# Acute tubular necrosis is characterized by activation of the alternative pathway of complement

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**Acute tubular necrosis is characterized by activation of the alternative pathway of complement.**

*Background.* Studies in animal models have shown that the alternative pathway of complement is activated in the kidney after ischemia/reperfusion. In addition, mice deficient in complement factor B, a necessary component of the alternative pathway, are protected from ischemic acute renal failure. The purpose of this study was to determine whether alternative pathway activation also occurs during the development of ischemic acute tubular necrosis in the human kidney.

*Methods.* Biopsies were identified from nine patients with morphologically normal kidneys and seven patients with evidence of acute tubular necrosis by light microscopy. Immunofluorescence microscopy was used to quantify and localize the complement activation products C3d and C4d. The results were correlated with available clinical data.

*Results.* Similar to mice, small amounts of activated C3d were present along the tubular basement membrane in normal kidneys. However, kidneys from patients with acute tubular necrosis had C3d complement deposition along a significantly greater number of tubules, and many of the tubules were completely circumscribed. In contrast, C4d was not detectable, indicating that complement activation occurred primarily via alternative pathway activation.

*Conclusion.* Complement activation occurs in human ischemic acute tubular necrosis. As in rodents, complement activation along the tubular basement membrane after ischemia appears to occur principally via the alternative complement pathway. Because of this, an inhibitor of the alternative pathway might limit complement activation and inflammation after ischemia/reperfusion, thereby protecting the kidney from ischemic acute renal failure.

Studies in mice have demonstrated that the alternative complement pathway is activated during renal ischemia/reperfusion (I/R), and that mice deficient in this pathway are protected from ischemic acute renal failure (ARF). Activation of the classic pathway of complement

occurs after the I/R of the intestine [1, 2], skeletal muscle [3, 4], and heart [5]. Rodent studies have shown, however, that complement activation after I/R of the kidney occurs almost exclusively via the alternative pathway [6, 7]. Ischemic acute tubular necrosis (ATN) is one of the most common causes of renal failure in humans [8], and alternative pathway inhibition may be an effective method of preventing renal inflammation and injury after I/R.

The pathogenic role of complement activation in glomerular disease has been deduced from reductions of plasma levels of complement components during disease activity, animal models, and glomerular deposits of complement components as seen by immunofluorescence [9]. Although the deposition of C3 and C4 in glomerular diseases is well established, little has been published describing the pattern of complement deposition in ATN. We felt that it is important to corroborate the findings from animal models by examining complement activation in the human kidney. Although the presence of complement deposits in diseased kidneys does not in and of itself demonstrate a pathogenic role for complement activation, it does provide evidence that complement activation has occurred and supports the relevance of animal models that show a similar pattern of deposition. Therefore, we retrospectively undertook an immunohistologic analysis of kidney tissue from patients with clinical and pathologic evidence of ATN.

## METHODS

### Patient selection

Renal biopsies evaluated at the University of Colorado Health Sciences Center from 2002 to 2003 were reviewed with the approval of the Colorado Multiple Institutional Review Board. Biopsies were fixed in 10% zinc-formalin and embedded in paraffin by vacuum infiltration. Biopsies were then serially sectioned at 4 μm intervals, and alternate sections were stained with hematoxylin and eosin, trichrome, periodic acid–Schiff, and silver (Jones' method). A second biopsy was frozen for immunofluorescence studies. Of those biopsies for which frozen
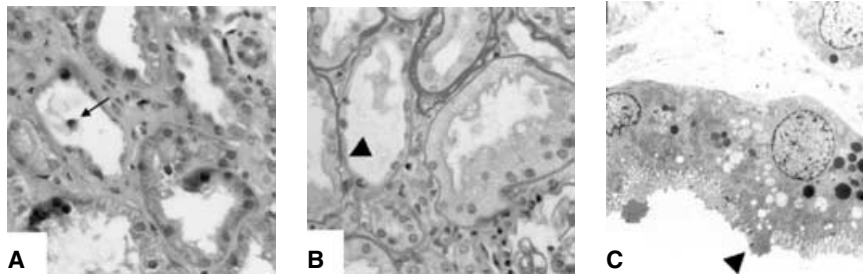
**Fig. 1. Histologic diagnosis of acute tubular necrosis.** Available biopsies were reviewed, and those with normal morphology or a pathologic diagnosis of ATN were identified. These images are from patient 8 in Table 1, demonstrating the changes characteristic of ATN. The ATN biopsies demonstrated epithelial sloughing (arrows), epithelial simplification (arrowhead), loss of brush border, tubular dilatation, and cast formation. All had acute rises in the serum creatinine before biopsy, and all had clinical data consistent with a diagnosis of ATN. Electron microscopy of renal tubules was available for some of the biopsies. (*A*) is stained with trichrome, and (*B*) is stained with periodic acid Schiff. (*C*) The epithelial cells were characterized by vacuolization, apical blebbing (arrowhead), reduced apical brush border, and basal infoldings (*C*). Original magnification was ×200 for (A) and (B).

