Overload proteinuria is followed by salt-sensitive hypertension caused by renal infiltration of immune cells

VIOLETA ALVAREZ, YASMIR QUIROZ, MAYERLY NAVA, HÉCTOR PONS, AND BERNARDO RODRÍGUEZ-ITURBE

Overload proteinuria is followed by salt-sensitive hypertension caused by renal infiltration of immune cells. *Am J Physiol Renal Physiol* 283: F1132–F1141, 2002. First published July 24, 2002; 10.1152/ajprenal.00199.2002.—Recent evidence suggests that salt-sensitive hypertension develops as a consequence of renal infiltration with immunocompetent cells. We investigated whether proteinuria, which is known to induce interstitial nephritis, causes salt-sensitive hypertension. Female Lewis rats received 2 g of BSA intraperitoneally daily for 2 wk. After protein overload (PO), 6 wk of a high-salt diet induced hypertension [systolic blood pressure (SBP) = 156 ± 11.8 mmHg], whereas rats that remained on a normal-salt diet and control rats (without PO) on a high-salt diet were normotensive. Administration of mycophenolate mofetil (20 mg·kg⁻¹·day⁻¹) during PO resulted in prevention of proteinuria-related interstitial nephritis, reduction of renal angiotensin II-positive cells and oxidative stress (superoxide-positive cells and renal malondialdehyde content), and resistance to the hypertensive effect of the high-salt diet (SBP = 129 ± 12.2 mmHg). The present studies support the participation of renal inflammatory infiltrate in the pathogenesis of salt-sensitive hypertension and provide a direct link between two risk factors of progressive renal damage: proteinuria and hypertension.

interstitial nephritis; oxidative stress

PREVIOUS STUDIES FROM OUR laboratories have shown that arterial hypertension develops if a high-salt diet is administered after recovery from short-term angiotensin II infusion (37) as well as after several weeks of nitric oxide synthesis inhibition (30). The pathogenetic relevance of the renal inflammatory infiltrate present in these experimental models is evidenced by the demonstration that reducing the immune infiltration prevented or markedly ameliorated the subsequent salt-sensitive hypertension (30, 37). More recently, we have extended these observations to a genetic model of hypertension in which the reduction of renal immune cell infiltration results in an improvement of hypertension (39).

The role played by the inflammatory infiltrate may be the result of the local generation of vasoactive mediators, particularly angiotensin II, which could induce focal ischemia and oxidative stress (reviewed in Refs. 36 and 40), creating a self-perpetuating reactivity that favors sodium retention by the combined effects of increased tubular sodium reabsorption and glomerular vasoconstriction, which results in reduced filtered sodium load (11).

The model of protein overload proteinuria is suitable to explore further the participation of immune cells in the development of salt-sensitive hypertension. Systemic protein overload has been studied for more than half a century (21) to evaluate structural changes in the glomeruli resulting from large doses of exogenous protein (7, 20). Seminal studies by Eddy (8) were the first to focus on the tubulointerstitial nephritis in this model, and subsequent studies by several research groups have analyzed these findings in the context of the role of proteinuria in the progression to chronicity of renal disease (reviewed in Refs. 6, 9, 33, 34, and 49). Increased mRNA expression of monocyte chemoattractant protein-1 and transforming growth factor-β (10, 50), tubular activation of NF-κB (13, 51, 55), RANTES production (51), and upregulation of angiotensin-converting enzyme (18) have all been demonstrated in protein overload experiments.

To our knowledge, the development of hypertension has not been studied in this model, but we speculated that in light of our laboratory’s recent work as mentioned before (30, 37, 39) and as reviewed recently (15), overload proteinuria should result in salt-sensitive hypertension. Furthermore, if such were the case, the role of the immune inflammatory infiltrate could be investigated to study what effect, if any, the prevention of interstitial nephritis would have on salt-driven hypertension. The present studies were done to answer these questions. Our results indicate that, in fact, a high-salt diet given after 2 wk of protein overload in the rat induces hypertension and that the administration of the immunosuppressive drug mycophenolate mofetil (MMF) (1) during the period of protein overload

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markedly reduces the tubulointerstitial immune infiltrate and prevents the development of salt-sensitive hypertension.

