Mechanisms of Glomerular Immune Injury: Effects of Antioxidant Treatment

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Abstract. Significant glomerular vasoconstriction and production of reactive oxygen species has been known to occur with exposure to anti–glomerular basement membrane antibody (AGBM-Ab) in the rat model. Previously published studies have demonstrated that such effects can be reduced by therapy with phenololamine, an α-adrenergic antagonist. It was hypothesized that antioxidant pretreatment with water-soluble probucol would improve glomerular hemodynamics 60 to 90 min after the administration of AGBM-Ab. These relationships were examined with both in vivo renal micropuncture and in vitro studies in rats. Single-nephron GFR (SNGFR) decreased markedly in untreated rats after AGBM-Ab as a result of afferent and efferent arteriolar vasoconstriction with consequent reductions in nephron plasma flow (SNPF) and decreases in the glomerular ultrafiltration coefficient (LpA). Basal SNGFR was increased, and SNGFR was significantly higher after AGBM-Ab in probucol-treated versus untreated rats. This finding was due solely to higher values for SNPF and prevention of afferent arteriolar constriction. A reduction in LpA after AGBM-Ab was not prevented by probucol treatment. In vitro analyses of glomeruli revealed reduced myeloperoxidase activity in antioxidant-treated rats. Lipoxigenase activity and leukotriene products, however, were not changed by antioxidant therapy. Reactive oxygen species generation before and after formyl-methionyl-leucyl-phenylalanine stimulation was significantly reduced before and after AGBM-Ab in glomeruli harvested from rats that were treated with the antioxidant. Antioxidant therapy in this model of AGBM-Ab injury did not prevent reductions in LpA, an index of glomerular membrane damage, but did prevent afferent arteriolar vasoconstriction. Reactive oxygen species generation was reduced by probucol. The specific mechanisms whereby antioxidant therapy ameliorates glomerular hemodynamic effects will be defined in additional studies and is likely to involve either enhanced vasodilator or diminished vasoconstrictor activity.

Antibodies directed to the antigens on the glomerular basement membrane (GBM) produce a rapid inflammatory reaction characterized by accumulation of polymorphonuclear leukocytes and later by transition to a mononuclear cell response (1,2). The rapidity of the glomerular capillary inflammatory reaction is related to the site of the antigen/antibody reaction, the endocapillary luminal surface, where the endothelium is fenestrated, allowing easy access of the antibody to its antigenic site. This model of proliferative glomerulonephritis resulting from the administration of anti-GBM antibody (AGBM-Ab) has been used to examine the early glomerular hemodynamic events and the mediators on which this inflammatory reaction is based (3–7). Past studies have demonstrated the important role of polymorphonuclear leukocytes within the first 60 to 90 min after antibody administration and have also characterized the important role of activation of the complement system (8–10). Additional humoral factors, including leukotrienes (7), other arachidonic acid products (6), and even adrenergic and angiotensin systems, may influence the magnitude and severity of this inflammatory response (11,12). The polymorphonuclear leukocyte (PMNL) may contribute both an enzymatic and a humoral component to this inflammatory process (1,13).

Probucol is a cholesterol-lowering agent that is also a remarkably potent antioxidant and yet is nontoxic (14,15). The lipophilic probucol molecule has been esterified, producing a hydrophilic compound that is more likely to enter cells because of higher plasma levels than are possible with commercially available lipophilic compounds (16) (Figure 1). Once inside the cell, the water-soluble probucol is deesterified, trapped, and can act as a local antioxidant without toxic end products.