**Table 1.** Patient characteristics

| Patient | Age | Diagnosis | *SCr* *mg/dL* | Serum C3 (82-180) | Serum C4 (14-39) | Proteinuria | Percentage of tubules demonstrating C3d (Grade C3d) | Percentage of tubules demonstrating C4d |
|---|---|---|---|---|---|---|---|---|
| 1 | 46 | Proteinuria, normal kidney morphology | 1.5 | "Normal" | "Normal" | 1.2 g/24 h | <10 (2+) | N/A |
| 2 | 49 | Proteinuria, normal kidney morphology | 0.8 | 178 | 17 | 1 g/24 h | 0 | N/A |
| 3 | 17 | Thin GBM disease | 0.7 | 94 | N/A | 0.5 g/24 h | 10 (2+) | 0 |
| 4 | 23 | Proteinuria, normal kidney morphology | 0.9 | 156 | 20 | 6.2 g/24 h | 0 | 0 |
| 5 | 30 | Proteinuria, normal kidney morphology | 0.7 | 67 | 33 | 3.4 g/24 h | 10 (2+) | 0 |
| 6 | 22 | Proteinuria, normal kidney morphology | 0.8 | 169 | N/A | 4+ | 20 (1+) | 0 |
| 7 | 20 | Acute tubular necrosis | 3.8 | N/A | N/A | 100 mg/dL | 15 (3+) | N/A |
| 8 | 80 | Acute tubular necrosis | 3.9 | "Normal" | N/A | Trace | 40 (3+) | 0 |
| 9 | 22 | Acute tubular necrosis; 10 year history of MCD, now with focal segmental glomerulosclerosis. | 1.1 | "Normal" | N/A | 700 mg/24 hr | 10 (2+) | 0 |
| 10 | 73 | Acute tubular necrosis; moderate nephrosclerosis | 6.7 | 116 | 28 | 4152 mg/24 hr | 70 (2+) | 0 |
| 11 | 45 | Acute tubular necrosis | 2.8 | N/A | N/A | 4+ | 20 (2+) | 0 |
| 12 | 49 | Acute tubular necrosis | 6.9 | 102 | 26 | Negative | 0 | 0 |
| 13 | 21 | Acute tubular necrosis | 2.9 | 158 | 47 | 2474 mg/24 hr | 30 (1+) | 0 |

N/A indicates that the data were not available. "Normal" indicates that the referring provider had stated that the value was normal, but the actual value was not available.

tissue was also available, seven biopsies with a pathologic diagnosis of ATN were available for further analysis. Biopsies were classified as having ATN if they demonstrated epithelial simplification, loss of brush border, apical blebbing, epithelial cell sloughing, tubular dilatation, and cast formation (Fig. 1). Nine biopsies with no abnormalities by light microscopy (minimal change disease or thin basement membrane disease) were chosen as controls. A biopsy from a patient with type 1 MPGN was used as a positive control for C3d and C4d staining as well as C3d and C8 staining.

