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Accelerated Nephrotoxic Nephritis Is Exacerbated in C1q-Deficient Mice

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C1q deficiency strongly predisposes to the development of systemic lupus erythematosus in humans and mice. We used the model of accelerated nephrotoxic nephritis in C1q-deficient mice to explore the mechanisms behind these associations. C1q-deficient mice developed severe glomerular thrombosis within 4 days of induction of disease, whereas wild-type mice developed mild injury. These findings suggest that C1q protects from immune-mediated glomerular injury. This exacerbated thrombosis was also seen in mice triply deficient in C1q, factor B, and C2, excluding a major pathogenic role for the alternative pathway of complement in this phenomenon. However, these mice did not develop elevated creatinine levels. No exacerbation of accelerated nephrotoxic nephritis was observed in mice doubly deficient in factor B and C2, suggesting a protective role for C1q against renal inflammation that is proximal to C2 activation. There were increased murine IgG deposits, neutrophil numbers, and apoptotic cells in the glomeruli of C1q-deficient mice compared with wild-type mice. Renal expression of genes encoding procoagulant proteins was also enhanced in C1q-deficient mice. The increased IgG deposits and apoptotic cells in the glomeruli of C1q-deficient mice suggest that the exacerbation of disease may be due to a defect in the clearance of immune complexes and/or apoptotic cells from their kidneys. The Journal of Immunology, 2001, 166: 6820–6828.

Deposition of complement proteins in glomeruli is a common finding in the inflammatory glomerulonephritides, including that caused by systemic lupus erythematosus (SLE). Studies using a variety of animal models and experimental systems have indicated that the complement system contributes to inflammatory tissue injury in the kidney (1–10). However, in humans, there is also epidemiologic evidence that complement may protect from the development of autoimmune renal disease. For example, patients deficient in classical pathway complement components are predisposed to developing SLE. The association with SLE is strongest for deficiencies of C1q and C4, with 92 and 75% of patients developing disease, respectively (11). One mechanism, which has been thought to play a role in the development of SLE in patients with complement deficiency, is abnormal clearance of immune complexes. We have demonstrated previously that immune complex handling is defective in patients with SLE who have acquired complement deficiency secondary to disease activity or genetic deficiency of C2 (12, 13). However, this mechanism does not explain why deficiencies of C1q or C4 should predispose to SLE more strongly than genetically determined deficiency of C2, or why patients deficient in C3, which has a critical role in immune complex modification and the facilitation of the transport of complexes to the fixed macrophage system, rarely develop SLE. It has also been suggested that another mechanism that may contribute to autoimmunity in complement deficiency is abnormal clearance of apoptotic cells. A role for C1q in the clearance of apoptotic cells is suggested by observations that C1q bound to apoptotic cells (14), that increased numbers of apoptotic cells were found in the glomeruli of C1q-deficient mice (15), and that there was a defect in the clearance of apoptotic cells in C1q-deficient mice and humans (16).

Accelerated nephrotoxic nephritis is a model of immune complex-mediated glomerular inflammation. The role of complement has been explored in this model by depleting mice of C3 using cobra venom factor (CVF). In BALB/c mice, which showed a Th2-biased immune response and a thrombotic pattern of injury, disease was complement dependent. However, the severity of disease in C57BL/6 mice, which showed a Th1-biased response and a crescentic pattern of nephritis, was not influenced by experimental complement depletion (17). We developed C1q-deficient mice to explore the mechanism by which C1q deficiency may predispose to SLE and previously reported that a proportion of these mice develop spontaneous glomerulonephritis (15). In this study, we used the model of accelerated nephrotoxic nephritis in mice to investigate further the role of complement, and C1q in particular, in immune-mediated renal disease. We studied three groups of animals: C1q-deficient (C1qa−/−) mice, mice deficient in both factor B and C2 (H2-Bf/C2−/−), and animals triply deficient in C1q, factor B, and C2, (C1qa/H2-Bf/C2−/−), to dissect the relative contributions of the classical and alternative pathways to pathogenesis.
Materials and Methods

