</td>
<td>0.4 ± 0.09</td>
</tr>
<tr>
<td>S&lt;sub&gt;Alb&lt;/sub&gt;, g/dl</td>
<td>3.2 ± 0.22 (30)</td>
<td>3.3 ± 0.27 (30)</td>
<td>3.4 ± 0.22 (20)</td>
<td>3.5 ± 0.32 (20)</td>
</tr>
<tr>
<td>S&lt;sub&gt;Na&lt;/sub&gt;, meq/l</td>
<td>142 ± 1.3 (25)</td>
<td>141 ± 0.8 (30)</td>
<td>143 ± 1.8 (25)</td>
<td>144 ± 1.8 (20)</td>
</tr>
<tr>
<td>Total WBC, cells/mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>7,557 ± 2,026 (22)</td>
<td>7,950 ± 2,468 (24)</td>
<td>7,600 ± 1,492 (19)</td>
<td>8,000 ± 1,665 (20)</td>
</tr>
<tr>
<td>Lymphocytes, cells/mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4,771 ± 1,162 (22)</td>
<td>5,402 ± 1,834 (24)</td>
<td>5,268 ± 1,272 (19)</td>
<td>4,792 ± 1,082 (20)</td>
</tr>
<tr>
<td>Urine protein, mg/24 h</td>
<td>2.1 ± 0.84</td>
<td>2.1 ± 1.03</td>
<td>4.1 ± 2.17 (30)</td>
<td>2.8 ± 1.66 (28)</td>
</tr>
</tbody>
</table>

Values are means ± SD, with nos. in parenthesis indicating a different no. of determinations from that listed for a treatment group. MMF, mycophenolate mofetil; S<sub>Cr</sub>, S<sub>Alb</sub>, and S<sub>Na</sub>: serum creatinine, albumin, and sodium, respectively; WBC, white blood cells.

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*AJR Renal Physiol • VOL 282 • FEBRUARY 2002 • www.ajprenal.org*
one-fourth of that in vehicle-treated rats ($P < 0.001$, Fig. 4). When data from all three periods of study are used, the renal infiltration of CD5-positive cells correlated with the blood pressure levels ($r = 0.473$, $P < 0.01$). Representative microphotographs are presented in Fig. 2, C and D.

Activated lymphocytes expressing the IL-2 receptor (CD25-positive cells) were present in tubulointerstitium at the end of period I (4 wk) in the vehicle-treated group ($3.3 \pm 1.4$ CD25-positive cells/mm$^2$) and were significantly reduced in the MMF-treated group ($0.38 \pm 0.8$ positive cells/mm$^2$, $P < 0.01$). At the end of period II (week 7), 3 wk after MMF was stopped, the number of CD25-positive cells was comparable in rats previously receiving vehicle ($5.2 \pm 2.3$ CD25-positive cells/mm$^2$) and those previously receiving MMF ($4.0 \pm 2.4$ CD25-positive cells/mm$^2$). An example is shown in Fig. 2E.

There were few macrophages infiltrating the glomeruli ($0.7$ to $1.2$ ED1-positive cells/gcs). In tubulointerstitial areas, the macrophage infiltration in vehicle-treated rats was essentially similar at 4, 7, and 11 wk ($P > 0.05$, Fig. 5). MMF treatment significantly reduced macrophage infiltration in period I ($P < 0.001$, Fig. 5). Interstitial macrophages increased after the discontinuation of MMF (7 week in Fig. 5) and decreased during the second period of MMF treatment ($P < 0.001$, Fig. 5). Macrophage infiltration correlated with the levels of SBP ($r = 0.552$, $P < 0.01$). An example of ED1-positive cells is shown in Fig. 2F.

