The alternative pathway of complement is activated in the glomeruli and tubulointerstitium of mice with adriamycin nephropathy

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1Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado; 2Department of Pathology, University Hospital Dubrava, Zagreb, Croatia; 3Department of Medicine, Medical University of South Carolina and Medical Research Service, Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina

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Lenderink AM, Liegel K, Ljubanović D, Coleman KE, Gilkeson GS, Holers VM, Thurman JM. The alternative pathway of complement is activated in the glomeruli and tubulointerstitium of mice with adriamycin nephropathy. Am J Physiol Renal Physiol 293: F555–F564, 2007. First published May 23, 2007; doi:10.1152/ajprenal.00403.2006.—The complement system effectively identifies and clears invasive pathogens as well as injured host cells. Uncontrolled complement activation can also contribute to tissue injury, however, and inhibition of this system may ameliorate many types of inflammatory injury. Several studies have demonstrated that the filtration of complement proteins into the renal tubules, as occurs during proteinuric renal disease, causes tubular inflammation and injury. In the present study, we tested the hypothesis that activation of the complement system in the urinary space requires an intact alternative pathway. Using a model of adriamycin-induced renal injury, which induces injury resembling focal segmental glomerulosclerosis, we examined whether mice deficient in factor B would be protected from the development of progressive tubulointerstitial injury. Complement attenuation was attenuated in the glomeruli and tubulointerstitium of mice with congenital deficiency of factor B (Fb−/−) compared with wild-type controls, demonstrating that complement activation does occur through the alternative pathway. Deficiency in factor B did not significantly protect the mice from tubulointerstitial injury. However, treatment of wild-type mice with an inhibitory monoclonal antibody to factor B did delay the development of renal failure. These results demonstrate that complement activation in this nonimmune complex-mediated model of progressive renal disease requires an intact alternative pathway.

IN PROGRESSIVE RENAL diseases, the degree of injury in the tubulointerstitial correlates with the degree of renal dysfunction, and it is one of the best predictors of disease prognosis (5, 6, 15, 20, 28). Injury to the tubulointerstitial is felt to be a final common pathway of disease progression (26), and many different glomerular diseases likely involve common mechanisms of tubulointerstitial injury. The degree of proteinuria also correlates with disease progression, and proteinuria itself is associated with tubulointerstitial scarring (27). Large molecular weight factors, which are toxic to the tubular epithelial cells (TECs), are filtered into the urine in proteinuric diseases. These factors thus link the development of proteinuria with the subsequent development of tubulointerstitial injury and progressive renal decline, even when the primary glomerular insult is quiescent. Factors that have been demonstrated to be injurious or proinflammatory when in contact with TECs include albumin (31), albumin-associated fatty acids (13), transferrin (31), and complement proteins (11).

The role of the complement system in glomerular injury has been established in several disease models (2, 24, 30, 41), and a number of recent studies have also linked the complement system with the development of tubulointerstitial scarring in progressive renal diseases (10, 11, 19, 22). Complement activation fragments are detectable in the urine of patients with proteinuria, particularly diabetic nephropathy and focal segmental glomerulosclerosis (FSGS) (17). Tubular complement deposition has also been demonstrated in the biopsies of patients with proteinuric diseases (1, 4, 8). A study using C6-deficient rats (which are unable to form the membrane attack complex) demonstrated that the C6-deficient animals developed milder tubulointerstitial disease than wild-type controls when they had equivalent degrees of proteinuria (18). Another study demonstrated that a complement inhibitor prevented tubulointerstitial injury and loss of renal function in a similar model (22). C6-deficient rats were not protected from progressive renal injury in nonproteinuric models (25), however, supporting the theory that complement components must reach the tubular lumen to injure the TECs.