Mice

C1qa−/−, H2-Bf/C2−/−, and C1qa/H2-Bf/C2−/− mice were developed as previously described (15, 18, 19). Age-, strain-, and sex-matched mice were used in all experiments. All complement-deficient and wild-type animals used in these studies were of mixed genetic background 129/SvC57BL/6 and aged 6–12 wk. C1qa−/− mice on this mixed genetic background have been shown to develop spontaneous glomerulonephritis and autoantibodies at 8 mo of age (15). However, at 3 mo only 4 of 120 C1qa−/− mice on this background had a weakly positive anti-nuclear Ab (1/80) compared with 1 of 80 wild-type mice. Renal histology of untreated 129/Sv × C57BL/6 wild-type and C1qa−/− mice, aged 2–3 mo, was examined histologically (n = 20 and n = 16, respectively), and none was abnormal. Mice were kept in a standard animal house environment, and experiments were performed according to institutional guidelines.

Preparation of rabbit nephrotoxic globulin

Whole mouse kidneys were passed through two sieves (250 and 160 µm pore size) and collected on a 63-µm pore size sieve. The material collected was washed twice in cold PBS (pH 7.2) with centrifugation at 700 × g for 5 min between wash, resuspended, and sonicated at 8-µm amplitude in 30-s bursts on ice. Disruption of glomeruli was verified by microscopy. The material was washed in cold PBS twice and in distilled water once with centrifugation at 700 × g for 15 min before being resuspended in distilled water. Both the pellet was resuspended in distilled water, washed twice, with centrifugation at 700 × g for 5 min between washes, and frozen onto the surface of a centrifuge tube in liquid nitrogen. It was lyophilized overnight at −70°C and stored at −20°C. Male and female New Zealand White rabbits were immunized s.c. with 1 mg of this preparation of lyophilized glomeruli in CFA (Sigma, Poole, U.K.). Two weeks later they were immunized again with 1 mg in IFA (Sigma), and after an additional 2 wk they were exsanguinated by cardiac puncture. The serum was heat inactivated at 55°C for 30 min, and a globulin fraction was obtained by precipitation in 50% ammonium sulfate. The precipitate was resuspended in PBS and extensively dialyzed against PBS. It was sterilized using a 0.2-µm pore size filter and stored at −70°C. The concentration of rabbit IgG in the preparation was measured by radial immunodiffusion using a sheep anti-rabbit IgG Ab (Serotec, Oxford, U.K.) and rabbit IgG standards (Sigma).

Induction of glomerulonephritis in mice

Mice were initially immunized with 200 µg of rabbit IgG (Sigma) in CFA (Sigma) administered i.p. Seven days later the animals were injected with 1 mg of rabbit nephrotoxic globulin prepared as described above via the tail vein. At selected times up to 4 days after injection of nephrotoxic Ab, mice were anesthetized by i.p. injection of midazolam and fentanyl and exsanguinated before harvesting the kidneys. Whenever possible, mice were sacrificed in groups to allow comparison at identical time points. However, some C1qa−/− mice died prematurely.

Histological studies and quantitative immunofluorescence

Kidneys were fixed for 4 h in Bouin’s solution, transferred to 70% ethanol, and embedded in paraffin or were snap-frozen in isopentane and stored at −70°C. Sections embedded in paraffin were stained with periodic acid-Schiff (PAS) reagent. Frozen sections were cut at a thickness of 5 µm. All light microscopic analysis and quantitative immunofluorescence were performed by an observer without knowledge of the sample identity. Glomerular thrombosis was assessed by grading the degree of PAS-positive material with a scale of 0–3: grade 0 = no glomeruli with PAS-positive material; grade 1 = 1–25%; grade 2 = 25–50%; grade 3 = 50–75%; and grade 4, 75–100%. Glomerular permeability was assessed by visualizing cells under phase contrast microscopy. The fluorescence intensity over each glomerulus was measured. For each section, 20 glomeruli were examined, and the mean fluorescence intensity was recorded, with results expressed in arbitrary fluorescence units (AFU).