We conducted studies that examined the effects of administration of this water-soluble form of probucol after the acute administration of AGBM-Ab to determine whether there is an amelioration of adverse glomerular hemodynamic events. We also attempted to determine with in vitro and in vivo studies the role of polymorphonuclear leukocytes after AGBM-Ab was not prevented by probucol treatment. In vitro analyses of glomeruli revealed reduced myeloperoxidase activity in antioxidant-treated rats. Lipoxigenase activity and leukotriene products, however, were not changed by antioxidant therapy, yet vasoconstriction was prevented. H2O2 generation before and after formyl-methionyl-leucyl-phenylalanine stimulation was significantly reduced before and after AGBM-Ab in glomeruli harvested from rats that were treated with the antioxidant. Antioxidant therapy in this model of AGBM-Ab injury did not prevent reductions in LpA, an index of glomerular membrane damage, but did prevent afferent arteriolar vasoconstriction. Reactive oxygen species generation was reduced by probucol. The specific mechanisms whereby antioxidant therapy ameliorates glomerular hemodynamic effects will be defined in additional studies and is likely to involve either enhanced vasodilator or diminished vasoconstrictor activity.

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mechanisms whereby these potential benefits take place. The highly site-specific activity of PMNL, which are adherent to the GBM, and the subsequent release of reactive oxygen species (ROS) and other materials precludes the use of extracellular or circulating antioxidants such as superoxide dismutase. Extracellular molecules in low concentrations should not be effective in this setting. These studies focus on the effects of water-soluble probucol, an antioxidant, on the early injury phase of this immune model.

Materials and Methods
All studies were performed with male Munich-Wistar rats (200 to 265 g body wt) obtained from Simonsen Laboratories (Gilroy, CA) and housed in the Animal Research Facility of the Veterans Affairs Medical Center (San Diego, CA). Rats were randomized to either antioxidant or control and exposed to AGBM-Ab on the morning of the experiment 60 to 90 min before micropuncture and hemodynamic evaluation. Two treatment groups were examined: (1) Control rats \( (n/6) \) received only 0.5 cc of Ringers with sodium bicarbonate intraperitoneally twice daily for 2 d before the experiment and 0.5 cc intravenously on the morning of the experiment and (2) probucol pretreated rats \( (n/6) \) received 2.5 mg of water-soluble probucol in 0.5 cc Ringers sodium bicarbonate intraperitoneally twice daily for 2 d before the experiment and 1.0 mg intravenously on the morning of the experiment. All rats were permitted free access to rat diet and water. In vitro studies of isolated glomeruli from identically treated rats were performed as described below.

Probuco1
The probucol used was a water-soluble form of the commercially available lipid-soluble agent (Marion Merrill Dow, Cincinnati, OH) manufactured in a process described previously (16). Briefly, probucol diglutarate was formed by treating probucol with glutaric anhydride at 130°C for 24 h in the presence of catalytic amounts of 4-dimethyl-aminopyridine (Figure 1). Purity of the product was confirmed by thin-layer chromatography and could, upon alkaline hydrolysis, yield free probucol.

Preparation of AGBM-Ab
AGBM-Ab was produced by methods described previously by this laboratory (2,8,17). Briefly, rabbits received repeated injections of rat GBM in complete Freund adjuvant. At the onset of proteinuria, serum was collected and absorbed with rat plasma and peripheral red cells. The \( \gamma \)-globulin fraction was separated and concentrated by precipitation at a final concentration of 50% saturated ammonium sulfate. The volume of AGBM-Ab from a specific batch delivered during experiments was tailored to the intensity of proteinuria produced in healthy rats and typically was on the order of 0.3 to 0.4 cc intravenously.

