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Lack of a Functional Alternative Complement Pathway Ameliorates Ischemic Acute Renal Failure in Mice

Joshua M. Thurman,* Danica Ljubanovic,‡ Charles L. Edelstein,* Gary S. Gilkeson,§ and V. Michael Holers²†

Ischemia/reperfusion (I/R) injury in the kidney is a common cause of acute renal failure (ARF) and is associated with high morbidity and mortality in the intensive care unit. The mechanisms underlying I/R injury are complex. Studies have shown that complement activation contributes to the pathogenesis of I/R injury in the kidney, but the exact mechanisms of complement activation have not been defined. We hypothesized that complement activation in this setting occurs via the alternative pathway and that mice deficient in complement factor B, an essential component of the alternative pathway, would be protected from ischemic ARF. Wild-type mice suffered from a decline in renal function and had significant tubular injury, particularly in the outer medulla, after I/R. We found that factor B-deficient mice (fB−/−) developed substantially less functional and morphologic renal injury after I/R. Furthermore, control wild-type mice had an increase in tubulointerstitial complement C3 deposition and neutrophil infiltration in the outer medulla after I/R, whereas fB−/− mice demonstrated virtually no C3 deposition or neutrophil infiltration. Our results demonstrate that complement activation in the kidney after I/R occurs exclusively via the alternative pathway, and that selective inhibition of this pathway provides protection to the kidneys from ischemic ARF. The Journal of Immunology, 2003, 170: 1517–1523.

Studies of renal I/R injury have also demonstrated that mice deficient in complement were protected from injury (10, 11). Deposition of C3 occurred primarily at the tubular basement membrane, and the areas of deposition corresponded with morphologic injury (11). Mice that were deficient in C4 did not derive the same protection as those deficient in C3, suggesting that complement activation during I/R occurs via the alternative pathway (11). A study by Park et al. (12) demonstrated that mice subjected to I/R of the kidneys did not have IgG or IgM deposition. They also showed that in recombination-activation gene-1 deficient (RAG-1−/−) mice (which do not develop mature B and T cells or have serum Ig) that were subjected to I/R, complement deposition was still seen in the kidneys. Thus, in contrast to intestinal I/R injury, complement activation in renal I/R injury does not appear to be due to natural Ab. However, complement activation in the kidney does correlate with morphologic injury, and both complement C3 deficiency and membrane attack complex (MAC) blockade appear to confer protection from renal I/R injury (11).

The alternative pathway of complement is a potent line of defense against invading organisms (13). The alternative pathway is usually initiated by bacteria, parasites, viruses, or fungi, although IgA Abs and certain Ig L chains have also been reported to activate this pathway (14). Factor B is an essential protein in the alternative pathway. Factor B also helps solubilize immune complexes, has been reported to act as a B cell growth factor, and can activate monocytes (15–17). Bb, one of its split products, may induce apoptosis (18). Factor B is predominantly produced by hepatocytes, but it can be produced by other cells such as epithelial and endothelial cells, and its production can be up-regulated by cytokines such as IFN-γ (19). Although only one human patient with congenital deficiency of factor B has been reported (20), studies of factor B knockout mice (fB−/−) have not yet demonstrated an immune-modulating effect for this factor (21). The lymphoid organs, IgG1 Ab response to T-dependent Ags, and sensitivity to endotoxic shock appeared normal in fB−/− mice. In contrast, patients with congenital deficiencies of classical pathway components appear to
have an increased risk of infection (most commonly Staphylococcus and Streptococcus) and of systemic lupus erythematosus (22). Thus, inhibition of the alternative pathway may be better tolerated than classical pathway complement inhibition. The only study to date that has specifically examined the role of the alternative pathway in renal disease showed that in the MRL/lpr model of lupus nephritis, B6−/− mice developed milder renal disease than wild-type B F +/+ controls (23).