Peripheral blood leukocyte counts

Blood was collected into potassium EDTA as described above, followed by 1/10 dilution in 2% acetic acid and counting the total white cells using a hemocytometer. Neutrophil numbers as a percentage of the total leukocyte count were assessed by visualization of cells under phase contrast microscopy.

Serum creatinine and albumin

Serum creatinine and albumin were measured at Department of Chemical Pathology, Hammersmith Hospital, using an Olympus AU600 analyzer (Olympus Diagnostics, London, U.K.), with albumin analyzed by the bromocresol green method. Baseline measurements in untreated mice showed that the normal ranges of serum creatinine and albumin did not differ between the wild-type and C1qa−/− mice used in the study. Serum albumin levels in 15 wild-type and 11 C1qa−/− age- and sex-matched mice were 25 (range, 9–28), and 24 (range, 19–28) g/L, respectively. Baseline serum creatinine levels in these mice were 43 (range, 36–52) and 44 (range, 39–54) µmol/L, respectively.

Albumin clearance

Mice were housed in metabolic cages for 24 h for urine collection. The albumin concentration was measured by radial immunodiffusion. Samples and standards (mouse albumin; Sigma) were placed in wells (6 µL/well) in 1.2% agarose in PBS containing rabbit anti-mouse albumin (Biogenesis, Poole, U.K.). Gels were dried and stained with Coomassie Blue. Concentrations in samples were calculated with reference to a standard curve. Albumin clearance was calculated from the formula: (urinary albumin concentration × plasma volume)/plasma albumin concentration. Baseline urinary albumin measurements in wild-type, C1qa−/−, and C1qa/H2-Bf/C2−/− mice showed concentrations of <50 µg/m in all cases (n = 10, 10, and 5, respectively).

Measurement of the murine anti-rabbit IgG immune response

ELISA plates (Nunc Maxisorb, Life Technologies, U.K.) were coated overnight at 4°C with 10 µg/mL of rabbit IgG (Sigma) or 5 µg/mL goat anti-mouse IgG3 (Biotechnology Associates, Birmingham, AL) (Southern Biotechnology Associates, Birmingham, AL), and C1qa/H2-Bf/C2−/− (Sigma) (18, 19). Plates were blocked with 1% BSA in PBS. Blocking and all subsequent incubations were performed for 1 h at 37°C. Plates were washed twice in PBS-0.075% Tween and twice in PBS (pH 7.2). Wells coated with goat anti-mouse Ig were incubated with 50 µL of serial dilutions of mouse IgG1, IgG2a, IgG2b, or IgG3 (Sigma) to generate a standard curve. Wells coated with rabbit IgG were incubated with 50 µL of sera, diluted as required in 1% BSA-PBS-0.05% Tween. Plates were washed as before, incubated with alkaline phosphatase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 diluted 1/2000 to 1/20000 in 1% BSA-PBS-0.05% Tween (Southern Biotechnology Associates). Following further washing, and developed using the substrate p-nitrophenyl phosphate (Sigma). The OD of the reaction mixture at 405 nm wavelength was measured using an ELISA reader (Titertek-Multiskan, Flow, Costa Mesa, CA). The concentration of mouse IgG subclasses in the samples was calculated with reference to the standard curve.

