Acute nephrotoxic serum nephritis in complement knockout mice: relative roles of the classical and alternate pathways in neutrophil recruitment and proteinuria

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Abstract

Background. The importance of complement in the pathophysiology of renal disease is still being appreciated. To further address the role of this mediator system, we evaluated the influence of absolute deficiency of C3 and C4 on acute nephrotoxic serum nephritis (NSN).

Methods. Selective ‘knockout’ of C3 and C4 was routinely confirmed in null mice by ELISA. NSN was induced by intravenous injection of a sheep anti-rat nephrotoxic serum that cross-reacts with murine glomerular antigens. Deposition of heterologous immunoglobulin in wild-type glomeruli was associated with rapid complement deposition and neutrophil infiltration, and followed by the development of proteinuria.

Results. Neutrophil infiltration was markedly inhibited in C3-deficient mice indicating a role for complement in PMN recruitment. In contrast, C3 deficiency afforded only partial protection against proteinuria. NSN was studied further in C4 null mice to probe the relative roles of the classical and alternate pathway in disease pathophysiology. C3 and C4 deficiency were associated with equivalent inhibition of PMN recruitment and proteinuria.

Conclusions. In aggregate, the data support a major role for complement in PMN recruitment in this model and point to complement-independent mechanisms of proteinuria in antibody-mediated glomerulonephritis. These ‘knockout’ mice should prove valuable for defining the complement-activated mediator systems that regulate leukocyte recruitment and tissue injury in renal diseases.

Key words: complement; glomerulonephritis; knockout; neutrophils; proteinuria

Introduction

Since its first description in the late 1890s, complement activation has been implicated in the pathogenesis of various immune-complex diseases including glomerulonephritis. Depletion of experimental animals of complement protects against most forms of immune-complex mediated glomerular injury [1–4]. Recent studies using gene ‘knockout’ of complement components in non-renal systems yielded different results to pharmacological complement depletion and have questioned the role of complement in the pathophysiology of inflammation [5]. To re-evaluate the relative roles of the classical and alternate pathways in glomerulonephritis, we induced acute immune complexes glomerulonephritis in complement C3 and C4 ‘knock-out’ mice.

Subjects and methods

Derivation of C3 and C4 deficient (‘knockout’) mice

Mice deficient in C3 (C3–/–) and C4 (C4–/–) were constructed, as previously described [6,7], using the approach of homologous recombination in embryonic stem cells. C3−/− bear a deletion of 121 amino acids (Δ620–741) which includes the C-terminal region of the beta-chain and the N-terminal region of the alpha gene [6]. Mice have two highly homologous and tandemly arranged C4 loci, i.e. C4Slp and C4Ss. C4Slp is not activated by C1 and it is not expressed in all strains of inbred mice including the ones used in this study. The C4Ss gene was disrupted by targeting the locus with a replacement vector which substituted a PGK-Neo cassette for 620 nucleotides of coding sequence, i.e. exons 23–27, which included the thioester region [7]. Selective ‘knockout’ of C3 and C4 was routinely confirmed in null mice by ELISA as described previously [6,7].
Induction of experimental immune complex-mediated glomerulonephritis

Nephrotoxic serum nephritis (NSN) was induced in C3 or C4 knock-out mice and in control littermates from the same breeding stock and same background (female, 6–8-week-old mice) by intravenous tail-vein injection of 50 μl of sheep anti-rat nephrotoxic serum heat-inactivated and diluted in 450 μl of PBS (NS) [8]. This non-fractionated complement-fixing antiserum cross-reacts with murine glomerular antigens and induces proteinuria and PMN infiltration in mice (vide infra). Tissue fixation and staining, light, immunofluorescence and electron microscopy were performed as reported previously [9,10]. Assessment of glomerular neutrophil infiltration was performed by the dichloroacetate esterase reaction and 40 glomerular cross-sections per mice were evaluated by an observer blinded to the experimental conditions. Urinary protein excretion was measured at 2, 4 and 24 h following induction of NSN, using the Bradford method (Biorad, Hercules, CA). Urinary creatinine levels were measured with a colorimetric assay (Sigma, St Louis, MO) based on the reaction of creatinine with picric acid which, under alkaline conditions, form a yellow-orange complex that can be measured at 500 nm by ELISA.