Based on the apparent lack of protection seen in mice deficient in classical pathway components, we hypothesized that complement activation in renal I/R injury occurs primarily via the alternative pathway (11). If this were true, blockade of this pathway would protect mice from ischemic ARF (i.e., ATN). To evaluate the role of the alternative pathway in renal I/R injury, we induced ARF in B F −/− mice, which exhibit no alternative pathway activity. By comparing I/R injury in B F −/− mice to wild-type controls, we were able to specifically demonstrate the key contribution of the alternative pathway in ischemic ARF.

Materials and Methods

Experimental animals

As previously described, mice deficient in factor B were intercrossed at F1 following an initial cross to the C57BL/6 strain (21). Because the factor B-deficient mice were created with Sv129 strain embryonic stem cells and were then crossed with C57BL/6 mice before expansion of the colony at F1, B6129F1, hybrid mice (Taconic Farms, Germantown, NY) were used as wild-type controls for this experiment. Only male mice were used in this study because they demonstrate a higher level of complement activity and are more vulnerable to complement-mediated renal injury (10). Nine B F −/− mice aged 10–12 wk and nine B6129F1 control mice aged 10–12 wk were subjected to I/R as described below. Sham surgery was performed on two B6129F1, mice. Serum was also obtained from three unmanipulated B6129F1, and three unmanipulated B F +/+ mice. The mice were housed and maintained in the University of Colorado Center for Laboratory Animal Care (Denver, CO) in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Protocol for induction of ischemic ARF

Mice weighing 20–25 g were anesthetized with 300 μl of 2,2,2-trifluoroethanol (Sigma-Aldrich, St. Louis, MO) injected i.p. and placed on a heating pad to maintain their body temperature during surgery. Laparotomies were then performed, and the renal pedicles were located and isolated by blunt dissection. The pedicles were clamped with surgical clips (Miltex, Bethpage, NY), and occlusion of blood flow was confirmed by visual inspection of the kidneys. The clamps were left in place for 22 min and then released. The time of ischemia was chosen to obtain a reversible model of ischemic ARF with a minimum of vascular thrombosis, and to avoid animal mortality. The kidneys were observed for ~1 min to ensure blood reflow, then fascia and skin were sutured with 4-0 silk (U.S. Surgical, Norwalk, CT). Sham surgery was performed in an identical fashion, except that the renal pedicles were not clamped. The mice were volume resuscitated with 0.5 ml of normal saline and kept overnight in an incubator at 29°C to maintain body temperature. After 24 h the mice were anesthetized, and blood was obtained by cardiac puncture. Laparotomy was performed and the kidneys were harvested.

Serum urea nitrogen (SUN) measurements

SUN was determined for each mouse using a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA).

Renal morphology

After the kidneys were removed from the mice, sagittal sections were fixed in 4% paraformaldehyde. After being embedded in paraffin, 4-μm sections were cut and stained with H&E. The sections were evaluated by a renal pathologist (D.L.) in a blinded fashion. The cortex and outer stripe of the outer medulla were assessed for epithelial necrosis, loss of brush border, tubular dilatation, and cast formation. At least 10 fields (×400) were reviewed for each slide, and the percentage of tubules displaying these findings was calculated. The kidney sections were scored as follows based on the percentage of affected tubules: 0, none; 1, <10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; 5, >75%.

A renal pathologist (D.L.) quantitatively assessed the neutrophil infiltration by counting the number of neutrophils per high powered field (×400). To 10 fields were counted in the outer stripe of the outer medulla on H&E-stained slides, and the number of neutrophils were averaged for each slide.