RNase protection assay (RPA)

The genes analyzed were protease-activated receptor-1 (PAR-1), tissue factor pathway inhibitor (TFPI), tissue factor (TF), early growth response-1 (Egr-1), vascular endothelial growth factor (VEGF), uridine-phosphoribosyltransferase-1 activated (uP3A), and plasminogen activator inhibitor-1 (PAI-1). cDNA fragments of different size for each of the chosen genes were recovered by RT-PCR and subsequently subcloned into the phGE-T-PLasmid (Promega, Madison, WI). Positive clones with antisense orientation with respect to the T7 promoter were identified by colony PCR, and their complete sequences were verified by DNA sequencing. Radioactive multihybrid probe preparation and the RPAs were performed according to standard protocols (21). Briefly, antisense [32P]UTP-labeled riboprobes were synthesized by in vitro transcription of the DNA template set, which contained all the linearized plasmid cDNAs pooled in equimolar amounts. DNase I treatment was performed to remove the DNA templates, and riboprobes were purified by phenol/chloroform extraction, followed by ethanol precipitation using
glycogen as carrier. For each sample ~5 μg of total RNA was hybridized overnight at 52°C with 3 x 10⁶ cpm of the labeled multiprobe. Samples were first treated with an RNase cocktail (Ambion, Austin, TX). RNase was then removed by proteinase K treatment, and samples were purified by phenol/chloroform extraction, followed by ethanol precipitation using glycogen as carrier. Protected fragments were resolved through a 5% sequencing gel. Quantification of all the experimental samples for all time points was done using a Phosphorimager and Optical Progenesis. Results were expressed as the median (range). Nonparametric tests were applied throughout. The Mann-Whitney U test was used to analyze the differences between two groups of mice. Where three groups of mice were compared, the Kruskal-Wallis test was used, with Dunnett’s multiple comparisons test (p values given are from Dunnett’s test). GraphPad Prism (GraphPad Software, San Diego, CA) was used to analyze the data. Differences were considered significant when p < 0.05.

Results

C1q deficiency caused increased glomerular injury in accelerated nephrotoxic nephritis independently of alternative pathway complement activation

The patterns of injury that developed following the induction of accelerated nephrotoxic nephritis were compared in wild-type, C1qa−/−, and C1qa/H2-Bf/C2−/− mice. There were premature deaths in C1qa−/− mice. In the experiment shown in Fig. 1, a–c, six C1qa−/− mice died on day 2, and one died on day 3. The remaining three C1qa−/− mice and all wild-type and C1qa/H2-Bf/C2−/− mice were sacrificed on day 4. Glomerular histology in C1qa−/− and C1qa/H2-Bf/C2−/− mice showed extensive PAS-positive material representing glomerular thrombosis, whereas wild-type mice typically showed little thrombosis and only mild hypercellularity. Representative glomeruli from each group of mice are shown in Fig. 2. Significantly more thrombosis was seen in C1qa−/− mice than in wild-type mice, as shown in Fig. 1a (p < 0.001 and p < 0.01 for C1qa−/− and C1qa/H2-Bf/C2−/−, respectively). More severe renal injury in C1qa−/− and C1qa/H2-Bf/C2−/− mice was also evident from analysis of serum albumin and creatinine levels, with serum albumin used as an indirect index of proteinuria. Four days after injection of nephrotoxic Ab, serum albumin levels were significantly lower in both C1qa−/− and C1qa/H2-Bf/C2−/− mice than in wild-type animals (Fig. 1b). Results (expressed as the median in grams per liter) were: wild-type, 21.5 (range, 14–31); C1qa−/−, 11.5 (range, 10–14); C1qa/H2-Bf/C2−/−, and 11 (range, 8–18; p < 0.01 for each compared with wild-type mice). Serum creatinine was higher in C1qa−/− than in wild-type mice (Fig. 1c). Results were: wild-type, 41.5 μmol/l (range, 33–54); C1qa−/−, 81 μmol/l (range, 61–179); C1qa/H2-Bf/C2−/−, 42 (range, 28–45; p < 0.001 for C1qa−/− compared with wild-type mice).