Hemodynamic Studies and Micropuncture before and after AGBM-Ab
Preparation for surgery was standard protocol as described previously (2,8,18). Rats were anesthetized with Inactin (100 mg/kg intraperitoneally) on the morning of study. A tracheotomy tube (PE-240) was placed and PE-50 catheters were inserted in the jugular vein, femoral artery, and bladder. Arterial BP was monitored with a pressure transducer and recorded on Statham Instruments SP2000 Chart Recorder (Oxnard, CA). Animal temperature was maintained on a heated micropuncture platform with a servocontrolled heating unit connected to a rectal thermometer. Rats were allowed a 1-h equilibration period and plasma volume expanded with a 3.75% body wt infusion (2.5% body wt donor plasma + 1.25% body wt Ringer) after which time rats were maintained at a constant Ringer infusion of 80 ml/h. A \( ^{3} \text{H} \) inulin infusion (mixed with either probucol or Ringer as described above) was administered during and after plasma volume expansion at a rate of 1.5 \( \mu \)l/min. A baseline GFR was measured over
the next 30 min, and micropuncture was performed as previously published in a paired manner in control and probucol-treated rats (2,18). Proximal tubules were located randomly for micropuncture using dye injection with a 3- to 5-mm beveled-tip pipette. The tubule was then injected with mineral oil, and a 3-min collection was obtained. Hydrostatic pressures were measured in the glomerular capillary and Bowman space of multiple surface glomeruli using a servo-nulling device with 1-μm tip pipettes. Pressure measurements and the mechanics of the pressure monitoring devices have been previously described from this laboratory (19). Efferent peritubular capillary samples were obtained with 1- to 3-mm tip micropipettes. The protein method was modified and has been described previously by this laboratory for micropuncture (3). After completion of control period measurements, AGBM-Ab was injected at a dose of 0.3 to 0.4 cc, determined previously based on quantitative proteinuria in normal rats over a period of 5 min and allowed to circulate for 30 min. Measurements of glomerular capillary pressure, blood samples for protein concentration, nephron filtration rate, and total GFR were repeated within 1 h.

Calculations of single nephron plasma flow (SNPF), afferent arteriolar resistance (AR), and efferent arteriolar resistance (ER) have been described extensively in previous publications from this laboratory (20). After measurements were completed, the kidney was removed and bisected longitudinally. Samples of tissue were obtained for electron microscopy (2,8). One half was snap-frozen in liquid nitrogen for immunofluorescence and the other half was fixed in formalin for histology. Histologic study was undertaken without knowledge of treatment group.

In Vitro Studies in Isolated Glomeruli

Pretreatment of control (n = 6) and experimental probucol-treated (n = 6) groups, surgery, plasma volume expansion, and AGBM-Ab administration were performed in the same manner as described for micropuncture studies above. Animals were paired for all studies, and time intervals were identical to those for micropuncture. At the conclusion of the second period of GFR measurement, bilateral nephrectomies were performed and 5 to 7 cc of whole blood was collected in heparinized syringe via aortic puncture. One kidney was collected as described above was aliquoted into each of five test tubes and 5 μL aliquots of these fractions with assay buffer for a final concentration of 100 mM Tris, 0.35% BSA (pH 7.8). LTC₄ synthase activity was assessed in 15,000 × g supernatant in an assay buffer of 100 mM Tris, 0.35% BSA, 1 mM reduced glutathione at pH 7.8. Samples were then prewarmed to 37°C for 5 min and then incubated for 15 min in the presence of 15 μM LTA₄ at 37°C in a circulating water bath. The incubations were terminated abruptly by quenching with 100 μL of cold methanol containing 1 μg/ml PGB₂ (as an internal standard) and mixing. Samples were then acidified to pH 4.5 with 1 N HCl and evaporated to dryness under nitrogen. The residues were redissolved in methanol and stored under argon at −70°C until further analysis. The percentage recovery of PGB₂ and leukotrienes and standards using this extraction ranged from 75 to 95%.

Extracts were dried under nitrogen and reconstituted in chromatography solvent for analysis by reversed-phase HPLC. The analysis was performed using a liquid chromatograph developed at a flow rate of 1.0 mL/min using a gradient program and a methanol/water/phosphoric acid/ammonium hydroxide solvent system, as previously reported in detail (24). Metabolites were quantified by UV absorbency using molar extinction coefficients of 50,000 at 270 nm, 27,500 at 235 nm, and 49,400 at 280 nm for LTB₄, HETEs, and LTC₄, respectively. Enzymatic activity was expressed as picomoles of product formed per milligram of tissue protein.