Statistics

Data are expressed as mean ± SEM. Differences between group means were determined by using a two-tailed Student’s t test. A P value of < 0.05 was taken to represent a statistically significant difference between group means.

Results

Immune-complex mediated glomerulonephritis in normal and C3 deficient mice

NSN was induced by intravenous injection of NS in wild-type C57 B6 mice. Control animals received PBS intravenously. Urinary protein excretion and PMN infiltration of the kidneys were evaluated at baseline and 2, 4 and 24 h following the injection of nephrotoxic serum. Deposition of heterologous immunoglobulin in wild-type glomeruli was associated with rapid complement deposition (Figure 1) and neutrophil infiltration (maximal 2 h following the injection of nephrotoxic serum (Figure 2 upper panel)), and followed by the development of proteinuria (maximal 24 h following the injection of nephrotoxic serum (Figure 2 lower panel)). Baseline levels of proteinuria and kidney PMN infiltration were similar in C3 deficient and normal wild-type litter mates (data not shown). Injection of nephrotoxic serum into C3 deficient mice resulted in rapid deposition of heterologous immunoglobulin without deposition of C3 (Figure 1). Neutrophil infiltration was markedly inhibited in C3 deficient mice (Figure 3 upper panel), indicating a role for complement in PMN recruitment. In contrast, C3 deficiency afforded significant but only partial protection against proteinuria (Figure 3 lower panel).

Contribution of classical and alternate pathways in NSN: comparison between C3 and C4 deficient mice

NSN was studied further in C4-deficient mice to probe the relative roles of the classical and alternate pathways in the pathophysiology of NSN. Injection of nephrotoxic serum into C4-deficient mice resulted in a glomerular immunofluorescence pattern similar to that found in C3-deficient animals (i.e. intense glomerular staining for IgG and absence of C3, data not shown). C3 and C4 deficiency were also associated with equivalent inhibition of PMN recruitment (Figure 3 upper panel) and proteinuria (Figure 3 lower panel). These results suggest a predominant role of the classical pathway for complement activation and for PMN recruitment in NSN.

Discussion

Most forms of glomerulonephritis are immunological in origin; however, the precise mechanisms responsible for initiating and perpetuating the immunological insult are still unclear. Experimental models of immune glomerular disease have proved valuable in identifying several mediators of injury such as the complement pathway, polymorphonuclear and mononuclear leukocytes and their secretory products, and various lipid and peptide mediators of inflammation released by glomerular or infiltrating cells. Neutrophils are currently thought to be key effectors in the mediation of immune glomerular injury [2,3,11] and complement activation is considered a central stimulus for neutrophil recruitment as determined through complement depletion experiments [3,4,11,12]. The complement cascade is broadly divided into three operational pathways: the classical and alternate pathways that initiate the activation of complement cascade, and the common pathway leading to formation of the membrane-attack...
Fig. 2. Time-course of PMN infiltration and proteinuria in wild-type animals. NSN was induced by injection of NS i.v. Control animals received PBS i.v. PMN infiltration and proteinuria were measured at baseline and 2, 4 and 24 h following the injection. Upper panel. The induction of NSN was associated with a significant recruitment of PMN infiltrate maximal at 2 h with normalization at 24 h. n of at least 3 animals per group. Lower panel. NSN was associated with a progressive proteinuria maximal at 24 h. n of at least three animals per group.