**ANG II-positive cells.** Increased numbers of ANG II-positive cells were found in the tubulointerstitial areas of vehicle-treated SHR (Fig. 6). MMF treatment reduced the number of ANG II-positive cells in periods I and III, to one-half and one-third, respectively, of the number found in paired vehicle-treated SHR (Fig. 6). The number of tubulointerstitial ANG II-positive cells correlated with the SBP ($r = 0.427$, $P < 0.05$). Double-staining studies demonstrated that $26$–$38\%$ of the CD5-positive cells and $20$–$40\%$ of the ED1-positive cells stained positive for ANG II. Figure 3 shows representative examples of the reduction in ANG II-positive cells induced by MMF treatment (Fig. 3, A and B) and of the double-staining studies (Fig. 3, C and D).

**Oxidative stress.** Urinary MDA increased in the vehicle-treated rats, whereas in the MMF-treated rats urinary MDA was reduced to levels similar to those in WKY rats (Fig. 7). Urinary MDA excretion did not correlate with SBP levels ($P = 0.239$).
The renal content of MDA, GSH, GSH-PX, and catalase was examined in kidneys harvested at the end of the experiment. The results are shown in Table 3. The renal content of MDA was higher in both groups of SHR than in the WKY rats. However, MMF treatment was associated with a significant reduction ($P < 0.001$) in the MDA content of the kidney (Table 3). There was a highly significant correlation between the renal MDA content and the levels of SBP ($r = 0.85$, $P < 0.001$). The renal content of GSH, GSH-PX, and catalase was comparable in both groups of SHR and in WKY controls.

Intracellular superoxide staining was done in the biopsies taken at the end of both periods of treatment. Vehicle-treated rats showed an increment in tubulointerstitial $O_2^-$-positive cells: the values at 11 wk are significantly higher ($P < 0.001$) than the values at 4 wk (Fig. 8). MMF drastically reduced the number of superoxide-positive cells in the kidney (Figs. 8 and 3, E and F).

Table 2. Histology scores

<table>
<thead>
<tr>
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<th>Glomerular Score</th>
<th>Tubulointerstitial Score</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>MMF</td>
</tr>
<tr>
<td>Period I (4 wk)</td>
<td>8.0 ± 5.8</td>
<td>4.5 ± 3.6</td>
</tr>
<tr>
<td>Period III (11 wk)</td>
<td>8.8 ± 7.5</td>
<td>4.7 ± 3.0</td>
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Values are means ± SD. Glomerular and tubular damage scores are defined in the text. There are no significant differences between the vehicle- and MMF-treated groups.

Fig. 3. ANG II- and superoxide-positive cells. A and B: ANG II-positive cells in untreated (A) and MMF-treated SHR (B). C and D: double-staining studies of rhodamine-stained ANG II-positive cells (C), 3 of which are lymphocytes (fluorescein-labeled CD5-positive cells; D). E and F: large numbers of superoxide-positive cells in the kidney of SHR (E) that are found only occasionally in MMF-treated rats (F).
In periods I and III, the mean values of O$_2^-$-positive cells in MMF-treated rats were about one-third and one-sixth, respectively, of the values in the vehicle-treated rats ($P < 0.001$, Fig. 8). The number of superoxide-positive cells in the kidney had a highly significant correlation with the SBP levels ($r = 0.780$, $P < 0.0001$, Fig. 9).

**Afferent and efferent arteriolar media thickness.**
There were no significant differences in the afferent arteriolar thickness (vehicle-treated group = 3.0 ± 0.81, MMF-treated group = 2.7 ± 0.47 μm) nor in the efferent arteriolar thickness (vehicle-treated group = 1.8 ± 0.39, MMF-treated group = 1.6 ± 0.21 μm).

**DISCUSSION**

The central finding of this study is that intermittent administration of MMF to SHR resulted in a reversible reduction in SBP and that the blood pressure changes were associated with corresponding fluctuations in the intensity of lymphocyte infiltration, intrarenal ANG II-positive cells, and oxidative stress in the kidney. Four weeks of MMF treatment induced a progressive reduction in SBP, and cessation of MMF administration was associated with a progressive increase in SBP to hypertensive levels similar to those in paired vehicle-treated SHR. Then, a new period of treatment with MMF induced a new reduction in SBP, with essentially the same characteristics observed in the initial period of treatment (Fig. 1). In both periods of 4 wk of MMF administration, SBP decreased to levels almost within the 95% confidence interval of those in control WKY.