MATERIALS AND METHODS

Animals, diets, drugs, and protein overload. Experiments were done, as in other investigations of protein overload proteinuria (8, 10, 17, 46), in female Lewis rats (Instituto de Investigaciones Científicas, Los Teques, Venezuela), weighing 190–230 g at the beginning of the experiment, and were handled in accordance with institutional guidelines of animal care and with unrestricted access to food and water. Diets used were regular rodent feed (Protnal, Valencia, Venezuela) containing 0.4% NaCl and a similar diet but with a high-salt content (4% NaCl) as described in previous communications (30, 37, 39). MMF (CellCept, Roche Laboratories) was given suspended in 500 µl of water by vigorous agitation immediately before administration by gastric gavage (30, 37, 39). Protein overload was done by daily intraperitoneal injections of 2 g of BSA (fraction V, endotoxin-free, Sigma, St. Louis, MO). BSA was given as 3 ml of a 66.6% albumin solution in 0.9% saline.

Experimental design and groups. The experimental design included two phases during an 8-wk period. The first phase, protein overload, lasted 2 wk, during which the rats in the experimental groups received daily intraperitoneal injections of BSA and a regular rodent chow with normal sodium content. During this phase and for 1 additional wk (total of 3 wk), rats received 20 mg/kg body wt of MMF or vehicle (500 µl water) as described below. Protein overload did not result in significant proteinuria in all rats; therefore, we excluded from the study animals that had <65 mg/24 h of urinary protein excretion in both initial determinations (days 3 and 7; see below). The second phase was designed to uncover salt sensitivity. It started immediately after the protein overload phase and lasted for 6 wk, during which the animals were either given a high-salt diet or remained on a normal-salt diet.

Two experimental groups and two control groups were studied (see Fig. 1). The PO+HSD group (n = 15) consisted of rats that received protein overload (2 wk) and vehicle (3 wk) and were given a high-salt diet afterward. The PO+MMF+HSD group (n = 14) consisted of rats that received protein overload (2 wk) and MMF (3 wk) and were given a high-salt diet afterward. The control groups consisted of one group of rats that did not receive intraperitoneal BSA but 0.9% saline and remained on a normal-salt diet throughout the experiment (Control-S group, n = 10) and another (Control-PO group, n = 10) that was included to demonstrate the salt dependence of hypertension and consisted of rats that received protein overload but were maintained on a normal-salt diet afterward.

Blood pressure was taken weekly. Weight and serum samples for serum creatinine were taken at baseline, at the end of the protein overload phase, and at the end of the experiment. Rats were placed in metabolic cages, and 24-h urine samples were collected on days 3, 7, and 14 (protein overload phase) and 3–4 wk after protein overload was finished (weeks 5–6 of the experiment) to document that during the salt-sensitivity phase the urinary protein excretion in the animals in all groups had returned to normal (≤10 mg/24 h) levels. Animals were euthanized by aortic dissection under ether anesthesia at the end of the protein overload phase and at the end of the experiment (end of the salt-sensitivity phase). On each occasion, one kidney from six to seven rats per group were used for histological and immunohistological studies. The contralateral kidneys were used to determine renal content of malondialdehyde (MDA) and GSH. Rats from the control groups were killed at the end of the experiment.

Blood pressure determinations. Before the experiments were begun, the rats were conditioned to handling and blood pressure measurements three or four times. Systolic and mean blood pressure were measured by tail-cuff plethysmography (ITC, Life Scientific Instruments, Woodland Hills, CA). The value recorded for each week represented the mean of three to four determinations, as described in previous communications (30, 37, 39).

Biochemical determinations. Urinary protein determinations in 24-h urine collections were done by the sulfosalicylic acid technique. Serum creatinine determinations were done by autoanalyzer methodology (Express Plus, Ciba Corning, Oberlin, OH).

Histological studies. As in previous studies (30, 37, 39), kidneys selected for histology were divided into two parts. One part was fixed in methyl Carnoys and embedded in Paraplast Plus (Monoject, Sherwood Medical Scientific Division, St. Louis, MO). Light microscopy evaluation was done in 4-μm sections stained with hematoxylin and eosin and period acid-Schiff (PAS) reagents. The other part of the kidney was frozen in dry ice and acetone and stored at −70°C.