Serum contains high concentrations of the complement components. Ordinarily, these proteins do not cause injury to host cells because of complement inhibitory proteins expressed on cell surfaces (14). The alternative pathway of complement is continually activated in the fluid phase, however, and inadequate inhibition can permit injury via alternative pathway activation (33). The presence of complement inhibitors is, therefore, critical for the prevention of inappropriate inflammation. Although several complement inhibitors are expressed in the different resident renal cell types, inhibitors are not expressed in high density on the apical surface of TECs (12, 36). Consistent with this, the alternative complement pathway can be activated on the brush border of TECs (7). Ordinarily, inhibition is not necessary within the tubular lumen, because complement proteins are excluded from this space by the size and charge selectivity of the glomerular filtration apparatus. During proteinuria, however, these proteins gain access to this surface. Furthermore, with a progressive loss of nephrons, the concentration of ammonia in the remnant nephrons increases, an adaptation that may further favor alternative pathway activation (21).

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We hypothesized that tubulointerstitial complement activation in proteinuric renal disease is the result of uncontrolled activation through the alternative pathway. To examine the tubular effects of the alternative pathway, we chose to study a model of proteinuric renal disease that is not immune complex mediated, thus avoiding the effects of classical pathway activation by immunoglobulin. Adriamycin nephropathy causes acute injury of the glomeruli followed by proteinuria and progressive loss of renal function (40). Studies in rats have demonstrated that complement activation does not influence the development of proteinuria in this model (18, 22). We predicted that mice lacking an intact alternative pathway would incur glomerular injury equivalent to that of wild-type controls, but that they would be protected from the development of tubulointerstitial injury over the ensuing weeks. To test this hypothesis, we induced adriamycin nephropathy in wild-type mice and in mice deficient in factor B (fB⁻/⁻), an essential component of the alternative pathway (39). We also treated wild-type mice with an exogenous inhibitor of the alternative pathway activity in wild-type mice, 1 mg of an inhibitory monoclonal antibody (MAb) to mouse factor B (34) was injected intraperitoneally every day starting 1 wk after the administration of the adriamycin. Antibody (MAb) to mouse factor B (34) was injected intraperitoneally every day starting 1 wk after the administration of the adriamycin.

METHODS

Mice. Factor B-deficient mice were generated as previously described (16). The adriamycin model has been characterized in Balb/c mice, and C57BL/6 mice are resistant to this model (40). Therefore, fB⁻/⁻ mice were back-crossed onto a Balb/c background for seven generations, and congenic fB⁻/⁻ and fB⁺/+ mice were compared. Because the factor B gene is located in the major histocompatibility complex (MHC) class II locus on chromosome 17 (41), fB⁻/⁻ mice on the Balb/c background mice will carry the H-2b MHC. Balb/c mice, in contrast, have the H-2d MHC. To determine whether the differences in MHC could account for differences in disease severity, Balb/cJ mice were also compared with CB10-H2b/LilMcJ mice (a Balb/c strain crossed with C57BL/10 mice to generate a strain with the H-2b MHC). Both groups were obtained from the Jackson Laboratory. A total of 71 mice were injected with adriamycin: 16 fB⁻/⁻ mice (of which 4 died before completing the study), 47 fB⁺/+ mice (of which 15 died before completing the study), and 8 C.B10-H2b/LilMcJ mice (of which none died before the end of the study). The mice were housed and maintained in the University of Colorado Center for Laboratory Animal Care in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and all animal procedures were in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Adriamycin nephropathy. Disease was induced in 8- to 10-wk-old male mice with a single intravenous injection of 10–11 mg/kg of adriamycin (Bedford Laboratories, Bedford, OH) (40). Mice were followed for 4–5 wk after disease induction and were then killed for serum and tissue collection. Control groups of fB⁺/+ and fB⁻/⁻ mice received intravenous injections of PBS. To inhibit alternative pathway activity in wild-type mice, 1 mg of an inhibitory monoclonal antibody (MAb) to mouse factor B (34) was injected intraperitoneally every day starting 1 wk after the administration of the adriamycin. Control mice were injected with an isotype-matched mouse anti-human antibody that does not react with murine protein (MAb 171 (9)]. These mice were killed after 4 wk due to concerns that the control-treated group appeared moribund.

Renal function. Serum was collected at weekly intervals and stored at −80°C. Renal function was assessed at the end of the study by measurement of the serum urea nitrogen (SUN) using a Beckman Autoanalyzer (Beckman, Fullerton, CA).