Immunofluorescence and immunohistochemistry

For immunofluorescence, sagittal sections of the kidneys were snap-frozen in OCT 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. Slides were stained with FITC-conjugated anti-mouse C3 (Cappel Laboratories, Durham, NC) diluted 1/50 with PBS or with rabbit anti-mouse C9 (generously provided to us by Dr. S. Tomlinson, Medical University of South Carolina, Charleston, SC) diluted 1/500. Sections stained with the anti-C9 Ab were then stained with FITC-conjugated F(ab)′2 goat anti-rabbit IgG (Cappel Laboratories) diluted 1/100. The slides were counterstained with DAPI (Vector Laboratories, Burlingame, CA). C3 deposition along the tubular basement membrane was scored according to the system used by Park et al. (10). Ten high power fields in the cortex and in the outer stripe of the outer medulla were assessed and scored as follows: 0, none; 1, <3 tubules with <30% of the circumference stained; 2, ≥3 tubules stained, of which at least one had ≥50% of the circumference stained in a continuous pattern; 3, >60% of the tubules stained, of which the majority had ≥75% of the circumference stained in a continuous pattern; 4, >90% of the tubules stained with the majority having >90% of the circumference stained. Because mice are known to have basal complement deposition within the kidneys, sections from unmanipulated B6129F1 mice were examined for comparison.

Statistical analyses

Multiple group comparisons were performed using ANOVA with posttest according to Newman-Keuls. Comparison between the control and B F −/− groups was performed with unpaired Student t testing. A value of p < 0.05 was considered statistically significant. Results are reported as mean ± SE.

Results

The role of the alternative complement pathway in ischemic ARF

To assess the role of the alternative complement pathway in ischemic ARF, SUN was measured in serum obtained from B F −/− mice and from B6129F1 controls 24 h after release of the renal pedicle clamps. The SUN following I/R was significantly lower in B F −/− mice when compared with B6129F1 controls (Fig. 1). In the B F −/− mice the SUN was 55 ± 7 mg/dl (n = 9) compared with 94 ± 11 for the control group (n = 9, p < 0.01). This result indicates that the alternative pathway contributes to the loss of renal function seen after I/R.

FIGURE 1. SUN after induction of I/R is lower in B F −/− mice. In mice subjected to I/R, both kidneys were subjected to 22 min of ischemia followed by 24 h of reperfusion. The results are shown as the mean ± SEM. and the number of animals in each group is described below each column. * p < 0.01 vs B6129F1, after sham surgery; ** p < 0.01 vs B6129F1, after I/R; *** p < 0.05 vs unmanipulated B F −/−.
Of note, the SUN in the $\beta B^{-/-}$ mice that underwent I/R was significantly greater than unmanipulated $\beta B^{-/-}$ mice (19 ± 1, $n = 9, p < 0.05$). The higher SUNs in the $\beta B^{-/-}$ mice subjected to I/R suggests that alternative pathway complement activation is not solely responsible for the loss of renal function after I/R injury.

Histologic analysis
Histologic injury was less severe in the $\beta B^{-/-}$ mice compared with wild-type controls. Kidneys from wild-type mice subjected to I/R showed a greater degree of damage, particularly in the outer medulla where tubules showed epithelial cell necrosis and cast formation. (Fig. 2). The mean injury score in the outer medulla of B6129F1, control mice was 4.0 ± 0.4 vs 1.7 ± 0.7 for the $\beta B^{-/-}$ mice ($p < 0.01$) (Fig. 3). Injury to the cortical tubules was mild (as is usually the case in ischemic ARF), and it was not significantly different between the two groups (1.1 ± 0.2 for the wild-type mice vs 0.7 ± 0.3 for the $\beta B^{-/-}$ mice, $p = NS$).

The interstitium in each group were also evaluated for neutrophil infiltration. The number of neutrophils following I/R was strikingly less in the $\beta B^{-/-}$ group compared with the B6129F1 group (0.8 ± 0.6 vs 22.6 ± 7.3, $p < 0.01$) (Fig. 3). For example, the one $\beta B^{-/-}$ mouse that had a histological injury score of 3 (26–45% of the tubules showing injury) had <1 neutrophil per high powered field, whereas the only B6129F1, mouse with an equivalent injury score had 14.1 PMNs per high powered field. The scarcity of neutrophils may be an important mechanism by which complement deficiency protects from renal failure, as infiltration of the kidney by activated neutrophils is probably an exacerbating factor in ARF (24).