**Clinical data**

Charts were reviewed to obtain clinical data for the patients whose biopsies were chosen for the study. Biopsies with histologic evidence of ATN were not included in the study if the clinical data conflicted with the diagnosis of ATN. Clinical data were incomplete for some of the biopsies that had been sent to UCHSC from other facilities. Available measurements of serum creatinine, complement levels, and proteinuria are reported for 13 of the 16 biopsies evaluated in this study (Table 1).
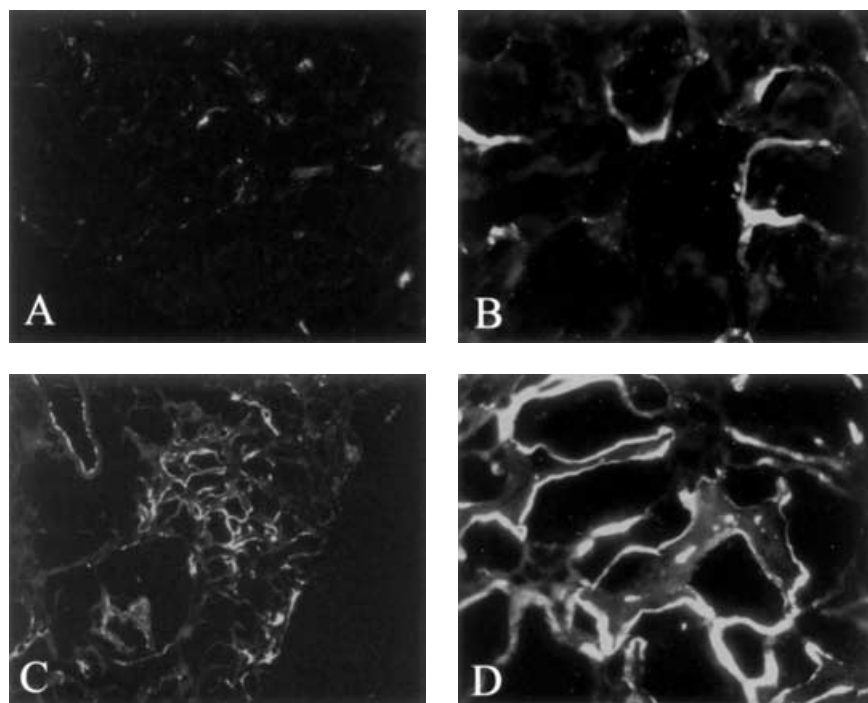
**Fig. 2. C3 deposition along tubules of normal kidneys and those with ATN.** Low power views (*A*) and high power views (*B*) of C3d in the tubulointerstitium of morphologically normal kidneys showed sparse, discrete patches of C3d deposition. Low (*C*) and high (*D*) power views of C3d deposition in kidneys with morphologic evidence of ATN demonstrated regions of heavy C3d deposition involving nearly all of the tubules, and individual tubules were completely circumscribed by C3d. (C) and (D) were from the same biopsy in Figure 1, panels B and D (patient 8 in Table 1). Original magnification was ×100 for (A) and (C), ×400 for (B) and (D).

## Immunofluorescence

Tissue sections were stained with antibodies to C3d (DakoCytomation, Carpinteria, CA, USA), C4d (Quidel Corporation, Santa Clara, CA, USA), and C8 (Quidel Corporation). The antibody to C8 (Quidel product #A249) binds to the membrane attack complex (MAC). Four μm sections were air dried and then fixed with acetone for 10 minutes. Nonspecific binding was blocked with 10% whole goat serum (Cappel, Aurora OH, USA) in phosphate-buffered saline (PBS). The sections were incubated overnight at 4°C with the primary antibodies diluted 1:150 in PBS. They were washed three times in PBS, and then incubated with fluorescein isothiocyanate (FITC) or Rhodamine-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA). The slides were imaged with a Nikon T-2000 inverted microscope (Nikon Instruments, Melville, NY, USA) and slidebook software (Intelligent Imaging Innovations, Denver, CO, USA).