Urine albumin measurement. Spot urine samples were collected 1 wk after injection of the adriamycin and at the end of the study (week 5 for studies comparing the fB⁺/+ and fB⁻/⁻ mice). Albumin was measured by an enzyme-linked immunosorbent assay, according to the manufacturer’s instructions (Bethyl Laboratories, Montgomery, TX). Urine creatinine was measured using a Beckman Autoanalyzer (Beckman). To normalize urine albumin excretion, the values are reported as micrograms albumin per milligram creatinine, and values <25 μg/mg were considered normal (24).

Renal morphology. After the kidneys were removed from the mice, sagittal sections were fixed, embedded in paraffin, and 4-μm sections were cut and stained with periodic acid Schiff. The sections were stained with periodic acid Schiff. The sections were stained with periodic acid Schiff. The sections were stained with periodic acid Schiff. The sections were stained with periodic acid Schiff.
evaluated by a renal pathologist (DL) in a blinded fashion. To evaluate tubulointerstitial injury, 10 cortical fields were examined, and the percentage of tubules demonstrating injury was assessed by the method described by Rangan et al. (25). Kidney sections were scored as follows based on the percentage of affected tubules: 0, none; 1, 25–45%; 3, 51–75%; and 4, >75%. To quantitate the degree of glomerular injury, 100 glomeruli were examined, and the number of segmental or globally sclerosed glomeruli was expressed as a percentage.

**Immunofluorescence.** For immunofluorescence, sagittal sections of the kidneys were snap frozen in optimum cutting temperature compound (Sakura Finetek, Torrance, CA). Four-micrometer sections were cut with a cryostat and stored at −70°C. The slides were later fixed with acetone and stained with FITC-conjugated or unconjugated anti-mouse C3 (Cappel, Durham, NC) diluted 1:150, anti-mouse C4 diluted 1:10 (Hycult Biotechnology, Uden, the Netherlands), anti-mouse collagen 1 diluted 1:100 (Chemicon, Temecula, CA), and anti-mouse IgM and IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Species-appropriate secondary antibodies were also from Jackson ImmunoResearch Laboratories, and sections were counterstained with hematoxylin (Vector Laboratories, Burlingame, CA).

**Western blot analysis.** Examination of C3 in the urine was performed using Western blot analysis. Urine volumes were normalized to the creatinine concentration and separated by SDS-PAGE. After transferring the proteins to nitrocellulose membrane, C3 was detected using a peroxidase-conjugated polyclonal goat anti-mouse C3 antibody (Cappel) at a dilution of 0.3 μg/ml.

**In vitro analysis of alternative pathway inhibition.** Alternative pathway activity in the serum of mice treated with MAβ 1379 or MAβ 171 was measured using an in vitro analysis of C3 deposition on zymosan A particles (Sigma-Aldrich), as previously described (37). Briefly, 10 μl of serum from each mouse to be tested were incubated with 109 zymosan particles at 37°C for 30 min in a master mix containing 5 mM MgCl2 and 10 mM EGTA (to prevent classical pathway activation). C3 deposition on the particles was analyzed by flow cytometry. The alternative pathway activity was calculated by normalizing the amount of C3 deposited on the zymosan particles to the average values obtained using the initial serum obtained from control-treated mice.

**Statistical analyses.** Comparison between groups was performed with unpaired T-testing. To compare groups of mice that were treated with a factor B inhibitor or control antibody, weekly measurements of the SUN were also compared using a repeated-measures ANOVA in which the main effects examined were the treatment group and the time point. A P value of <0.05 was considered statistically significant. Results are reported as means ± SE.

### Table 1. C3 immunofluorescence in glomeruli and tubules (0–5)

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Glomerular C3</th>
<th>Tubular C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>fB+/+ PBS week 5</td>
<td>4</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>fB+/− PBS week 5</td>
<td>4</td>
<td>0 ± 0*</td>
</tr>
<tr>
<td>fB+/+ adriamycin week 5</td>
<td>4</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>fB+/− adriamycin week 5</td>
<td>4</td>
<td>0 ± 0*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. fB, factor B. *P < 0.05 vs. fB+/+; †P < 0.01 vs. fB+/+ PBS; ‡P < 0.001 vs. fB+/+ adriamycin.
RESULTS