The potential development of gastrointestinal side effects associated with MMF treatment was carefully evaluated because lack of food intake and diarrhea could result in a volume-depleted state that, in turn, would result in lower blood pressure. The dose of MMF used in this study (20 mg·kg$^{-1}$·day$^{-1}$) was based on preliminary data that suggested that this dose was effective (48) and, in fact, it was lower than the dose of 30 mg·kg$^{-1}$·day$^{-1}$ we had used without significant side effects in previous experiments (49, 58). Diarrhea did not occur, and, although we did not measure food consumption, there was no apparent reduction in food intake, and the weight changes in the MMF-treated and vehicle-treated rats were similar. Also similar were the biochemical parameters, including serum albumin and sodium (Table 1). It is therefore unlikely that MMF was inducing significant side effects with systemic repercussions causing hypotension in the SHR. Direct hypotensive effects of MMF are also unlikely because 4 wk of MMF administration did not induce blood pressure changes in the WKY rats.

The reduction in lymphocyte infiltration by MMF treatment is an expected result from its activity as a

![Graph](image)

**Fig. 5. Tubulointerstitial infiltration of macrophages (ED1+ cells) was significantly suppressed with MMF treatment (filled bars). Highly significant statistical differences (***$P < 0.001$) from the vehicle-treated group (open bars) were found for both periods of treatment (4 and 11 wk).**

![Graph](image)

**Fig. 6. Tubulointerstitial infiltration with ANG II-positive cells was significantly suppressed with MMF treatment (solid bars). There are highly significant statistical differences (***$P < 0.001$) from the vehicle-treated group (open bars) for both periods of treatment (4 and 11 wk).**

![Graph](image)

**Fig. 7. Urinary MDA excretion studied at the end of period I (after 4 wk of MMF treatment) was increased in SHR (hatched bar) and reduced by MMF treatment to levels comparable to those found in WKY rats. *$P < 0.05$.**

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**Table 3. Renal content of malondialdehyde, glutathione, and catalase**

<table>
<thead>
<tr>
<th></th>
<th>4-Wk Treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WKY (n = 10)</td>
</tr>
<tr>
<td>Renal MDA, nmol/mg protein</td>
<td>0.44 ± 0.07</td>
</tr>
<tr>
<td>Renal GSH, nmol/mg protein</td>
<td>9.43 ± 2.65</td>
</tr>
<tr>
<td>Renal catalase, μg protein</td>
<td>0.68 ± 0.22</td>
</tr>
</tbody>
</table>

Values are means ± SD. MDA, malondialdehyde. *$P < 0.001$ and †$P < 0.001$, MDA vs. GSH and catalase.
selective lymphocyte-immunosuppressive agent (3) in association with the drug-induced downregulation of the expression of intercellular adhesion molecules (21, 58), which are constitutively increased in SHR (31). However, the beneficial effects of MMF could, conceivably, be due to other effects of the drug. For example, MMF could improve hypertension in the SHR by preserving structural and functional integrity of the kidney, as it has been shown to do in models of extensive renal ablation (19, 52, 58). It has been argued that the beneficial effects of this drug may result from a variety of actions, including antiproliferative effects on mesangial cells (22, 69), inhibition of collagen deposition (5) and of lymphocyte- and macrophage-derived transforming growth factor-β, tumor necrosis factor-α, and interferon-γ, and suppression of Th1 and Th2 cytokine responses (14, 37, 45).

In the present studies, the mean glomerular scores found in the vehicle-treated SHR group were higher than in the MMF-treated group, but the variability was large and the differences were not statistically significant (Table 2). Furthermore, it must be realized that glomerular sclerosis was infrequent and, when present, usually comprised <10% of the glomerular tuft. Correspondingly, the glomerular scores are very low in all instances, a fact that may be better appreciated when the values shown in Table 2 are compared with the maximal possible glomerular score (all glomeruli with >75% of sclerosis) of 400. Tubulointerstitial injury was also minor and similar in the vehicle-treated and the MMF-treated rats (Table 2). These mild histological findings, taken in conjunction with the lack of changes in serum creatinine and urine protein excretion observed during MMF treatment (Table 1), appear to offer insufficient explanation for the normalization of blood pressure induced by the drug.