Whole kidney tissue myeloperoxidase (MPO) activity was measured using a chromometric assay of the oxidation of O-dianisidine hydrochloride at 460 nm (26,27). Briefly, 200 mg of minced kidney pole was homogenized using a Polytron (Brinkman, Westbury, NY) for 30 s at a setting of “6-5” and sonicated using a cell disrupter (Heat Systems, Inc., Farmingdale, NY) with a 3-mm probe. Samples were suspended in PBS (pH 7.4) before the assay, and measurements were done in triplicate. The molar absorbency of the oxidized O-dianisidine (1.13 × 10⁵/cm) was used to calculate the moles of H₂O₂ decomposed. One unit of H₂O₂ activity was defined as that degrading 1 μmol of H₂O₂/min at 37°C.

Glomerular and Polymorphonuclear H₂O₂ Production

Approximately 5000 glomeruli in a glomerular suspension of 500 μL collected as described above was aliquoted into each of five spectrophoroumeter cuvettes that contained 845 μL of Kreb’s buffer. The samples were equilibrated for 15 min in a 37°C water bath. Five microliter aliquots of 4.2 mM 2, 7-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) was added to each sample (10 μmol final) and they were capped and incubated for 16 min. Baseline fluorescence was read on an Elmer-Perkins Spectrophotometer using an excitation wavelength of 488 nm and an emission wavelength of 530 nm (28). A total of 150 μL of formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma, St. Louis, MO) was added (0.1 μmol final) to activate each sample, and fluorescence was remeasured after 1 h of incubation.

Four milliliters of whole blood collected by aortic puncture in a heparinized syringe was diluted with 2 mL of DMEM (Sigma) and vortexed gently. Immediately after this, 1.5 mL was aliquotted into each of four test tubes and 5 μL of 2', 7'-dichlorofluorescin diacetate (10 nM
final) was added to two of four samples while 150 μl of Kreb’s buffer was added to the other two. All samples were again incubated for 30 min. Twenty-three microliters (1.67 mg/ml) of the nuclear dye stain LDS-751 (Exciton, Dayton, OH) was added 5 to 10 min before flow cytometric analysis (29). A FACStarPLUS cell sorter (Becton Dickinson, San Jose, CA) with a 2-W argon laser set at a 488 line was used to identify a total of 10,000 events (cells) and gate for LDS-751 stained white blood cells in the whole blood samples to identify nucleated cells (30,31). A real-time sorted population dot plot was used to gate for polymorphonuclear cell fluorescence. Data obtained was analyzed using FACStarPLUS Research Software (Becton Dickinson).

Statistical Analyses

Statistical significance between groups of animals was evaluated by unpaired t test, χ² analysis, and ANOVA. Two-way ANOVA was used to evaluate changes in paired studies (i.e., between first and second micropuncture periods). When only one value for a measurement was obtained in a measurement period, a paired t test was used. χ² analysis was used in MPO studies to determine the presence or absence of activity. Statistical significance was considered to be P < 0.05. All data are presented as means ± SE (except discrete numbers for χ² analysis of MPO data) (32).

Results

In vivo Evaluation

Micropuncture Study. The kidney surface appeared normal after antibody administration in both control and probucol-treated animals. There was no evidence of pallor on the surface and no blood within tubules. Probucol pretreatment produced a higher value for nephron filtration rate (SNGFR) in the baseline condition (control, 42.9 ± 1.6; probucol, 48.9 ± 2.2 nl/min). The value for SNGFR was also significantly higher for probucol-treated animals after antibody administration (Table 1). The reduction in nephron filtration rate after antibody administration in control rats was largely attributable to significant renal vasoconstriction. The major reason for the partial amelioration of this reduction in SNGFR by probucol treatment was the prevention of the reduction in single nephron plasma flow. The nephron plasma flow rate was lower quantitatively than pre-AGBM-Ab values after the antibody in probucol-treated rats, but the values were not different statistically. However, the nephron plasma flow rate was different in the control animals (121 ± 7 nl/min versus 51 ± 7 nl/min; P < 0.05). This prevention of vasoconstriction by probucol treatment was primarily the consequence of an elimination of the increase in AR (Table 1). The increase in afferent resistance observed in control animals after anti-GBM antibody was the primary reason for the reduction in nephron plasma flow after anti-GBM antibody as has been observed in previous publications using this model (2,8,11,13). Efferent arteriolar resistance increased after anti-GBM antibody in both groups (19 ± 1 to 31 ± 4 Gdynes sec⁻¹ cm⁻² in controls and 16 ± 1 to 25 ± 4 Gdynes sec⁻¹ cm⁻² in probucol-treated rats). Glomerular capillary hydrostatic pressure gradient did not change in either group (35 ± 1 to 31 ± 2 mmHg in control and 34 ± 2 to 32 ± 3 mmHg in probucol-treated).