Factor B-deficient mice develop milder proteinuria than controls in response to Adriamycin. We measured albumin/creatinine in PBS-treated $fB^{+/+}$ (46 ± 16 μg/mg, $n = 5$) and PBS-treated $fB^{-/-}$ Balb/c mice (27 ± 6 μg/mg, $n = 5$, $P =$ nonsignificant vs. $fB^{+/+}$ mice). Values for these mice were similar to those previously reported as normal on the C57BL/6 background (24). Disease was then induced in $fB^{-/-}$ and $fB^{+/+}$ mice by injection of adriamycin, and after 1 wk urine samples were collected. Urinary albumin/creatinine was measured (Fig. 1), and both groups showed a high degree of albuminuria (45,623 ± 12,435 μg/mg in the $fB^{+/+}$, $n = 14$; 16,716 ± 5,275 μg/mg in the $fB^{-/-}$, $n = 8$). Contrary to our expectation, however, albuminuria in $fB^{-/-}$ mice was significantly lower than in $fB^{+/+}$ mice 1 wk after disease induction ($P < 0.05$). By week 5, the albuminuria in both groups was not significantly different (Fig. 1).

Fig. 3. IgM, C3, C4, and collagen I in the glomeruli of $fB^{+/+}$ and $fB^{-/-}$ mice 5 wk after injection with PBS or with adriamycin. A: immunofluorescence microscopy was performed after dual-staining kidney sections for C3 (green) and IgM (red). IgM was present in the glomeruli of mice treated with PBS or with adriamycin and colocalized with glomerular C4d (appearing yellow in the overlay). B: immunofluorescence microscopy was also performed after dual-staining of kidney sections for C3 (green) and C4 (red). C4 was present in the glomeruli of all mice, probably due to classical pathway activation by IgM. The glomeruli in $fB^{+/+}$ mice injected with adriamycin also demonstrated deposition of C3 that only partially colocalized with C4d, however, suggesting that some of the C3 activation did not involve the classical pathway. Virtually no tubulointerstitial C4 was seen in any of the mice. C3 deposits in the glomeruli of adriamycin-treated mice were seen in areas of sclerosis (arrows), which also showed collagen I deposition (C). Original magnification ×200.
The alternative pathway is activated in the glomeruli of adriamycin-treated mice. We initially chose the adriamycin model as suitable for examination of complement-mediated injury of the tubulointerstitium, since it is not an immune complex-mediated model. Given the milder degree of proteinuria in \( fB^{-/-} \) mice compared with \( fB^{+/+} \) controls, however, we examined the glomeruli for evidence of complement activation. As has been reported before (35), control mice demonstrated some deposition of C3 in the glomeruli and along the tubular basement membrane (Fig. 2A). In control \( fB^{-/-} \) mice (Fig. 2B) and adriamycin-treated \( fB^{-/-} \) mice (Fig. 2D), little C3 deposition could be detected in the glomeruli, and it was virtually undetectable in the tubules (Table 1).

The relative lack of C3 in the adriamycin-treated \( fB^{-/-} \) mice suggests that the greater initial injury seen in \( fB^{+/+} \) mice after treatment with the adriamycin is due to alternative pathway activation. Glomeruli in all groups also contained IgM deposits (Fig. 3A), which can activate the classical pathway, secondarily activating the alternative pathway through the amplification loop. IgG was not detected in the glomeruli of adriamycin or control-treated animals. We performed dual-staining for C3 and for C4 (Fig. 3B). C4 can be seen in all glomeruli, probably due to classical pathway activation by the deposited IgM. The C4 did not colocalize with the C3 deposited in the glomeruli of wild-type mice, however, suggesting that deposition of this additional C3 does not involve the classical pathway. The \( fB^{-/-} \) mice also developed glomerulosclerosis, but did not have C3 deposited in the scarred regions of the glomeruli. Thus treatment with adriamycin causes selective activation of the alternative pathway within the injured glomeruli.