All analyses were done in a blinded fashion. The entire cortical and juxtaglomerular regions were studied in each biopsy. Glomerulosclerosis was defined as PAS-stained material without cellular elements and collapse of the capillary lumen with or without adhesion to Bowman’s capsule. It was graded by the severity score described initially by Raij et al. (32) and detailed in previous communications (30, 39). Tubulointerstitial injury was classified according to the extent (%) of the tubular (dilatation, disruption of basement membrane) and interstitial (cellular infiltration, widening, sclerosis) damage as described initially by Pichler et al. (29) and used by our laboratory in earlier communications (28, 38): 0 = no changes present; 1+ = <10%; 2+ = 10–25%; 3+ = 25–50%; 4+ = 50–75%; and 5+ = 75–100% of the renal tubulointerstitial area showing injury.

Immunohistology and antisera. Avidin-biotin peroxidase methodology was used to identify lymphocytes and macrophages and angiotensin II-positive cells. Cellular counts inside the glomerular tuft are given as positive cells per glomerular cross section and in tubulointerstitial areas as...
positive cells per square millimeter (30, 37, 39). Indirect immunofluorescence was used to identify OX42-positive cells. Anti-CD5 antibody (clone MRCOX19, Biosource, Camarillo, CA) was used to identify lymphocytes. Anti-ED1 (Harlan Bioproducts, Indianapolis, IN) and anti-OX42 (clone MRC OX-42, Accurate Chemical and Scientific, Westbury, NY) were used to identify macrophages. The anti-OX42 antibody recognizes macrophages and dendritic cells, granulocytes, and microglial cells in the central nervous system. Angiotensin II-positive cells were identified using rabbit anti-human angiotensin II with cross-reactivity to rat angiotensin II (Peninsula Laboratories) as described previously (30, 37, 39). Secondary rat anti-mouse and donkey anti-rabbit biotin-conjugated antibodies with minimal cross-reactivity to rat serum proteins were obtained (Accurate Chemical and Scientific). Glomerular immune deposits were investigated with anti-rat C3, IgG, and IgM (Cappel Research Products, Durham, NC). Double-staining studies were done to evaluate angiotensin II expression by infiltrating lymphocytes and macrophages as described previously (30, 37, 39). Readings of immunohistology were done at ×63 magnification.

Superoxide-positive cells. Cryostat sections of the kidneys were used to study intracellular superoxide production by the cytochemical method of Briggs et al. (5) with minor modifications previously reported (25). Sections were fixed in formalin and counterstained with 1% methyl green. Results are given as positive cells per square millimeter.

Renal content of MDA and GSH. Determination of renal content of MDA and GSH were done in supernatants of kidney slices placed in a cold mixture of 100 mM KCl and 0.003 M EDTA, homogenized, and centrifuged at 600 g (25). Renal content of MDA was determined by the method of Ohkawa et al. (27), and results are expressed as nanomoles of MDA per milligram of kidney homogenate protein. Renal GSH content was measured by the method of Beutler et al. (3) and expressed as nanomoles of GSH per milligram of kidney homogenate protein. Specific details of our laboratory methods have been reported before (25, 39).

Statistical analysis. Comparisons among groups were done with ANOVA and Tukey-Kramer posttests. Repeated-measures ANOVA was used to evaluate serial determinations. Associations were evaluated with linear (Pearson) and non-parametric (Spearman) correlations. Results are expressed as means ± SD, and two-tailed P values <0.05 were considered significant. A commercial statistical package (Instat, GraphPad, San Diego, CA) was used for statistical calculations.

RESULTS

Weight, renal function, and proteinuria. During the period of protein overload, all groups that received intraperitoneal BSA injections failed to gain weight, in contrast to rats in the Control-S group, which gained weight normally (Fig. 2). During the subsequent period in which the PO+HSD and PO+MMF+HSD groups received either a normal-salt or a high-salt diet, the rats gained weight, but they did not reach the weight of the rats in the control group.

Serum creatinine was similar in all groups and unchanged from baseline levels (0.4 ± 0.06 mg/dl) after 2 wk of protein overload (PO+HSD group = 0.5 ± 0.12, PO+MMF+HSD group = 0.4 ± 0.07, Control-S group = 0.5 ± 0.08, Control-PO group = 0.5 ± 0.10 mg/dl) and during the subsequent high- or normal-salt diet (PO+HSD group = 0.4 ± 0.05, PO+MMF+HSD group = 0.4 ± 0.05, Control-S group = 0.5 ± 0.07, Control-PO group = 0.4 ± 0.07 mg/dl).