Renal biopsy sections stained for C3d and C4d were assessed by a renal pathologist in a blinded fashion. The percentage of tubules with C3d or C4d deposition along the tubular basement membrane (TBM) was determined. The intensity of C3d deposition was also graded (0–3+).

## Statistical analysis

Data are expressed as mean ± SEM. The unpaired Student *t* test and Mann-Whitney test were used to compare values between the ATN and non-ATN groups. *P* values of less than 0.05 were considered statistically significant.

## RESULTS

### Human ATN is characterized by C3d deposition along the tubular basement membrane

Human ATN is characterized by epithelial injury and tubular dilatation (Fig. 1). Similar to what has been described in mice [6], morphologically normal kidneys have patches of C3d deposition along the TBM of approximately 10% of their tubules (Fig. 2). Kidney sections with morphologic evidence of ATN, in contrast, had complement deposition on a greater percentage of tubules ($26.4 \pm 9.0$ vs. $8.3 \pm 2.0$, $P < 0.05$ by unpaired Student *t* test) (Figs. 2 and 3). When analyzed as nonparametric data with the Mann-Whitney test, the *P* value did not quite reach significance ($P = 0.055$). Although on average the kidneys with ATN had more extensive C3d deposition, the degree of C3d deposition in approximately half of the kidneys was similar to that seen in morphologically normal kidneys.

Morphologic injury in ATN is patchy in nature [10]. C3d deposition in kidneys with ATN was also heavy in some regions, while other regions were relatively spared (Fig. 2). Injury by light microscopy, as well as C3d deposition by immunofluorescence, primarily involved the proximal tubules of affected kidneys. The intensity of C3d seen along the basement membrane trended toward being greater in those kidneys with morphologic evidence
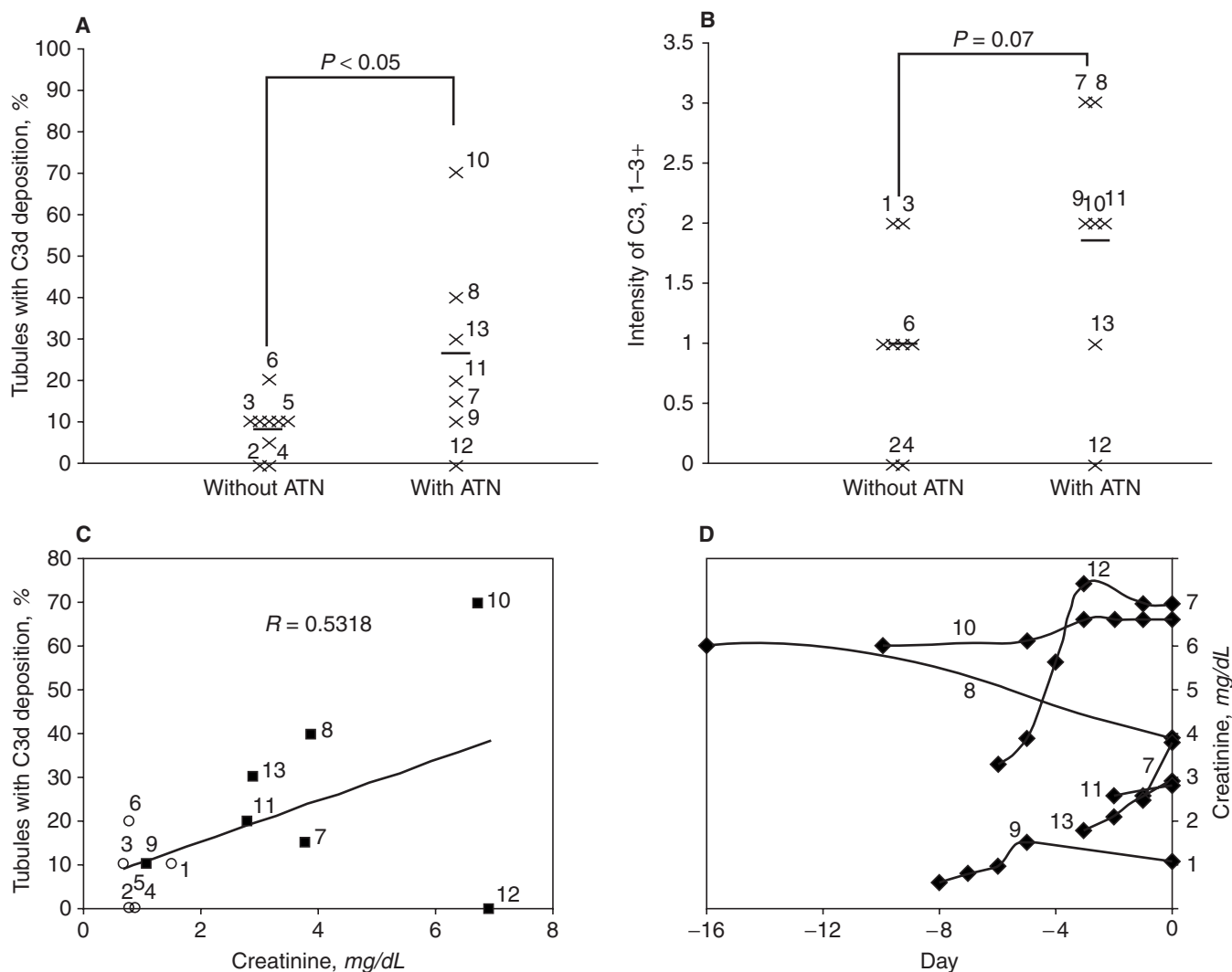
**Fig. 3. Kidneys with ATN demonstrated greater complement activation than morphologically normal kidneys.** A renal pathologist assessed the percentage of tubules in each biopsy with C3d deposition (*A*). Those in kidneys with morphologic evidence of ATN had C3 deposition along a significantly greater percentage of their tubules than those with normal kidneys by light microscopy. The number next to each data point indicates which patient in Table 1 it represents. As seen in Figure 2, normal kidneys demonstrated some C3d along the tubular basement membrane. The intensity of C3d deposition was also graded (*B*), and those kidneys with morphologic evidence of ATN trended towards a greater intensity of C3d deposition on affected tubules. The extent of C3d deposition is plotted versus the serum creatinine for patients without ATN (○) and with ATN (■) (*C*). For patients with ATN, the available serum creatinine values before the biopsy are plotted (*D*). The number next to each line indicates which patient in Table 1 it represents.