Alternative pathway-deficient mice are not protected from progressive injury in this model. After induction of injury, the mice were followed for 5 wk. Our initial hypothesis was that \( fB^{-/-} \) mice would develop an equal degree of proteinuria, but would develop milder tubulointerstitial injury and retain greater renal function than \( fB^{+/+} \) mice. Even with the lesser degree of initial proteinuria seen in the \( fB^{-/-} \) mice, however, the progression of renal injury appeared comparable between the two groups. SUNs were not significantly different at the end of the study (Fig. 4). Two mice in each group developed severe renal failure (SUN > 90) and died before the end of the study. Differences between the groups were not significant at any time point, however. Both groups of mice demonstrated tubular injury (Fig. 5), and the degree of injury was not significantly different between the two groups when graded in a blinded fashion (Table 2). There was a trend toward more severe injury in Balb/c mice with H-2\(^{b}\) MHC compared with Balb/c mice with H-2\(^{d}\) MHC (Table 2), suggesting that the MHC may influence disease outcome, but these differences were not statistically significant. Ten of the 45 mice injected with adriamycin for these experiments died before completing the study. Of these, four had severely elevated SUNs (>90 mg/dL). The causes of death for the other mice were not determined, but may have been due to nonrenal toxicity of the adriamycin.

Treatment with an inhibitor of factor B after the onset of disease delays the onset of renal failure. We next treated mice after the onset of disease with a MAb to factor B (34) to block the tubular effects of filtered alternative pathway proteins. Our laboratory previously used this antibody in a model of renal ischemia-reperfusion and found that it prevented complement-mediated injury of the tubules (37). In the present study, we started treating mice with daily injections of the MAb starting 1 wk after injection with the adriamycin. SUNs for these mice remained relatively stable over the next 2 wk, and they were significantly lower than the control-treated group at the 3-wk time point (Fig. 6B). By the end of the study, the SUNs in the anti-factor B-treated group had begun to rise, however, and were no longer significantly different than the control-treated group. Analysis of the rate of rise of SUNs with the repeated-measures ANOVA showed a significant worsening of renal function over time, but disease progression was not significantly attenuated by treatment, even when tested by an additional model, which included an interaction term. Average weights for the 1379 and control-treated mice did not change between weeks 3 and 4 (18.3 and 18.4 g for the 1379-treated mice at weeks 3 and 4, respectively, and 17.2 and 17.1 g for the control-treated mice), so the changes in SUNs are unlikely to be due to dehydration. Pathological scores were not significantly different between the groups at the end of the study (Table 2). Unfortunately, treatment with MAb 1379 did not significantly reduce serum alternative pathway activity compared with control-treated mice (Fig. 6C). We have effectively used this antibody to block alternative pathway activity in acute models (32, 34, 37), and why inhibition was unsuccessful in this model is not clear. Unexpectedly, both groups had a higher percentage of glomeruli with segmental sclerosis and showed a trend toward worse tubulointerstitial injury compared with the \( fB^{+/+} \) and \( fB^{-/-} \) mice injected with adriamycin. This may be explained by immune activation by the injected antibodies, but it could also be a consequence of the daily intraperitoneal injections. Nine of the 26 mice injected with adriamycin for this experiment died before completing the study, and two of these had elevated SUNs (>90 mg/dL). These results also suggest that some mice died of nonrenal toxicity from the adriamycin.

Filtration of C3 into the urinary space is variable in this disease model. To further characterize this model, we performed Western blot analysis for C3 on the urine from \( fB^{+/+} \) and \( fB^{-/-} \) mice at the 5 wk time point (Fig. 7). All
of the mice in both groups had albuminuria, although the degree varied broadly (Fig. 1). We found that C3 was scarcely detectable in the urine of one-half of the mice from both groups. C3 is a large molecule [apparent molecular mass of 185 kDa (29)]. Given the varying amount of C3 that reaches the tubular lumen, it is likely that the degree of complement-mediated injury to the TECs also varied from mouse to mouse in the study. In support of this, the SUN values in wild-type mice did correlate with the amount of C3 detected in the urine (Fig. 7B).