Baseline urinary protein excretion was <5 mg/day in all rats. Proteinuria was determined on days 3, 7, and 15 during protein overload. By experimental design, the lower level of urinary protein excretion was ≥65 mg/day (range 65–473 mg/day). As demonstrated in Fig. 3, peak proteinuria (mg/24 h) was usually found on day 3 and did not differ significantly in the experimental groups. Mean levels of proteinuria decreased at day 7 and remained essentially at similar levels at day 14 (last day of protein overload). One week after protein overload was discontinued, proteinuria had returned to almost normal levels, and 2 wk later urinary protein excretion was reduced to values <10 mg/24 h.

Blood pressure. Figure 4 shows the serial systolic blood pressure determinations during the 8 wk of the studies. Rats were normotensive during the protein overload phase. Rats in the Control-PO group remained normotensive, whereas PO+HSD rats had a progressive increment in blood pressure that was evident in the second week of the high-salt diet (week 4 in Fig. 4). In contrast, rats in the PO+MMF+HSD group remained normotensive during the period of the high-salt diet (Fig. 4). Rats from the Control-S group (not shown in Fig. 4) were normotensive throughout the experiment.

Light microscopy and glomerular immunostaining. After 2 wk of protein overload, there were no significant glomerular changes. Glomeruli were either normal or showed only mesangial expansion. Focal hypercellularity was occasionally seen, but glomerular sclerosis was only rarely present. The glomerular sclerosis score (range 0–400 in the method used) was similar in the rats that received vehicle (PO+HSD group = 2.82 ± 0.41) to that in the rats that received MMF (PO+MMF+HSD group = 2.66 ± 0.51). In contrast to the scarcity of glomerular findings, there were
The tubulointerstitial infiltration of CD5-positive lymphocytes and the effects of MMF treatment are shown in Fig. 6. Tubulointerstitial infiltration of ED1-positive macrophages and the effects of MMF treatment are shown in Fig. 7. There is a significant infiltration of lymphocytes (Fig. 6) and macrophages (Fig. 7) after 2 wk of protein overload in the rats in the PO+HSD group, and this infiltrate remained essentially unchanged in the subsequent 6 wk of a high-salt diet, during which this group of rats developed salt-sensitive hypertension. Unexpectedly, the number of infiltrating macrophages was not higher than the number of infiltrating lymphocytes.

As expected, the number of tubulointerstitial OX42-positive cells was higher than the number of ED1-positive cells. In studies at the end of protein overload, the PO+HSD group had 75.2 ± 3.4 OX42-positive cells/mm² and 67 ± 6.5 ED1-positive cells/mm², whereas rats in the PO+MMF+HSD group had 44.9 ± 6.6 OX42-positive cells/mm² and 27 ± 9.3 ED1-positive cells/mm².

MMF treatment (PO+MMF+HSD group) reduced the severity of the infiltrate to levels similar to or slightly above control levels (Figs. 6 and 7). Examples of immunohistology are shown in Fig. 8 (A and B). There were significant correlations between the number of superoxide-positive cells (see below) and the number of ED1-positive cells (P < 0.001) and CD5-positive cells (P < 0.001).

Angiotensin II-positive cells were not detected in the Control-S group. In contrast, rats from the PO+HSD group had important numbers of angiotensin II-positive cells in tubulointerstitial areas after 2 wk of BSA-induced proteinuria (Figs. 8C and 9). The number of angiotensin II-positive cells in the PO+HSD group decreased by 30–40% during the salt-sensitive phase with respect to the proteinuric phase (P < 0.05). MMF treatment resulted in a reduction of angiotensin II-

Prominent tubulointerstitial changes, the most remarkable of which were areas of tubular dilatation that had a focal distribution and surrounded normal glomeruli (Fig. 5A). Dilated tubules in the deeper medullary region often showed protein casts and brush-border damage, but the tubular basement membrane was preserved (Fig. 5B). The tubulointerstitial damage was 34.89 ± 6.30% in the vehicle-treated rats in the PO+HSD group and 30.0 ± 12.8% in the PO+MMF+HSD group (P not significant). There was moderate cellular infiltrate (Fig. 5C) with focal distribution, frequently localized near glomeruli or in areas in the proximity of the dilated tubules. Widening of interstitial regions was present usually in association with the cellular infiltrate. There were no chronic fibrotic changes that could be evidenced by PAS or trichromic stains.