of ATN compared to those with normal morphology ($1.9 \pm 0.4$ vs. $1.0 \pm 0.2$, $P = 0.07$ by unpaired Student *t* test; when analyzed by the Mann-Whitney test, $P = 0.091$). The extent of C3d deposition in patients also appeared to correlate with the serum creatinine level (Fig. 3C and Table 1).

Of the biopsies with ATN, several had evidence of other pathologic processes. The biopsy with the most extensive deposition of C3d also had evidence of nephrosclerosis (Table 1). It has been proposed that a loss of renal mass can cause complement activation within the remaining kidney by increased ammoniagenesis in the remnant nephrons [11], and it is possible that this process

contributed to the degree of C3 deposition seen in this kidney. When the two kidneys with nephrosclerosis or glomerulosclerosis were excluded from the analysis, the percentage of tubules with C3d deposition was no longer significantly different between the ATN and non-ATN groups ($20 \pm 7.1$ vs. $8.3 \pm 2.0$, $P = 0.07$ by unpaired Student *t* test; when analyzed by the Mann-Whitney test, $P = 0.19$).

Serum C3 and C4 levels were only reported for seven of the non-ATN kidneys and five of the ATN kidneys (Table 1). C3 levels were normal in all of the patients with biopsy-proven ATN for whom this information was available.