Table 2. Histological injury scores

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Tubulointerstitial Injury (0–5)</th>
<th>n</th>
<th>Glomeruli Globally Sclerosed, %</th>
<th>n</th>
<th>Glomeruli Segmentally Sclerosed, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>fB+/+ PBS week 5</td>
<td>0±0</td>
<td>5</td>
<td>0±0</td>
<td>5</td>
<td>0±0</td>
<td>5</td>
</tr>
<tr>
<td>fB−/− PBS week 5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>fB+/+ adriamycin week 5</td>
<td>1.4±0.3</td>
<td>13</td>
<td>18.2±6.3</td>
<td>13</td>
<td>7.0±1.8</td>
<td>13</td>
</tr>
<tr>
<td>fB−/− adriamycin week 5</td>
<td>1.6±0.5</td>
<td>11</td>
<td>23.6±8.7</td>
<td>11</td>
<td>7.2±2.6</td>
<td>11</td>
</tr>
<tr>
<td>Balb/c H-2b week 5</td>
<td>1.75±0.5</td>
<td>4</td>
<td>23.5±7.3</td>
<td>4</td>
<td>15.25±6.8</td>
<td>4</td>
</tr>
<tr>
<td>Balb/c - anti-factor B week 4</td>
<td>2.3±0.3</td>
<td>7</td>
<td>22.1±5.3</td>
<td>7</td>
<td>28.1±6.4*</td>
<td>7</td>
</tr>
<tr>
<td>Balb/c- control IgG1 week 4</td>
<td>2.3±0.3</td>
<td>11</td>
<td>28.5±6.3</td>
<td>11</td>
<td>21.3±3.2†</td>
<td>11</td>
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</table>

Values are means ± SE; n, no. of animals. *P < 0.001 vs. fB+/+ adriamycin. †P < 0.01 vs. fB+/+ adriamycin.

F560 COMPLEMENT ACTIVATION IN ADRIAMYCIN NEPHROPATHY
DISCUSSION

We examined the role of the alternative complement pathway in the development of renal injury in a mouse model of FSGS. Our original hypothesis was that activation of the alternative pathway within the tubules of proteinuric mice would cause progressive tubular injury. We chose the adriamycin model because it is not an immune-complex mediated model, and in rats complement activation did not seem to influence the initial glomerular injury (18, 22). We believed that the complement system would not significantly alter the development of proteinuria, but that alternative pathway activation would cause tubular injury and progressive renal failure. Contrary to this prediction, however, the alternative pathway did appear to be activated in the injured glomeruli, and $fB^{-/-}$ mice developed less albuminuria in the early stage of injury.

Tubulointerstitial complement deposition was largely prevented in the $fB^{-/-}$ mice, demonstrating that activation is due to activation of the alternative pathway. Despite the lesser degree of initial albuminuria and the absence of C3 deposition by immunofluorescence, however, the $fB^{-/-}$ mice did not appear to be protected from the development of tubular injury or progressive renal failure over the 5 wk of the study. We also treated wild-type mice with an exogenous inhibitor of factor B after the onset of proteinuria. This approach was undertaken to differentiate the role of the alternative pathway in the initial glomerular injury (characterized by albuminuria and minimal loss of renal function) and subsequent injury of the tubulointerstitium. Such an approach is also more clinically relevant, as the diagnosis of FSGS is typically made after the onset of proteinuria. In the mice treated with anti-factor B, we did see a delay in the rise in the SUN, but levels did not differ from the wild-type controls at the end of the study period. Tubulointerstitial injury was also not significantly improved by alternative

Fig. 6. Albuminuria, SUN, and alternative pathway activity in mice with adriamycin nephropathy that were treated with an inhibitory antibody to factor B. Starting 1 wk after induction of adriamycin nephropathy, mice received daily injections with monoclonal antibody 1379, an inhibitory antibody to factor B, or with an isotype-matched control antibody. A: albuminuria was not significantly different between the two groups at the end of the study. B: treatment with 1379 did delay the rise in SUN after injection with adriamycin, and values were significantly lower after 3 wk, but the difference was not significant at the end of the study. C: measurement of alternative pathway activity in the serum of mice that received 1379 demonstrated that daily injections of the antibody were unable to suppress activity in diseased mice compared with control-treated animals. *$P < 0.05.$
pathway inhibition. These findings do suggest that there is selective activation of the alternative pathway in the glomeruli and tubulointerstitium of mice with adriamycin nephrosis. Alternative pathway activation contributes to the initiation of injury in this model (as evidenced by decreased albuminuria after 1 wk) and may contribute to the progression of renal failure.