Direct measurement of albuminuria, as an index of renal damage, is preferable to quantification of serum albumin, as in serum the latter variable may be depressed for reasons other than proteinuria (e.g., impaired synthesis, loss from the gastrointestinal tract, or increased catabolism). In one experiment, albumin clearance was directly compared in wild-type and C1qa/H2-Bf/C2−/− mice following the induction of nephritis, and in agreement with the serum albumin data, proteinuria was significantly higher in the complement-deficient animals. Results were: wild-type, 0.368 μl/min (0.002–1.610); and C1qa/H2-Bf/C2−/−, 1.765 μl/min (0.468–2.031).
Creatinine was lower in C1qa/H2-Bf/C2−/− mice and albuminuria in this model. However, serum complement do not have a critical role in the development of histologic changes and albuminuria in this model. C5a production, and formation of the membrane attack complex of C1q, which is unable to activate the alternative pathway, indicates that C3 cleavage, C5a production, and formation of the membrane attack complex of complement do not have a critical role in the development of histologic changes and albuminuria in this model.

Increased glomerular injury was not seen in mice deficient in both factor B and C2

Wild-type and C1qa−/− mice were compared with H2-Bf/C2−/− mice after induction of nephritis to assess the effect of disrupting the classical pathway distally to C1q, at the level of C2. All mice were sacrificed on day 4. The degree of glomerular injury in H2-Bf/C2−/− mice was not significantly different from that in wild-type mice, but was significantly less severe than that in C1qa−/− mice (p < 0.01), which, as described above, developed severe glomerular inflammation and thrombosis. The histologic appearance of representative glomeruli is illustrated in Fig. 2, and quantitative data comparing thrombosis are shown in Fig. 1d. Serum albumin was significantly higher in H2-Bf/C2−/− animals than in C1qa−/− mice and was similar to that in wild-type mice as shown in Fig. 1e. Results (expressed as the median) were: wild-type mice, 23 g/L (range, 16–24); C1qa−/−: 12 g/L (range, 8–15); H2-Bf/C2−/−: 22 g/L (range, 10–25); p < 0.05 for H2-Bf/C2−/− compared with C1qa−/−. Serum creatinine was significantly lower in H2-Bf/C2−/− mice than in C1qa−/− mice (p < 0.05) and was not significantly different from that in wild-type mice, as shown in Fig. 1f. Serum creatinine levels were: wild-type mice, 41 μmol/L (range, 37–58); C1qa−/−: 58 (range, 37–88); H2-Bf/C2−/−: 37 (range, 35–44; p < 0.05 for H2-Bf/C2−/− compared with C1qa−/−).

These data showed that the increased glomerular injury seen in the C1qa−/− mice did not occur in H2-Bf/C2−/− mice, suggesting that the complement proteins that have a critical role in protecting from the development of renal inflammation in this model lie proximal to C2 in the classical pathway of complement activation.

Quantitation of glomerular complement proteins and glomerular mouse and rabbit IgG by immunofluorescence

Interpretation of immunofluorescence studies performed on histological material obtained on day 4 after the induction of nephritis, when severe glomerular thrombosis was present in C1qa−/− and C1qa/H2-Bf/C2−/− mice, proved unreliable. Therefore, we used material taken at baseline (before the induction of nephritis) and 1 day after induction of disease for all quantitative immunofluorescence studies. Following immunostaining of kidney sections, the site of protein deposition was determined by direct microscopy, and quantification was performed by computer-aided image analysis as described in Materials and Methods. We studied the deposition of complement proteins C1q, C4, and C3 and both rabbit and mouse IgG.

Complement proteins

Frozen sections of kidney from wild-type and C1qa−/− mice (n = 6/group) were stained for C1q, C4, and C3. Representative sections are shown in Fig. 3.

C1q. C1q was not detectable in the glomeruli of either wild-type or C1qa−/− mice at baseline. On day 1 after induction of glomerulonephritis, capillary wall C1q was seen in wild-type mice only (Fig. 3, top panel).