C3 and C9 deposition
Immunofluorescent microscopy was used to compare C3 deposition in unmanipulated B6129F1 controls, and in $\beta B^{-/-}$ and control B6129F1 mice after I/R injury. In agreement with previous reports, we found that unmanipulated mice have C3 deposition along the
number of neutrophils per high powered fields that the staining shown in Fig. 5 subjected to I/R, even in areas of cast formation. The latter point compared with B6129F1 mice (1.7 vs 4.0 × 0.4; *p < 0.01). The number of neutrophils per high powered field in the interstitium of each kidney was also counted. In the B6/F1 mice, significantly fewer neutrophils were seen than in the B6129F1 mice (0.8 ± 0.6 vs 22.6 ± 7.3; **p < 0.01).

FIGURE 3. Tubular injury and neutrophil infiltration after I/R are less severe in B+/− mice compared with B6129F1 controls. Renal pathology was graded on a scale from zero to five based upon the percentage of tubules demonstrating epithelial necrosis, loss of brush border, dilatation, and cast formation. Significantly less damage was seen in the B+/− mice compared with B6129F1 mice (1.7 ± 0.7 vs 4.0 ± 0.4; *p < 0.01). A small amount of C9 is present in the kidneys at baseline, probably a result of the basal complement activation described above. After I/R, the damaged tubules stain densely for C9, indicating that MAC formation is increased in the injured region of the kidney. No C9 was detected in the kidneys of B+/− mice subjected to I/R, even in areas of cast formation. The latter point confirms that the staining shown in Fig. 5B is specific for C9.

tubular basement membrane (Fig. 4). This has been attributed to complement activation by ammonia, which can modify C3 to form a C3 convertase and activate complement (25). C3 was present around the renal tubules indicating that there is a low level activation of the alternative pathway in ischemic ARF. At baseline there is C3 deposition around the renal tubules indicating that there is a low level activation in the interstitium despite these inhibitors (25). In rats, treatment with an Ab to Crry led to renal injury marked by tubulointerstitial damage and cast formation, demonstrating the necessity of complement inhibition to prevent spontaneous tubular injury (27). Therefore, complement dysregulation during I/R (due to a decline in the synthesis of Crry by the tubular epithelium, for example) could contribute to the pathologic findings seen in ATN. I/R leads to a heterogeneous pattern of injury, and it is possible that some of the increase in C3 seen after I/R is the result of an increase in ammonia generation by those nephrons still functioning to maintain acid base balance. An increase in ammoniagenesis by remnant nephrons has been proposed as a mechanism of progressive interstitial fibrosis in chronic renal disease (28). Given the key role of the alternative pathway in mediating renal I/R injury, determining the mechanism(s) of activation is an important undertaking.

Complement activation by the alternative pathway could contribute to ischemic ARF by numerous mechanisms. For example, the anaphylatoxins, C3α and C5α, can cause endothelial activation and increased expression of adhesion molecules leading to leukocyte chemotaxis (29). These factors may also lead to smooth muscle cell and vascular constriction, decreasing renal blood flow. The MAC can directly cause cellular damage, and mice that are deficient in MAC components (C5 and C6) have also been shown to be protected from renal I/R injury (11). Complement activation can also induce endothelial and epithelial cells to elaborate inflammatory cytokines such as IL-1 (29). The relation of complement activation to other known or postulated mediators of renal I/R injury, such as IL-18 production and caspase activation (30), is unknown.

Discussion

Ischemic ARF remains a serious cause of morbidity and mortality. Previous studies have implicated complement activation in the pathogenesis of I/R injury in the kidney (10, 11). These studies indirectly suggested that complement activation occurs via the alternative pathway by showing that C4−/− mice did not derive the same protection as C3−/− mice. We set out to directly examine the role of the alternative pathway in I/R injury of the kidney by inducing ischemic ARF in mice deficient in factor B and comparing them to wild-type controls subjected to the same injury.