Fig. 3. NSN in C3- and C4-deficient animals. NSN was induced by injection of nephrotoxic serum i.v. in C3- and C4-deficient mice and in wild-type littermates. Control animals received PBS i.v. Upper panel. PMN infiltration, measured at 2 h after injection of nephrotoxic serum. Statistically significantly less PMN infiltration was found in C3- and C4-deficient mice than in wild-type littermates (WT) injected with nephrotoxic serum. Control animals received PBS only. *P < 0.001 when compared to WT; n of at least four animals per group. Lower panel. The level of proteinuria, estimated 24 h after injection of nephrotoxic serum, was significantly attenuated in both C3- and C4-deficient mice. *P < 0.05 when compared with WT; n of at least five animals per group.

complex (MAC) [13]. The classical pathway is activated by antigen-antibody complexes and serial activation of early components C1, C4, C2 is followed by activation of C3. The alternate pathway does not require antigen–antibody complex for activation of C3. Activation through both pathways leads to formation of the membrane attack complex C5b-C9 and release of the potent chemotactic factors C3a and C5a [13].

Complement independent mechanisms of PMN recruitment are being recognized increasingly and were originally suggested when complement depletion did not protect against glomerular injury [14]. Interpretation of complement depletion experiments is complicated by the fact that depletion is preceded by massive complement activation [4]. The recent development of mice deficient in C3 or C4 (gene ‘knock-out’) has provided unique tools with which to evaluate
the selective absence of an individual complements component and to assess the relative contribution of classical and alternate complement pathways in immune complex-mediated glomerulonephritis. Our results show that glomerular neutrophil infiltration is markedly reduced in C3-deficient mice in NSN induced with a complement-fixing antiserum. A similar pattern of protection is found in C4-deficient mice. Together these results suggest that PMN infiltration is predominantly complement-dependent at sites of immune-complex mediated glomerular injury and that the classical pathway plays a dominant role in this phenomenon. The possible mechanisms by which complement recruits leukocytes include (i) stimulation of leukocyte chemotaxis and CD11/CD18 dependent leukocyte adhesion through C3a- and C5a-triggered activation of leukocytes, and (ii) enhancement of endothelial cell adhesiveness for leukocytes through C5b-9-mediated mobilization of P-selectin to the endothelial cell surface [15]. Intriguingly, complement-independent mechanisms coexist since C3 or C4 gene knock-outs are not completely protected from glomerular PMN infiltration. Potential complement-independent mechanisms include PMN-Fc receptor interaction and direct activation of glomerular endothelial, epithelial and mesangial cells by immune complexes with subsequent release of inflammatory mediators [16–19].

Complement also plays a central role in pathogenesis of proteinuria in many experimental models and human diseases [11]. An emerging body of evidence suggests that complement-independent components to proteinuria also exist [8,14]. Our results with C3 ‘knock-out’ mice support this contention. We found that C3 and C4 deficiencies were associated with decreased proteinuria, but that this protective effect was small and incomplete. Proteinuria was comparable in C4- and C3-deficient animals. Although C3 and C4 deficiencies were associated with significant and comparable reductions in PMN glomerular infiltrate, this did not translate into a major effect on proteinuria. This again suggests that PMN-independent, complement-independent mechanisms contribute to the development of proteinuria in NSN. A recent report by Sheerin et al. [20] also supports this hypothesis. Using high doses of anti-GBM IgG in mice, C4 deficiency was found to be associated with a major reduction in PMN infiltrate, but only with a partial protective effect on the proteinuric response. Possible complement-independent mechanisms include direct interaction of antibodies with antigen on glomerular epithelial cells (GEC) resulting in detachment of podocyte foot processes and/or GEC toxicity [8,21–23]. Alternatively, the binding of antibody to GEC may induce the production and secretion of inflammatory mediators or matrix-degrading proteases [19,24].

In summary, our findings support a role for activation of the classical pathway of complement as a key event in PMN recruitment in immune-complex-mediated GN, but suggest a major role for complement-independent mechanisms in the pathogenesis of proteinuria.

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References


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