To determine whether complement activation resulted in formation of the MAC, sections from four kidneys were also stained with an antibody to C8 (Fig. 4). MAC was deposited in a pattern similar to that of C3d. This demonstrates that complement activation with the tubulointerstitium results in formation of the MAC, as has been seen in animal studies [12].

## Activation of complement in ATN occurs primarily via the alternative pathway

Dual staining of human kidney sections for C3d and for C4d demonstrated that basal complement activation, as well as that which occurred in ATN, was the result of alternative pathway activation. Classic pathway activation, such as that which occurs in the glomerulus as a result of immune complex deposition, results in the cleavage of C4 and C3 generating C4d and C3d, respectively. C4d and C3d are therefore both generated and colocalize (Fig. 5). Alternative pathway activation occurs without the generation of C4d. C3d deposition along the TBM in kidneys with ATN did not colocalize with C4d (Fig. 5), demonstrating that cleavage of C3 has primarily occurred by alternative pathway activation. The low levels of complement activation in normal kidneys also occurred via the alternative pathway, as evidenced by the absence of C4d.

## DISCUSSION

Studies in animal models have shown abundant deposition of complement activation products in kidneys with ischemic ATN [12, 13], and there is evidence that complement inhibition can protect the kidneys from I/R-induced injury [6, 12–14]. In this study, we demonstrated that morphologically normal human kidneys had intermittent deposition of C3d along the tubular basement membrane, indicating that there is a low level of complement activation at baseline. In kidneys with ATN, however, the kidneys were characterized by more extensive activation of complement. Deposition of C3d in these kidneys was patchy, like the morphologic changes seen by light microscopy. The kidneys with ATN had more extensive C3d deposition on average, although deposition in some of the kidneys with ATN was similar to that of normal kidneys.

It is believed that ammonia, which can react with C3 to form an alternative pathway C3 convertase, activates complement within the tubulointerstitium of the kidney [11]. Indeed, the absence of C3 deposition in the kidneys of mice lacking factor B of the alternative pathway [6] demonstrates that basal complement activation in mice appears to occur via the alternative pathway. Given the absence of C4d in morphologically normal human kidneys, basal complement activation in humans may also be due to alternative pathway activation. The absence of C4d in the regions of intense C3d deposition within kidneys

with ATN suggests that the increased complement activation in the tubulointerstitium of these kidneys also results from alternative pathway activation. In contrast, humoral rejection of renal allografts is characterized by deposition of C4d in the peritubular capillaries [15], which reflects the key role of antibodies and classic pathway activation in this setting.

Because patients with suspected ATN do not routinely undergo renal biopsy, the number of biopsies available is necessarily limited. Furthermore, some of the patients we identified as having morphologic evidence of ATN also had other underlying renal abnormalities (Table 1). Unfortunately, we could not identify enough potential biopsies to restrict our examination to those kidneys without clinical or histologic evidence of other pathologic processes. A study of biopsies rigorously limited to those without clinical or histologic evidence of any other process would be difficult to conduct. Furthermore, given the heterogeneous patient population with the clinical diagnosis of ATN, such strict inclusion criteria for biopsy analysis would not necessarily be an accurate representation of patients considered to have ATN.

We have analyzed the data by both parametric and nonparametric methods because little has been reported regarding the nature of tubulointerstitial complement deposition in large populations. While statistically significant using a Student $t$ test, the percentage of affected tubules was not statistically significant when evaluated as nonparametric data using the Mann-Whitney test, although there was still a trend toward greater C3d deposition in the ATN group ($P = 0.055$). Furthermore, several of the kidneys with ATN had C3d deposition that resembled that of normal kidneys. ATN is by nature a patchy disease, and the biopsies were likely performed at various stages in the course of the disease (Fig. 3). A larger sample size would likely compensate for these variables. However, when considered together, the greater activation seen in biopsies with ATN, predominant activation via the alternative pathway, and the pattern of C3d deposition along the tubular basement membrane demonstrate that alternative complement pathway activation in ATN may parallel that seen in rodents subjected to renal I/R.