Probucol pretreatment prevented renal vasoconstriction, resulting in higher values for nephron plasma flow, but there were no significant membrane-protective effects observed, at least as indexed by alterations in LpA. The reductions in the glomerular ultrafiltration coefficient were similar in both control and probucol-treated animals after AGBM-Ab. In summary, antioxidant pretreatment prevented the reduction in nephron plasma flow after administration of AGBM-Ab by preventing the increase in AR.

In Vitro Evaluation

Glomerular Histology. Evaluation of PMNL within glomeruli revealed that there was no significant difference between the antioxidant-treated group and the control group after the administration of AGBM-Ab. There were 4.7 ± 0.4 PMNL per glomdulus in the control group and 4.4 ± 0.6 PMNL per glomerulus in the antioxidant-treated group.

Table 1. Effects of AGBM-Ab on MAP (mmHg), GFR (ml/min), SNGFR (nl/min), SNPF (nl/min), ΔP transcapillary hydrostatic pressure gradient (mmHg), LpA (nl/sec⁻¹/mmHg⁻¹), ER (Gdynes/sec/cm²), and AR (Gdynes/sec/cm²) in control rats and rats treated with water-soluble probucol

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + Ab</th>
<th>Probucol</th>
<th>Probucol + Ab</th>
</tr>
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<tbody>
<tr>
<td>MAP</td>
<td>105 ± 5</td>
<td>100 ± 8</td>
<td>98 ± 4</td>
<td>102 ± 3</td>
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<tr>
<td>GFR</td>
<td>1.05 ± 0.08</td>
<td>0.66 ± 0.21b</td>
<td>1.14 ± 0.05</td>
<td>0.49 ± 0.13b</td>
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<td>SNGFR</td>
<td>42.9 ± 1.6a</td>
<td>20.9 ± 2.7a,b</td>
<td>48.9 ± 2.2a</td>
<td>29.8 ± 2.8a,b</td>
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<tr>
<td>SNPF</td>
<td>121 ± 7a</td>
<td>51 ± 7a,b</td>
<td>153 ± 14a</td>
<td>117 ± 16a</td>
</tr>
<tr>
<td>ΔP</td>
<td>34.8 ± 0.09</td>
<td>31.0 ± 2.2</td>
<td>34.3 ± 2.2</td>
<td>31.7 ± 2.7</td>
</tr>
<tr>
<td>AR</td>
<td>20.8 ± 1.8</td>
<td>42.7 ± 4.7a,b</td>
<td>16.7 ± 2.1</td>
<td>21.3 ± 2.4a</td>
</tr>
<tr>
<td>ER</td>
<td>18.6 ± 1.2</td>
<td>31.2 ± 3.6b</td>
<td>16.0 ± 1.3</td>
<td>25.0 ± 4.3b</td>
</tr>
<tr>
<td>LpA</td>
<td>0.07 ± 0.01</td>
<td>0.04 ± 0.01b</td>
<td>0.06 ± 0.01</td>
<td>0.03 ± 0.002b</td>
</tr>
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</table>

MAP, mean arterial pressure; ΔP, transcapillary hydrostatic pressure gradient.