We have shown that mice deficient in the alternative complement pathway are substantially protected from the functional and morphologic signs of ischemic ARF. I/R injury leads to increased deposition of C3 along the tubular basement membrane as well as in the mesangium and along Bowman’s capsule. Mice deficient in factor B had no evidence of C3 deposition within any section of the kidney after I/R indicating that the alternative complement pathway exclusively accounts for the detectable complement activation that occurs after I/R. The B+/− mice also had virtually no interstitial neutrophil infiltration, whereas this is a prominent finding in the wild-type B6129F1 mice with ischemic ARF.

How the alternative pathway becomes activated in ischemic ARF is not known. Activation could occur either by the loss of inhibition, by the generation of a complement activator that overwhelms the endogenous complement inhibitors, or by the local generation of complement components. Complement activation is ordinarily tightly regulated. Crry is an endogenous murine inhibitor of C3 convertase, analogous to human membrane cofactor protein and decay-accelerating factor. Crry is heavily expressed within the glomerular mesangium of mice, as well as at the basolateral surface of the renal tubules (26), both of which are areas of C3 deposition in ischemic ARF. At baseline there is C3 deposition around the renal tubules indicating that there is a low level activation in the interstitium despite these inhibitors (25). In rats, treatment with an Ab to Crry led to renal injury marked by tubulointerstitial damage and cast formation, demonstrating the necessity of complement inhibition to prevent spontaneous tubular injury (27). Therefore, complement dysregulation during I/R (due to a decline in the synthesis of Crry by the tubular epithelium, for example) could contribute to the pathologic findings seen in ATN. I/R leads to a heterogeneous pattern of injury, and it is possible that some of the increase in C3 seen after I/R is the result of an increase in ammonia generation by those nephrons still functioning to maintain acid base balance. An increase in ammoniagenesis by remnant nephrons has been proposed as a mechanism of progressive interstitial fibrosis in chronic renal disease (28). Given the key role of the alternative pathway in mediating renal I/R injury, determining the mechanism(s) of activation is an important undertaking.
However, based on our results it is possible that these pathways are interlinked.

The marked paucity of neutrophils seen in the interstitium of the fB−/− mice may also be an important mechanism whereby these mice are protected from ARF. Although many other inflammatory pathways can lead to neutrophil priming and chemotaxis, virtually no neutrophils were seen in the fB−/− group, demonstrating that these other pathways are not playing a significant role in neutrophil recruitment in this model. Complement activation has been shown to increase expression of the adhesion molecules P-selectin, E-selectin, and ICAM-1 (31, 32). By increasing expression of these adhesion molecules, complement activation may initiate neutrophil infiltration.

Neutrophils can contribute to the decline in renal function by producing cytokines and oxidants, and they may obstruct the microvasculature, impeding blood flow to the kidney (4, 33). A study by Kelly et al. (34) demonstrated that mice deficient in neutrophils were protected from ischemic ARF, suggesting that neutrophils are an important mechanism of injury after I/R. This study also demonstrated that mice deficient in ICAM-1 had significantly fewer neutrophils 24 h after being subjected to I/R, and those mice were protected from ischemic ARF, suggesting that this adhesion molecule is integral to neutrophil infiltration and tissue injury after I/R.

The induction of adhesion molecules may be an important mechanism of complement induced injury after I/R, and future studies should look at the expression of adhesion molecules in wild-type and complement-deficient mice after I/R as a means of integrating these findings.

Because complete suppression of complement activation only partially protects fB−/− mice, or even C3−, C5−, or C6-deficient mice (11) from ARF, complement activation may occur downstream of or in parallel with other injurious events. Complement activation could explain many of the morphologic changes seen in ARF, but I/R injury is a complex process that likely triggers multiple deleterious pathways. Furthermore, ARF in the hospital setting is caused by a variety of different insults (drugs, infections,
surgies, etc.), and no single model will replicate all of these pathophysiologic processes. Investigators who have used a longer ischemia time also demonstrated significant protection when mice were complement-deficient (11). Whether inhibition of the alternative pathway is protective in other models of renal failure, such as endotoxin induced ARF, remains to be determined.