The presence of complement activation products in kidneys with ATN does not establish a causal role for complement activation in the pathogenesis of this disease. Such conclusions can only be extrapolated from animal studies or verified with a clinical trial of a complement inhibitor. The similar mechanisms of complement activation after ischemia, as well as the similar pattern of C3d deposition along injured tubules, however, do support the relevance of the animal studies to human disease. Taken together, therefore, the numerous animal studies demonstrating a pathogenic role for complement activation in ATN, as well as the presence of complement
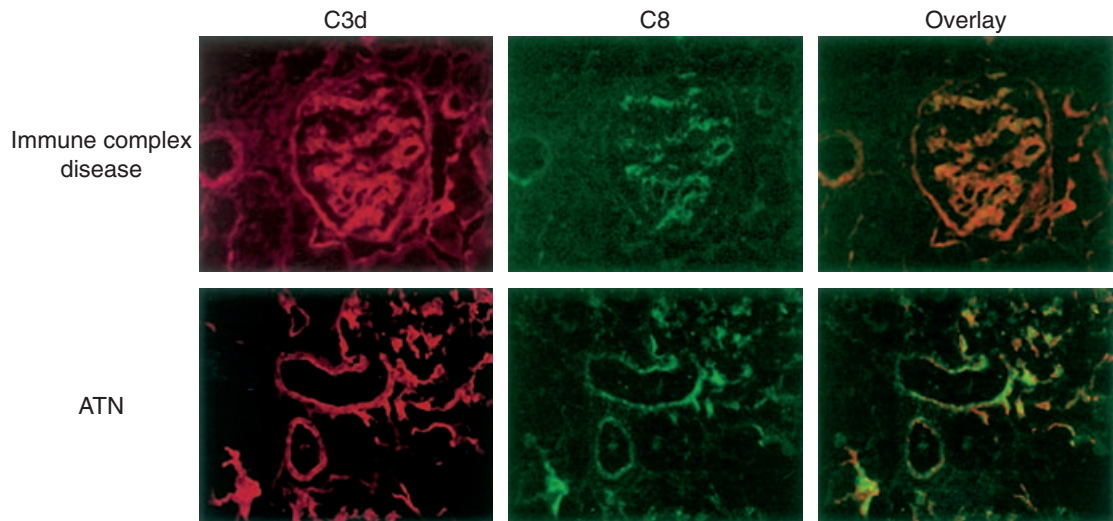
**Fig. 4. Complement activation in kidneys with ATN results in formation of the membrane attack complex.** Sections the kidneys were stained for C3d (red) and C8 (green). A kidney section from the patient with immune complex disease (type I MPGN) was also stained. C8 is evident within the glomerulus of the kidney with immune complex disease in a pattern similar to that of C3d (colocalization appears yellow). In kidneys with ATN (patient 10 from Table 1 shown), C8 was also seen along the tubules in a pattern similar to that of the C3d. Original magnification was ×200.
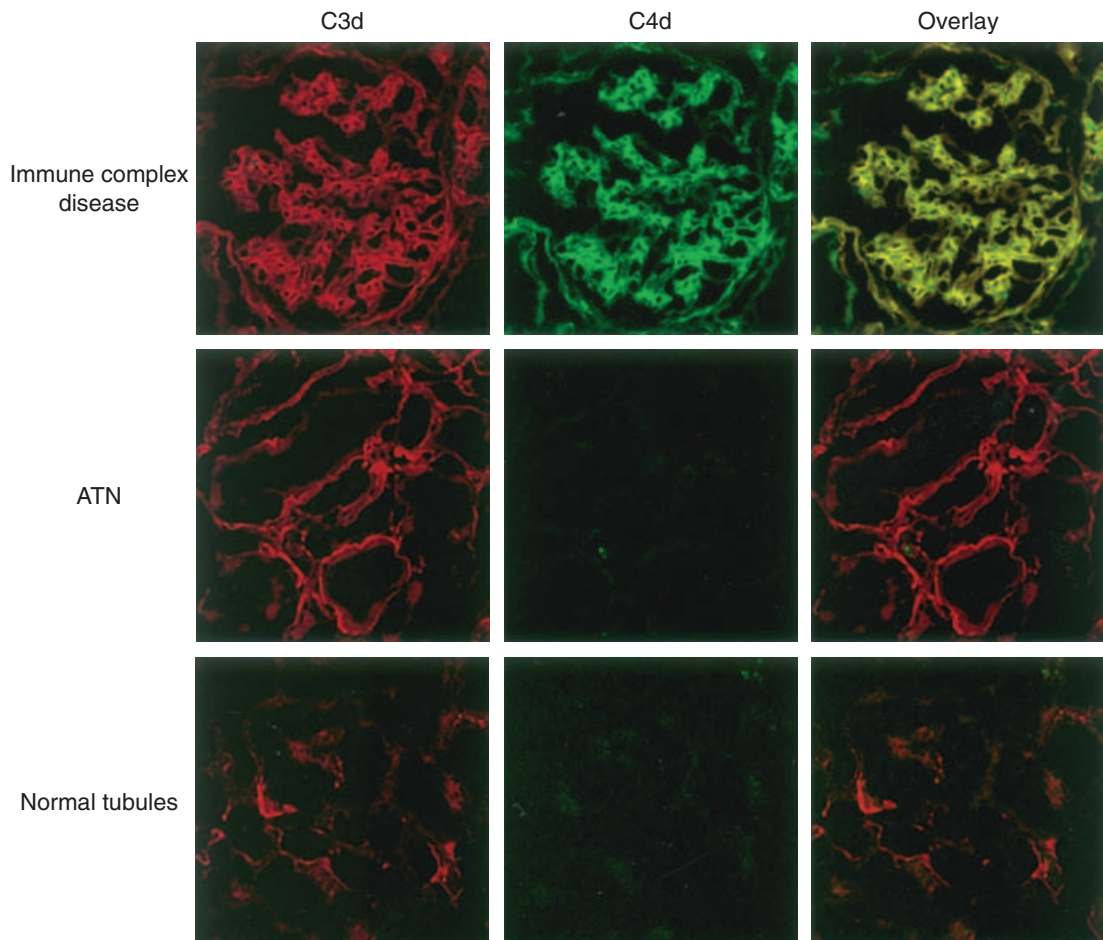