Conceivably, MMF could have an effect on afferent or efferent arterioles. Previous studies have reported that preglomerular vessels in SHR are thicker (25, 62) than in WKY rats and have narrower lumens and heightened vasoconstrictor responsiveness (8, 23, 29, 62). Nevertheless, the cross-sectional area of the media of afferent arterioles is reduced in SHR (61). In our studies, MMF treatment did not induce significant changes in the thickness of media of the afferent and efferent arterioles. Although estimates of diameter, lumen, and lumen-to-media ratios require renal perfusion and vascular cast models that are beyond the scope of this work, the present studies indicate that the reduction in blood pressure obtained with 4 wk of MMF treatment is not the result of changes in media thickness in the afferent or efferent arterioles.

Because similar blood pressure-lowering effects have been reported with other modalities of immunosuppression, such as treatment with cyclophosphamide (6, 27), daily injections of anti-thymocyte serum (7), and thymectomy (28), it is reasonable to assume that the observed effects with MMF are the result of its activity as a lymphocyte-suppressive agent (3).

The mechanism by which the lymphocyte suppression reduces the blood pressure in the SHR has not been adequately explained in previous studies. Although the careful studies of Khraibi et al. (28) documented a postthymectomy reduction in lymphocyte count in peripheral circulation, the investigations previously discussed (7, 21, 28) were not addressed to evaluate changes in renal infiltration with immune cells. This is understandable, because the prevailing view among those who championed the participation of immune mechanisms in the pathogenesis of hypertension in the SHR was that autoimmunity was responsible for systemic vasculitis and that immune dysfunction in the SHR was an adaptive mechanism that would “prevent [a] life threatening increment in blood pressure” (7).

In this work, we find that the genetic model of hypertension in the SHR presents some characteristics previously observed in acquired models of salt-sensitive hypertension. These characteristics offer some insights into the participation of immune cells in the hypertension in the SHR. The experimental design, which included intermittent MMF administration, per-
mitted the establishment of correlations between the severity of hypertension and the tubulointerstitial infiltration of lymphocytes, macrophages, and ANG II-positive cells and several manifestations of increased oxidative stress in the kidney. Although these correlations are not a proof of causation, they certainly emphasize the need for further investigations to clarify the role of these factors in the genesis of hypertension.

Some aspects deserve special mention. The infiltration of ANG II-positive cells is a mechanism for increased intrarenal ANG II activity, which could induce hemodynamic changes that favor sodium retention, including a reduction in single-nephron glomerular filtration rate and a shift to the right of the pressure-natriuresis relationship. Furthermore, chronic vasconstriction is likely to induce longstanding tubulointerstitial ischemia that eventually would result in the generation of oxidative stress and in structural changes. Rajagopalan et al. (51) have shown that ANG II-mediated hypertension increases superoxide production in vascular smooth muscle cells as a result of activation of the NADH/NADPH oxidase system. The relevance of oxidative stress in the damage induced by immunocompetent cells and increased ANG II activity has been reviewed recently (57), and its role in the modulation of nitric oxide synthesis in the SHR has been demonstrated in the careful studies of Vaziri et al. (67). Several studies have previously shown the participation of oxidative stress in the pathogenesis of hypertension in the SHR (60, 63, 65, 67); the present studies provide additional evidence of this pathogenetic mechanism, with the findings of increased urinary and renal peroxidation products and intracellular superoxide generation and their striking correlation with blood pressure levels.

In summary, these investigations provide evidence that renal infiltration with immunocompetent cells plays an important role in the maintenance of hypertension in the SHR and add further insight into the complex relationship among the immune system, the kidney, and blood pressure.

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