As we have shown, the alternative pathway accounts for all of the detectable C3 activation and MAC formation seen after I/R. Therefore, an inhibitor of the alternative pathway might be protective in patients who have sustained or who are at risk for ischemic ARF. In this setting, alternative pathway inhibition should offer renal protection that is equivalent to a more broadly active complement inhibitor, but would possibly have fewer side effects. One previous study examined a C3-convertase inhibitor in renal I/R injury. This inhibitor blocked both the classical and alternative pathways (10). Although C3-deficient mice were protected from I/R injury in that study, administration of the complement inhibitor did not protect the mice from renal I/R injury. However, mice in this study received heparin to prevent thrombotic complications of the renal artery clamping. Heparin itself can act as a complement inhibitor, and its use likely masked any benefit of treatment with the complement inhibitor (Crry-Ig). Another group of mice in this study was subjected to I/R without the use of heparin. This group had a slightly higher SUN than the group that received the inhibitor (157 ± 11.3 vs 127.7 ± 28.7 mg/dl), although this difference was not statistically significant. In contrast, heparin was not used in our study or the study by Zhou et al. (11), perhaps explaining why complement deficiency provided protection from ischemic ARF in these studies.

One obstacle to the use of an exogenous complement inhibitor is that it must reach the site of injury to be therapeutically useful. In the case of ischemic ARF, this means that the inhibitor must be able to reach therapeutic levels within the renal interstitium and at the tubular basement membrane. In the study by Park et al. (10), renal C3 staining negatively correlated with the SUN values in the animals that received the inhibitor. Therefore, it is possible that the large Crry-Ig molecule was only able to penetrate tissue and inhibit complement after significant injury had occurred, and at levels proportional to the degree of injury already sustained. Future studies using complement inhibitors may need to overcome technical aspects such as this to effectively prevent ischemic ARF.

In summary, our results demonstrate that factor B and the alternative complement pathway have an important role in the pathogenesis of functional and morphological injury to the kidney after I/R. Blockade of this pathway eliminates all of the detectable C3 deposition that occurs within the kidney as a result of I/R injury. Therefore, inhibition of this pathway may protect the kidneys from I/R injury and other forms of progressive renal disease in which the alternative pathway is activated.

Table 1.  C3 immunofluorescence staining intensitya

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Cortex</th>
<th>Outer Medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmanipulated B6129F1 (5)</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>B6129F1 after I/R (9)</td>
<td>2.1 ± 0.2</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>fB−/− after I/R (9)</td>
<td>0.0 ± 0.0</td>
<td>0 ± 0</td>
</tr>
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*a Tubular C3 deposition was graded on a scale from zero to four based upon the percentage of tubules with C3 along the basement membrane and the percentage of the circumference of individual tubules demonstrating C3. B6129F1 mice had significantly greater C3 in the outer medulla after I/R compared with unmanipulated B6129F1 mice (p < 0.001). No C3 was seen in any of the fB−/− mice following I/R (p < 0.001) compared with wild-type B6129F1 mice subjected to I/R. C3 in the cortex increased slightly after I/R, but this change was not significant compared with unmanipulated controls. n = number studied.

FIGURE 5.  Immunofluorescence staining for C9 in ischemic ARF demonstrates no deposition in fB−/− mice. A, A small amount of C9 can be seen outside of the tubules in the outer medulla of unmanipulated B6129F1 mice (×400). B, C9 deposition in the outer stripe of the outer medulla of a B6129F1 mouse with ischemic ARF (×200). C, C9 is present around damaged tubules and abundantly within the lumen of damaged tubules. C9 is not detected in fB−/− mice subjected to I/R (×200).
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References