A reduction in SNPF post-Ab was ameliorated as was an increase in AR in probucol-treated rats. Probucol treatment did not influence Ab effects on LpA.

a P < 0.05 between groups with the same AGBM-Ab exposure status.
b P < 0.05 between baseline and post–AGBM-Ab within a group.
5-Lipoxygenase Products and Enzymatic Activity. We evaluated lipoxygenase products and enzymatic activity after antibody administration in both control and probucol-treated rat whole kidneys and suspensions of glomeruli. Neither the upstream lipoxygenase products (5-, 12-, or 15-HETE) nor the downstream 5-lipoxygenase products (LTB₄, LTC₄, or LTD₄) were found in any directly extracted kidney or glomeruli samples (lower limits of detection approximately 20 pmol/mg tissue protein). Likewise, no specific 5-, 12-, or 15-lipoxygenase enzymatic activity was found in any sample (limits of detection approximately 1 pmol/mg tissue protein per min of enzymatic activity). No LTC₄ synthase activity was detected with similar limits of detection. However, LTA₄ hydrolase activity was found in all samples. After AGBM-Ab, LTA₄ hydrolase activity in whole kidney was not significantly different between controls and probucol-treated rats (106.5 ± 4.2 versus 103.2 ± 8.0 pmol/mg tissue protein per 15 min, mean ± SEM; n = 3; NS). Likewise, LTA₄ hydrolase activity in glomeruli was not significantly different between controls and probucol-treated rats (123 ± 22.6 versus 133.4 ± 18.8 pmol/mg tissue protein per 15 min, mean ± SEM; n = 6; NS).

MPO Activity. MPO activity was evaluated to determine whether the rate of conversion of H₂O₂ to hypohalous acids was different based on different enzyme activity. When the data were examined for the presence or absence of MPO activity using χ² analysis, a clear and significant pattern emerged. Six of eight rats in the control group displayed significant MPO activity, whereas only one of six rats in the probucol-pretreated group exhibited any detectable MPO activity, suggesting that probucol may inhibit this enzyme system as previously suggested. However, this finding does not immediately explain the hemodynamic results after probucol treatment because probucol prevented renal vasoconstriction, an event that one would not expect to be caused by altered MPO activity.

Glomerular and PMNL H₂O₂ Production. We examined H₂O₂ production using fluorescence techniques in both PMNL and isolated glomeruli before and after administration of the antibody. We assessed H₂O₂ production in the basal condition and after the administration of a nonspecific stimulant, fMLP. Circulating PMNL harvested 30 min after AGBM-Ab in both groups exhibited similar H₂O₂ production in both the basal and stimulated condition in comparison with preantibody PMNL (pre-fMLP, C = 47 ± 2, CAB = 43 ± 2, P = 40 ± 3, PAB = 39 ± 2; post-fMLP, C = 47 ± 1, CAB = 41 ± 1, P = 39 ± 2, PAB = 43 ± 2 in fluorescence units). In a single evaluation 90 min after AGBM-Ab, fMLP increased H₂O₂, but all values were lower in PMNL after the antibody was given in both control and probucol-treated rats. Pre- and post-fMLP values were as follows: control, 63 to 119; control after Ab, 39 to 49; probucol, 60 to 100; and probucol after Ab, 44 to 68 fluorescence units. When isolated glomeruli were examined for H₂O₂ production before and after fMLP, probucol-treated animals exhibited lower H₂O₂ production in both the basal and post-fMLP states in glomeruli both before and after antibody administration (Figure 2). However, stimulation with fMLP produced a large increase in H₂O₂ production in all groups, suggesting that the capacity to generate H₂O₂ was not totally impaired but that responses were lower in the animals that were treated with the antioxidant.

Discussion

After administration of AGBM-Ab, the nephron filtration rate falls precipitously, both as a consequence of reductions of the glomerular ultrafiltration coefficient and as a result of renal vasoconstriction producing reductions in renal plasma flow (2,8). The latter effect is primarily the consequence of afferent and, to a lesser extent, efferent arteriolar constriction.