C4. Mesangial C4 deposits were seen in both wild-type and C1qa−/− mice at baseline, with significantly more present in wild-type mice (Fig. 3). One day after induction of disease, wild-type mice showed the presence of additional linear mesangial capillary wall C4, which was not seen at baseline, although there was no increase in the amount of C4 deposited in wild-type mice on day 1 compared with baseline. The large and variable amount of C4 present at baseline may have prevented detection of the small increase in the total amount of C4 resulting from capillary wall deposition at day 1. There was no change in the very small amount of C4 present in the glomeruli of C1qa−/− mice following induction of disease compared with the baseline, compatible with failure to activate the classical complement pathway in these animals. On day 1, there was no change in the amount or pattern of C4 staining in C1qa−/− mice, but a linear capillary wall distribution was seen in wild-type mice. Sparse mesangial C3 (with intense tubular C3 staining) was seen at baseline in both groups of mice, with linear capillary wall C3 in both groups on day 1.
day 1, as at baseline, there was significantly more C4 in the glomeruli of wild-type mice compared with C1q-deficient animals. Results (expressed as the median in AFU) at baseline were: wild-type, 43.1 (range, 33.1–65); and C1qa\(^{-/-}\), 9.05 (range, 6.6–12; \(p < 0.005\)). Day 1 results were: wild-type, 45.35 (range, 23.8–56.2); and C1qa\(^{-/-}\), 11.55 (range, 11.3–45.9; \(p < 0.05\)).

C3. Both wild-type and C1qa\(^{-/-}\) mice had sparse mesangial C3 deposits at baseline. In both wild-type and C1qa\(^{-/-}\) mice on day 1 after disease induction, there was also linear capillary wall C3 (Fig. 3). The amount of C3 was quantified, and there was a significant increase in the amount of glomerular C3 in both wild-type and C1qa\(^{-/-}\) mice on day 1 compared with baseline levels. No significant differences were detected between wild-type and C1qa\(^{-/-}\) animals at either time point. Results (expressed as the median) at baseline were: wild-type, 19.9 AFU (range 15–32.4); and C1qa\(^{-/-}\), 17 AFU (range, 12.4–28). Day 1 results were: wild-type, 45.25 AFU (range, 32.9–51.3); and C1qa\(^{-/-}\), 36.9 AFU (range, 14.9–58.8; on day 1 compared with baseline: \(p < 0.005\) for wild-type and \(p < 0.05\) for C1qa\(^{-/-}\)). It seemed likely that the glomerular deposition of C3 following the induction of inflammation reflected alternative pathway activation, as we documented significantly increased amounts of the protein in C1qa\(^{-/-}\) mice. This was confirmed in studies performed in C1qa/H2-Bf/C2\(^{-/-}\) mice, unable to activate either the alternative or classical complement pathway. In sections from these mice stained for C3 at day 1 after injection of nephrotoxic Ab (\(n = 4\)), we were able to demonstrate only sparse mesangial C3. This was of a similar magnitude to that seen in wild-type, C1qa\(^{-/-}\), and C1qa/H2-Bf/C2\(^{-/-}\) mice at baseline.

**Immunoglobin**

Quantitation of rabbit IgG in the kidney was performed by immunofluorescence to examine the possibility that the increased disease in C1q deficient mice might be due to increased binding of nephrotoxic Ab. One day after induction of nephritis by the injection of rabbit nephrotoxic globulin no significant differences were observed between wild-type and C1qa\(^{-/-}\) mice (\(n = 5–8/\)group). Levels (expressed as the median) were: wild-type, 21.25 AFU (range, 13.8–29.55); and C1qa\(^{-/-}\), 25.75 AFU (range, 18.05–32.2).