**Fig. 5. Complement activation in kidneys with ATN occurs via the alternative complement pathway.** Sections the kidneys were stained for C3d (red) and C4d (green). A kidney section from a patient with immune complex disease (type I MPGN) was used as a positive control since immune complexes activate the classic complement pathway and cause deposition of both C3d and C4d. In the control section, C3d and C4d colocalized, appearing yellow when overlaid. C3d was present abundantly along the tubules of kidneys with ATN (patient 8 from Table 1 shown), and sparsely in morphologically normal kidneys (patient 6 from Table 1 shown). No C4d was present in any of the kidneys, however, demonstrating that complement activation in normal kidneys and those with ATN occurs by alternative pathway activation. Original magnification was ×200.

activation products in human kidneys with ATN, supports the concept that complement activation contributes to tissue injury in the human disease. Complement inhibitors have shown promise as therapeutics for cardiac ischemia [16], and recently for paroxysmal nocturnal hemoglobinuria [17]. We have recently developed a novel inhibitory monoclonal antibody (mAb) to mouse factor B that selectively inhibits the alternative pathway of complement [18]. This mAb also inhibits in vitro activation of the alternative pathway in human serum and may, therefore, provide a therapeutic means of inhibiting the alternative pathway in humans at risk of developing ischemic ARF.

## CONCLUSION

We have shown that ATN in humans is characterized by activation of the alternative pathway of complement. The pattern of activation is similar to that seen in animal models, in which abrogation of complement activation has ameliorated ischemic ARF. Decades of research have convinced many nephrologists that complement inhibition would benefit patients with glomerular disease [19, 20]. The similarities between human ATN and that seen in animal models also support serious consideration of the use of complement inhibitors in patients at risk of ATN.

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