Changes in whole-kidney GFR do not exactly parallel changes in SNGFR (Table 1). This has been observed in previous studies with the paired acute AGBM model and discussed in those manuscripts. We offer two explanations for why the changes in SNGFR in surface nephron micropuncture are often of greater magnitude than changes in the whole-kidney GFR (8). First, surface nephrons may behave differently because (1) they are more heavily innervated, (2) they are purported to have greater renin-angiotensin activity, and (3) they may even have different densities of critical GBM antigens for binding of antibody. This suggests that deeper cortical nephrons behave somewhat differently in a manner that cannot be defined directly by micropuncture techniques. Second—and perhaps more likely—changes in whole-kidney GFR are measured less accurately than SNGFR. SNGFR values and assessment of afferent resistance are highly accurate and believable. It is our experience with this model that during the 60- to
90-min period after AGBM, there is little increase in heterogeneity in SNGFR among the five samples at each time point. The reader will note the small SE as a percentage of the mean for SNGFR when compared with the parallel values for GFR.

It is known that activated PMNL are involved in this process through at least three primary functions: (1) release of degradative proteases and other enzymes; (2) release of inflammatory autacoids, major products being oxygenated metabolites of arachidonic acid; and (3) formation of ROS and other radicals (28,30,33–35). Experimental production of proteinuria with activation of MPO and generation of hypochlorous acid has suggested a role for ROS, but very little is known about the specifics of this form of immune-mediated injury (36). There is little specific information regarding the effectiveness of antioxidants in modulating the injury process in AGBM-Ab–related inflammation (37,38). Few publications reporting this model have demonstrated clearly whether, in the early phases of the inflammatory reaction, treatment with antioxidants might reduce or modify the role of ROS and provide benefit.

The reduction in filtration rate after exposure to AGBM-Ab is the consequence of both renal vasoconstriction with a reduction in plasma flow and a reduction in the glomerular ultrafiltration coefficient. The local release of ROS and other radicals by PMNL could affect this process by reducing the LpA, the glomerular ultrafiltration coefficient, through the release of local enzymes. This process could also be mediated by the generation of charged molecules or the generation of a variety of ROS, including superoxide anion, H$_2$O$_2$, and hydroxyl radicals. In addition, H$_2$O$_2$ can react with halides and MPO to produce the highly reactive hypohalous acids, primarily hypochlorous acid (36). Hypochlorous acid can react in turn with H$_2$O$_2$ to produce singlet oxygen, which also has the capacity to produce tissue damage (39). Therefore, antioxidant therapy, if effective, might be expected to ameliorate the acute inflammatory process by effecting alterations in LpA via changes in the hydraulic conductivity of the GBM and/or surface area.

Generation of ROS from circulating inflammatory cells and from local resident cells seems to play a role in the tissue damage associated with the acute inflammatory response (36–39). In the model of immune-induced glomerular injury, inflammatory events occur quickly and involve the attraction of PMNL into glomerular capillaries, at least when AGBM is present. The glomerular hemodynamic findings. We observed by histologic evaluations that there were no changes in the number of leukocytes observed per glomerulus after AGBM-Ab as a consequence of probucol therapy. H$_2$O$_2$ production in intrinsic glomerular cells was decreased, although this effect was reasonably modest. In addition, we observed that MPO activity, although present in six of eight rats in the control group after administration of the antibody, was present in only one of six rats in the probucol-pretreated group. This is an interesting finding but does not logically explain prevention of vasoconstriction. Diminished production of hypochlorous acids might have explained an amelioration of LpA, but this was not the case based on glomerular hemodynamic observations.