We also measured the deposition of mouse IgG using the same technique to explore the possibility that the increased disease in C1qa\(^{-/-}\) mice was due to the presence of more autologous Ab. Data on day 1 following the injection of nephrotoxic globulin (two separate experiments) are shown in Fig. 4. In an experiment comparing wild-type and C1qa\(^{-/-}\) mice (\(n = 5–7/\)group), there was significantly more IgG present in the glomeruli of C1qa\(^{-/-}\) mice. Results (median) were: wild-type, 19.95 AFU (range, 13.55–25.6); and C1qa\(^{-/-}\), 33.43 AFU (range, 17.65–72.85; \(p < 0.05\)). Data are illustrated in Fig. 4a. In a second experiment comparing wild-type, C1qa\(^{-/-}\), and C1qa/H2-Bf/C2\(^{-/-}\) mice (\(n = 6–11/\)group), there was a trend toward more IgG in the glomeruli of C1qa\(^{-/-}\) mice compared with wild-type, but this did not reach statistical significance. However, there was significantly more IgG in the glomeruli of C1qa/H2-Bf/C2\(^{-/-}\) mice compared with wild-type mice (Fig. 4b). Results were (AFU): wild-type, 17.6 (range, 12.5–24.9); C1qa\(^{-/-}\), 25.5 (range, 14.4–57); C1qa/H2-Bf/C2\(^{-/-}\), 30.5 (range, 18.4–65.4; \(p < 0.05\) for C1qa/H2-Bf/C2\(^{-/-}\) compared with wild-type). These data suggest that the increased disease in C1q-deficient mice may reflect the presence of more murine IgG in the glomeruli.

**FIGURE 4.** Glomerular mouse IgG 1 day after induction of disease in two separate experiments. Expt. a compared wild-type and C1q-deficient (C1qa\(^{-/-}\)) mice. Expt. b included a third group of mice triply deficient in C1q, factor B, and C2 (C1qa/H2-Bf/C2\(^{-/-}\)).

**Assessment of the humoral immune response to rabbit IgG**

We considered the possibility that the increased amount of murine IgG we observed in the glomeruli of C1q-deficient mice may reflect a difference in the immune response to rabbit IgG in these animals compared with wild-type. Serum levels of mouse anti-rabbit IgG of different subclasses on day 4 after induction of disease were measured by ELISA in C1qa\(^{-/-}\) and wild-type mice (\(n = 7/\)group). Levels (expressed as the median) on day 4 were as follows for wild-type and C1qa\(^{-/-}\), respectively: IgG1, 11.4 \(\mu\)g/ml (range, 4.4–27.8) and 5.43 \(\mu\)g/ml (range, 4.3–10.7); IgG2a, 0.215 \(\mu\)g/ml (range, 0.05–0.8) and 0.3 \(\mu\)g/ml (range, 0.03–0.66); IgG2b, 10.2 \(\mu\)g/ml (range, 2.6–17.7) and 3.56 \(\mu\)g/ml (range, 2.3–11.4); and IgG3, 0.11 (range, 0.04–0.226) and 0.04 \(\mu\)g/ml (range, 0.03–0.07). The only statistically significant difference observed between complement-deficient and wild-type mice was in IgG3 anti-rabbit IgG, where there was significantly less in the serum of the C1qa\(^{-/-}\) mice (\(p < 0.01\)), although total levels of this subclass were very low. This finding could not explain the increased disease in these mice.

**The early phase of nephritis in C1q-deficient mice was characterized by both increased neutrophil influx as well as thrombosis**