At the end of the salt-sensitive study period (6 wk after the end of the protein overload), the glomerular scores were not significantly different (PO+HSD group = 3.52 ± 2.81, PO+MMF+HSD group = 2.57 ± 0.79). Tubulointerstitial damage was still evident, with focal areas of dilated tubules in both experimental groups, but areas of fibrosis were not apparent (Fig. 5D). There was a reduction of tubulointerstitial damage in the PO+MMF+HSD group (23.9 ± 5.92%) with respect to the PO+HSD group (35.5 ± 10.3%, P < 0.05).

Immunofluorescent glomerular staining for C3, IgG, and IgM were negative in the kidneys harvested after 2 wk of protein overload and in those harvested at the end of the experiment.

Cellular infiltration. There was no significant infiltration of lymphocytes or macrophages in the glomeruli in any of the groups. Only occasionally was a CD5- or ED1-positive cell observed inside the glomeruli either at the end of protein overload or at the end of the salt-sensitive phase in the experimental groups, and these findings were essentially similar to those in the control groups (0.8 ± 0.7 positive cells/glomerular cross section).

**Fig. 3.** Proteinuria in 24-h urine collections during and after protein overload. Bars are defined as described in Fig. 2. Control-S rats remained free of proteinuria (<10 mg/24 h), and their data are not shown. Error bars, SD. There are no significant differences among the groups.

**Fig. 4.** Serial systolic blood pressure during the study. PO+HSD rats (○) developed progressive hypertension, whereas Control-PO rats (▪) remained normotensive. PO+MMF+HSD rats (■) also remained normotensive. Values are means ± SD. *P < 0.05. ***P < 0.001.

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positive cells in both experimental periods (PO/MMF/HSD group in Fig. 9). Double-staining studies indicated that 12–18% of the CD5-positive cells and 12–15% of the ED1-positive cells also stained positive for angiotensin II. The number of tubulointerstitial angiotensin II-positive cells correlated with the number of lymphocytes ($P < 0.05$), macrophages ($P < 0.01$), and superoxide-positive cells ($P < 0.05$).

**Oxidative stress.** The number of superoxide-positive cells were increased in tubulointerstitial regions 23 times above baseline control levels in rats in the

![Image](image-url)

**Fig. 5. Light microscopic findings in the kidney.** A: after 2 wk of protein overload, glomeruli were essentially normal, with distended capillary loops and areas of mesangial expansion surrounded by dilated tubules with brush-border loss and preserved basement membrane. B: tubular proteinaceous casts in distended tubules in deep medullary region. C: cellular infiltrate in widened interstitial areas near some dilated tubules that show cellular damage and focal loss of brush border. D: 8 wk after protein overload was discontinued (end of the salt-sensitivity phase), glomeruli remained normal and significant tubular dilatation was still present in focal areas such as that shown.

![Graph](graph-url)

**Fig. 6. Lymphocyte infiltration in tubulointerstitium.** Infiltration of CD5-positive cells (means ± SD) after 2 wk of protein overload and at the end of the high-salt-diet period is reduced by mycophenolate mofetil (MMF) treatment. Open bars, Control-S group; hatched bars, PO/MMF group (vehicle-treated group); filled bars, PO/MMF + HSD group (MMF-treated group). ***$P < 0.001$.

![Graph](graph-url)

**Fig. 7. Macrophage infiltration in tubulointerstitium.** Infiltration of ED1-positive cells (means ± SD) after 2 wk of protein overload and at the end of high-salt-diet period is reduced by MMF treatment. Open bars, Control-S group; hatched bars, PO + HSD group. Closed bars = PO + MMF + HSD group (MMF-treated group). ***$P < 0.001$. 

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PO/H11001 HSD group as a result of 2 wk of protein overload (Figs. 8D and 10). The number of superoxide-positive cells in these rats remained essentially unchanged as they developed salt-sensitive hypertension. In contrast, MMF treatment was associated with a drastic reduction of superoxide-positive cells in the PO/MMF/HSD group (Fig. 10).