To direct our activities toward systems that might have some effects on vascular smooth muscle or vascular resistance, we also examined glomerular lipoygenase activity in glomerular products and enzymatic activity in whole-kidney glomeruli. The lipoxygenase activity in glomeruli was undetectable within 60 to 90 min after antibody administration. Furthermore, LTA$_4$ hydrolase activity, the enzyme responsible for the synthesis of LTB$_4$, was not significantly different between groups. This metabolite of arachidonic acid has been suggested to play a role in altering glomerular hemodynamics, specifically LpA, after AGBM-Ab administration. It exerts no effects on renal plasma flow (7). It is not intuitively obvious how administration of the antioxidant might modify the activity of a vasoconstrictor (8). In the past, we have determined that vasoconstriction seems partially complement dependent in that complement depletion does ameliorate a portion of vasoconstriction. In previous studies, we have also observed that administration of α-adrenergic antagonists and previous renal denervation both ameliorate vasoconstriction observed after administration of AGBM-Ab (unpublished observations). As part of those studies we did determine that phenolamine, an α-adrenergic antagonist, modestly reduced the quantity of ROS generated. The present in vitro studies observed that glomerular H$_2$O$_2$ produc-
tion was diminished in probucol-pretreated animals both before and after fMLP stimulation. This finding might help to explain an alteration in membrane damage, but it is difficult to relate a reduction in ROS to diminished vasoconstriction.

Previous studies in this laboratory using the lipophilic probucol compound revealed that there was a consistent, although small, increase in nephron plasma flow rates in probucol-treated animals (14, 15). Probucol kidneys were vasodilated compared with the nontreated controls. These results may have been due to reduction in ROS as observed in the present study. A candidate system that might be influenced by lesser ROS would be the arginine/nitric oxide pathway (40–42). In addition, probucol has been suggested to be a 5-lipoxygenase inhibitor, but we found no major difference in leukotriene generation in the present study. An interaction of probucol with adrenergic systems has not been previously defined or suggested. However, the finding that phentolamine prevented renal vasoconstriction in two recent studies from this laboratory has raised the possibility that alterations in the oxidizing status of tissue might somehow modify adrenergic responses in vascular smooth muscle, but the specific mechanism has not been elucidated (11, 12). Oxidation of nitric oxide by ROS might enhance α-adrenergic vasoconstriction which then could be prevented by α-adrenergic blockade. Studies from our laboratory have suggested that the glomerular physiologic and tubular effects of inhibition of nitric oxide synthase with L-NMMA is greatly influenced by previous renal denervation (43). Additional studies have suggested that this may be due to an α2-adrenergic effect (44). Phentolamine blocks both α1- and α2-adrenergic activity. Probucol has also been suggested to alter the development of glomerulosclerosis in a subtotal nephrectomy model (44). Mechanisms that form the basis for this prevention of glomerulosclerosis have not been fully delineated.

Contrary to our predictions, antioxidant therapy did not prevent alterations in glomerular ultrafiltration coefficient, a reflection of membrane damage. Probucol seems to be an MPO inhibitor, but this effect alone does not explain the prevention of vasoconstriction. A complex series of interactions between vasodilators (including nitric oxide and prostacyclins) and vasoconstrictors (e.g., thromboxane A2, endothelin, angiotensin II, platelet activating factor, several others) influences glomerular hemodynamics. The most likely mechanism to account for our findings is that immune-induced events lead to release of both ROS and nitric oxide generation. Treatment with an effective antioxidant alters the balance between these two pathways and results in less oxidation of nitric oxide and presumably a greater cyclic GMP response, thereby preventing immune-induced vasoconstriction.

The studies presented here have examined the effect of a highly effective, nontoxic, water-soluble modification of probucol and have produced somewhat surprising results. The amelioration of glomerular hemodynamic events after AGBM-Ab in animals that were pretreated with probucol was specific. The parameters that reflect membrane damage were not modified; however, apparent arteriolar vasoconstriction was prevented. On the basis of in vitro data, the beneficial effects of antioxidant therapy may have resulted from a reduction in ROS and/or an alteration in the redox potential of inflammatory and resident cells. These beneficial affects, however, were not the consequence of any alteration in the number of PMNL adherent to glomerular capillaries. There was a reduction in H2O2 generation before and after fMLP stimulation of resident glomerular cells. Although the mechanisms have not been specifically delineated, it is likely that antioxidant therapy has either reduced the efficacy of vasoconstrictor or amplified the effects of a vasodilator substance associated with inflammation.

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