Since the original submission of this paper, another study examining the role complement in this model has been published (38). Very similar to our findings, these investigators also found that the development of albuminuria was delayed in complement-deficient mice. This study used factor D-deficient mice (fD−/−) to specifically test the role of the alternative pathway in this disease model. As we found with fB−/− mice, glomerular C3 deposition was not seen in fD−/− mice, indicating that glomerular complement activation in this model requires the alternative pathway. In contrast to our results, however, the fD−/− mice developed significantly milder disease. To our knowledge, the deficiency of factor B or factor D should both specifically block the alternative pathway. The more impressive protection seen in fD−/− mice compared with fB−/− mice is, therefore, likely due to differences in the experimental methods or subtle strain differences and is probably not due to differences in the complement activity of these animals.

There are several potential reasons why the protection was so modest in our study. The death of mice that did not have significant renal failure suggests that the adriamycin caused nonrenal toxicity to the mice. Similarly, the adriamycin may be directly toxic to the kidneys. Another possibility is that the variable amounts of filtered complement components limited the complement-dependent injury to just a subset of the mice. In patients with proteinuria, the type of underlying renal disease and the degree of proteinuria correlate with the levels of complement activation fragments found in the urine (17), probably reflecting the selectivity of the proteinuria. Activation of the alternative pathway depends on the presence of C3, factor B, factor D, and properdin (39). We examined levels of C3 in the urine, since it is the largest of these proteins, and levels in the urine of proteinuric mice did appear variable. We also did not achieve continual inhibition of the alternative pathway in the inhibitor-treated group. We suspect that loss of antibody in the urine accounted for the unsatisfactory inhibition, despite daily injections, and may highlight a potential obstacle to the use of MAbs in patients with proteinuric disease. Complement activation through the classical or lectin pathways could also account for incomplete protection in this study, although the paucity of C3 in the fB−/− kidneys by immunofluorescence makes this unlikely.

It has long been known that the complement system plays a crucial role in immune-complex-mediated glomerular injury, but several studies in rodents have also recently demonstrated that complement inhibition effectively prevents progressive tubulointerstitial injury in proteinuric renal disease (18, 19, 22). There is a relatively low expression of complement inhibitory proteins on the apical surface of TECs (36). Therefore, in proteinuric states, the complement proteins gain access to this vulnerable surface and may cause injury to the epithelial cells (18, 25). In vitro work has demonstrated that spontaneous activation on the apical surface occurs through the alternative pathway (3, 23), underscoring the potential for alternative pathway-mediated injury to surfaces with inadequate expression of complement inhibitors (33). Our results support the theory that this tubulointerstitial complement activation occurs through the alternative pathway and also demonstrate that the alternative pathway is activated within scarred glomeruli.

Our results further demonstrate that selective inhibition of the alternative pathway may delay the full development of proteinuria and delay the progression of renal failure, although the degree of protection was modest and temporary. Studies in other proteinuric models may help clarify whether this was because of the variability in this particular model or whether it reflects a limited role of the alternative pathway in tubulointerstitial injury. Given the weight of evidence supporting the importance of the complement system in tubulointerstitial injury, as well as the absence of C3 deposition in alternative pathway-deficient animals, however, we believe that alternative pathway inhibition can modulate the progression of proteinuric renal disease. Nevertheless, our results do suggest limitations to this approach and that such a therapy may require selection of patients based on evidence of alternative pathway activation in the urine or in the kidney biopsy.

In conclusion, we have found that the alternative pathway of complement is activated in the glomeruli and tubulointerstitium of mice after the induction of adriamycin nephrosis. Mice that lacked factor B demonstrated less initial proteinuria, and...
inhibition of the alternative pathway after the onset of proteinuria delayed the development of renal failure. Neither congenital deficiency of factor B nor treatment with an inhibitory MAb to factor B significantly attenuated the development of glomerular or tubulointerstitial scarring, however, perhaps because of the variable filtration of C3 into the urine in this model.

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DISCLOSURES

Three of the authors (J. M. Thurman, V. M. Holers, G. S. Gilkeson) hold stock in Taligen Therapeutics, which has licensed use of the anti-factor B antibody used in this paper. Our work was undertaken independently and was not submitted for their approval.

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