MDA and GSH content of the kidneys harvested after 2 wk of protein overload and at the end of the salt-sensitive hypertension phases are shown in Table 1. Rats in both experimental groups had increased

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Fig. 8. Immunohistological findings and superoxide-positive cells. A: infiltrating lymphocytes (CD5-positive cells). B: infiltrating macrophages (ED1-positive cells). C: angiotensin II-positive cells. Arrows, interstitial cells; arrowheads, intratubular cells. D: superoxide-positive cells after 2 wk of protein overload in the kidney of a PO/H11001 HSD group rat.

Fig. 9. Infiltration of ANG II-positive cells in tubulointerstitium. Protein overload (PO/H11001 HSD group; hatched bars) induced infiltration of ANG II-positive cells that decreased during the next high-salt-diet period (P < 0.05). The number of ANG II-positive cells were reduced by MMF treatment (PO/MMF/H11001 HSD group; filled bars). *P < 0.05. ***P < 0.0001.

Fig. 10. Superoxide-positive cells in tubulointerstitial areas. Protein overload (PO/H11001 HSD group; hatched bars) induced an increment of superoxide-positive cells that remained at similarly increased levels during the high-salt-diet period. MMF treatment (PO/MMF/H11001 HSD group; filled bars) reduced the number of superoxide-positive cells. Open bars, Control-S group. ***P < 0.001.
renal MDA during protein overload with respect to values in controls (0.40 ± 0.8 nmol/mg protein; Table 1), and there was a significant reduction of MDA renal content in the subsequent salt-sensitive phase (Table 1). MMF treatment reduced renal MDA content during protein overload. At the end of the high-salt-diet period, the normotensive rats of PO + MMF + HSD group had significantly (P < 0.05) reduced renal MDA content with respect to the hypertensive (untreated) counterparts of PO + HSD group (Table 1). GSH renal content increased in the MMF-treated group during the salt-sensitive phase, reaching values comparable to those in controls (9.6 ± 1.10 nmol/mg protein; Table 1).

DISCUSSION

Intraperitoneal injections of large doses of BSA induce a size-permselectivity defect (20, 51), resulting in massive proteinuria and tubulointerstitial nephritis (reviewed in Ref. 9). The glomerular filtration rate is largely preserved (20, 46, 52), and several studies have shown that immune complex formation does not play a role in salt-sensitive hypertension (reviewed in Ref. 38). Furthermore, this model uncovers a mechanism by which proteinuria may be followed by hypertension, which is important because both are independent risk factors for the progression of renal disease (28). In the experimental design, we avoided the uninephrectomy used by others to increase the sensitivity of the protein overload model (8, 10, 18) and to eliminate the contribution of uninephrectomy-derived hemodynamic factors, which was particularly desirable because other investigators (12, 35), as well as our laboratory (41), have shown that MMF has beneficial effects in the renal ablation model.

Proteinuria may be induced by the administration of BSA, ovalbumin, or homologous (rat) serum albumin. We selected BSA because previous investigations have shown that it induces a more intense proteinuria (8, 20) that may persist for 1–3 wk after discontinuation of protein administration (7, 22). Because sex and strain as well as BSA dose are important determinants of the proteinuric response (19), we selected female Lewis rats, a strain used by other investigators (8, 17). The weight of the rats during the protein overload phase did not increase normally and lagged behind that of control rats in the subsequent weeks. One must assume that experimental manipulations were responsible for these findings, but the MMF-treated and the vehicle-treated groups had similar weight changes throughout the experiment; therefore, differences between experimental groups cannot be attributed to this variable (Fig. 2).

The BSA dose administered (2 g/day) was similar to the dose given in other studies to rats with both kidneys (17, 46) and double the dose used in uninephrectomized animals (8). Nevertheless, we obtained a peak proteinuria that while unquestionably significant and occurring at the third day as in other studies (17, 46), was one-third to one-half the value obtained by other workers. The reasons for the lower proteinuria in our studies are not immediately apparent. Because other studies have used a more diluted BSA solution (17, 20), it is possible that the concentration of the BSA that we administered intraperitoneally (66% dilution) was too high. However, the magnitude and duration of the proteinuria were sufficient to obtain important tubulo-interstitial damage and tubulointerstitial infiltrate and resulted in the subsequent development of hypertension when rats in the PO + HSD group were challenged with a high-salt diet.

The administration of MMF resulted in a reduction of immune cell infiltration in the tubulointerstitium and prevented the development of hypertension in rats in the PO + MMF + HSD group. These effects are similar to those reported in salt-sensitive hypertension resulting from angiotensin II infusion (37), nitric oxide synthesis inhibition (30), and in spontaneously hypertensive rats (39). The salt resistance of the PO + MMF + HSD group cannot be attributed to the MMF because previous studies have shown that MMF does not affect blood pressure (30, 39), and, furthermore, the drug was not administered during the last 5 wk of the high-salt-diet period. Therefore, it is likely that the prevention of salt-sensitive hypertension induced by MMF is the result of a reduction of the lymphocyte and macrophage infiltration in the tubulo-interstitial areas. Some of these cells expressed angio-

Table 1. Renal content of malonaldehyde and GSH after 2 wk of protein overload and at the end of the high-salt-diet period

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<th>Protein Overload (2 wk)</th>
<th>High-Salt Diet (8 wk)</th>
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<tr>
<td></td>
<td>PO + HSD group</td>
<td>PO + MMF + HSD group</td>
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<td></td>
<td>PO + HSD group</td>
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<tr>
<td>MDA, nmol/mg protein</td>
<td>1.39 ± 0.33</td>
<td>0.94 ± 0.25*</td>
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<td>0.66 ± 0.13†</td>
<td>0.47 ± 0.07**†</td>
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<tr>
<td>GSH, nmol/mg protein</td>
<td>5.63 ± 1.15</td>
<td>5.38 ± 1.36</td>
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<td>7.59 ± 2.18</td>
<td>9.55 ± 1.54†</td>
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Values are means ± SD. MDA, malonaldehyde; PO + HSD group, rats that received vehicle during the period of protein overload; PO + MMF + HSD group, rats that received MMF during the period of protein overload. MDA values in control = 0.4 ± 0.08 nmol/mg protein. GSH values in controls = 9.6 ± 1.10 nmol/mg protein. *P < 0.05 vs. PO + HSD at the same time interval. †P < 0.01 vs. data at 2 wk.
tensin II, and treatment with MMF was associated with a reduction of angiotensin II-positive cells and oxidative stress. Other investigators have shown that intense proteinuria upregulates the expression of angiotensin-converting enzyme in the proximal tubules (15) and activates NF-kB as a result of increased angiotensin II and endothelin activity (13) as well as the generation of reactive oxygen species through a PKC-dependent pathway (23). Increased interstitial angiotensin II activity and the generation of reactive oxygen species are at a crossroads, promoting immune cell infiltration and sodium retention (36).

Tubulointerstitial infiltration of mononuclear cells persisted in the rats in the PO+HSD group after proteinuria had disappeared in the high-salt-diet phase. In contrast, interstitial nephritis did not develop during the high-salt-diet phase in rats in the PO+MMF+GSD group treated with MMF 6 wk earlier (data at 8 wk in Figs. 6 and 7). These findings are consistent with the interpretation that in this model, interstitial nephritis, once developed, may be maintained with a high-salt-diet that would tend to perpetuate the vicious cycle of hypertension and interstitial inflammation. Interestingly, interstitial angiotensin II activity in the kidney is unresponsive to systemic hemodynamic changes that cause suppression of plasma angiotensin II levels (26). On the other hand, our results indicate that if interstitial inflammation is prevented from occurring, as was done in with MMF treatment, a high-salt diet by itself is not sufficient to trigger an inflammatory reaction, and the rats remained normotensive (Fig. 4).

Lymphocyte and macrophage infiltration had a similar intensity in the tubulointerstitial regions after 2 wk of protein overload. This is in contrast to the results of the classic studies of Eddy (8), in which macrophages were the predominant cell type. However, the discrepancies may be more apparent than real. In fact, tubulointerstitial lymphocyte infiltration in our studies (93.5/mm²; data at 2 wk in Fig. 6) is comparable (on the assumption of 2,000 cells/mm²) to that reported by Eddy (40–45 lymphocytes/1,000 tubulointerstitial cells) in her work (8). Macrophage infiltration is lower in our studies likely because the proteinuria in our work (Fig. 3) was less intense than in other investigations. The correlation shown by Eddy in her work (8) indicates that for the proteinuria found in our studies at 2 wk (70–80 mg/24 h), one would expect ~40 OX42-positive cells/1,000 tubulointerstitial cells, which is very close to the number of 75 OX42-positive cells/mm² (or 37 OX42-positive cells/1,000 tubulointerstitial cells) in the present studies. In summary, our experiments resulted in a macrophage infiltration that was similar to that obtained by Eddy (8) for the corresponding intensity of proteinuria. Lymphocyte infiltration, which is unrelated to the proteinuria but related to the duration of the protein overload (8), corresponded to the expected values after 2 wk of intraperitoneal BSA administration.

As in other models of salt-sensitive hypertension studied by our laboratory (30, 37, 39), angiotensin II-positive cells were infiltrating the tubulointerstitial areas and the intensity of the infiltrate was reduced by MMF treatment (Figs. 8C and 9). Also in agreement with previous studies, 12–18% of lymphocytes and macrophages stained positive for angiotensin II. The role played by increased intrarenal angiotensin activity in the development of salt-sensitive hypertension has been emphasized in a recent study (38).

Studies by many investigators have emphasized the role of oxidative stress in the pathogenesis of hypertension (reviewed in Refs. 45 and 54) and in the modulation of nitric oxide synthesis (47, 48), yet it remains unclear whether oxygen-mediated damage is the cause or the consequence of hypertension (54). The relationship between angiotensin generation and oxidative stress has also been well documented (reviewed in Ref. 40). In the present studies, we have shown that generation of reactive oxygen radicals was increased by protein overload and, subsequently, during salt-driven hypertension. Infiltration and proliferation of immune cells may be induced by NF-kB, which is activated in tubular cells by proteinuria (13). Studies by several investigators have shown that metabolic oxygen radicals activate NF-kB (22, 40), and it has been postulated that oxygen free radicals may be a common second messenger for various pathways of activation of NF-kB (42–44).

MMF is known to suppress lymphocyte proliferation in a relatively selective manner as a result of a reversible inhibition of inosine phosphate dehydrogenase, which is a critical enzyme for the de novo purine synthesis (1). In addition, MMF limits mesangial cell and macrophage proliferation (1, 14) and reduces the expression of adhesion molecules (4, 31, 41). In this study, MMF treatment induced a reduction of the inflammatory infiltrate that was associated with an almost fourfold reduction of the number of superoxide-positive cells (Fig. 10). These results are in agreement with the MMF-induced reduction of the MDA content of the kidney in both phases of the study (Table 1). The relationship between oxidative stress and inflammation has been recently reviewed (38) and is suggested by the correlation between the number of superoxide-positive cells and intensity of macrophage and lymphocyte infiltration in the tubulointerstitial areas.

Proteinuria decreased after protein overload and reached normal levels 4 wk after discontinuation of BSA injections. Nevertheless, histological evidence of damage persisted, and focal areas of tubular dilatation and atrophy were present 6 wk after protein overload was stopped in both experimental groups. MMF treatment reduced tubulointerstitial damage, and the resistance to hypertension may have been partially related to the structural improvement. In fact, subtle structural damage in tubulointerstitial areas has been postulated to be a cause of salt-sensitive hypertension as it may impair the pressure-natriuresis response (16). However, tubular dilatation and atrophy were quite evident in the salt-resistant PO+MMF+HSD group, in which damaged areas covered about one-fifth of the cortical and juxtamedullary regions. However, these
rats remained normotensive when challenged with a high-salt diet. Clearly, no definite conclusions may be drawn from these observations, but it is likely that in the protein overload model, the salt resistance of the MMF-treated rats may be related more to the drastic reduction of oxidative stress, cellular infiltration, and intrarenal angiotensin II activity than to a 10% reduction of the extent of tubulointerstitial damage (35 ± 10.3% in the PO+HSD group and 23.9 ± 5.9% in the PO+MMF+HSD group).

Two important conclusions are derived from our work. First, salt-sensitive hypertension develops after overload proteinuria and, second, the interstitial mononuclear cell infiltrate caused by the proteinuria is likely involved in the pathogenesis of the salt-driven increment in blood pressure. The later conclusion may be drawn from the finding that reduction of the lymphocyte and macrophage infiltration with MMF abrogates the salt-dependent hypertension. Taken together with similar findings in other models of salt-sensitive hypertension (30, 37, 39), these results represent convincing evidence of the participation of the interstitial mononuclear infiltrate in the pathogenesis of salt-sensitive hypertension.

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