Groups of wild-type and C1qa\(^{-/-}\) mice (\(n = 5–7\)) were sacrificed every 24 h after induction of disease up to day 4 to explore the events preceding the development of glomerular thrombosis (Fig. 5). Although less severe disease was seen in C1qa\(^{-/-}\) mice in this experiment than in the experiments shown in Figs. 1 and 2, the experiment...
in Fig. 5 illustrates the histological changes that precede glomerular thrombosis. There were significantly more neutrophils in the glomeruli of C1qa−/− mice at all stages during the development of disease, and between one and three C1qa−/− mice at each time point showed glomerular thrombosis, which was not seen in the wild-type mice. Results (expressed as the median) for wild-type and C1qa−/− mice were: day 1, 0.06 neutrophils/glomerular cross-section (range, 0.04–0.3) and 0.58 neutrophils/glomerular cross-section (range, 0.1–1.1); day 2, 0.12 neutrophils/glomerular cross-section (range, 0–0.22) and 1.63 neutrophils/glomerular cross-section (range, 0.1–10); day 3, 0.1 neutrophils/glomerular cross-section (range, 0.02–0.68) and 1.16 neutrophils/glomerular cross-section (range, 0.18–11.3); day 4, 0.12 neutrophils/glomerular cross-section (range, 0.04–0.24) and 1.16 neutrophils/glomerular cross-section (range, 0.4–2.28; p < 0.05 for all except day 4, where p < 0.01, and day 2, where p = 0.059). Thus, it appears that thrombosis was preceded by increased glomerular neutrophil influx in C1q-deficient mice.

Albuminuria was also compared in C1qa−/− and wild-type mice on days 1–3. On day 1 six wild-type mice and five C1qa−/− mice had unrecordable albuminuria (<50 μg/ml), and one C1qa−/− mouse had 2.1 mg/24 h. On day 2 four wild-type mice and three C1qa−/− mice had unrecordable albuminuria, with one wild-type mouse having 1.1 mg/24 h, and three C1qa−/− mice having 1.7, 29.6, and 31 mg/24 h. On day 3 four wild-type mice and two C1qa−/− mice had unrecordable albuminuria, with two wild-type mice having 3.9 and 2.2 mg/24 h, and four C1qa−/− mice having 14.5, 13.1, 16.3, and 0.26 mg/24 h. Therefore, there was a clear trend toward more albuminuria in C1qa−/− mice on days 2 and 3.

This increased glomerular neutrophil number was not explained by coincident differences in blood neutrophil numbers, as there was no difference at baseline or on days 1 and 2. Measurements were made in blood taken from mice on days 1 and 2 of the experiment shown in Fig. 5 (n = 5–7/group). Results (expressed as the median) for C1qa−/− mice on days 1 and 2 were 895 (range, 590–2250) and 720 (range, 510–2620) neutrophils/μl, respectively. Results for wild-type mice on days 1 and 2 were 710 (range, 130–1140) and 590 (range, 200–1160) neutrophils/μl, respectively. There was no difference in neutrophil counts in untreated C1qa−/− mice and wild-type mice (data not shown).

The early phase of the disease was characterized by an increased number of apoptotic cells in C1q-deficient mice

Glomerular apoptotic cells were counted 1 day after induction of disease to determine whether the disease developing in C1q-deficient mice was associated with increased numbers of apoptotic cells. Data were obtained from three experiments. In the first, one wild-type animal had 2 apoptotic cells/50 glomeruli, and five had none. Results in six C1qa−/− mice were 0, 2, 2, 4, 10, and 10 apoptotic bodies/50 glomeruli. The difference between these two groups was significant (p < 0.05). In two additional experiments there was a trend toward more apoptotic cells in C1qa−/− mice compared with wild-type mice, but the differences did not reach statistical significance. There was a trend for apoptotic cells to be associated with increased neutrophil numbers, but this correlation reached statistical significance in only one of the three experiments performed (Spearman’s rank correlation coefficient = 0.7544; p < 0.05).

Assessment of factors that may cause glomerular thrombosis

Glomerular thrombosis was the most striking feature of disease in C1qa−/− and C1qa/H2-Bf/C2−/− mice (Fig. 2). As an additional and independent measure of thrombosis in the complement-deficient mice, we compared expression levels of genes encoding proteins involved in coagulation and fibrinolysis by RPA, using kidney RNA from wild-type and C1qa−/− mice on 1 and 4 days after induction of glomerulonephritis. No differences were found in the expression of any of the above genes in C1qa−/− compared with wild-type mice at 1 day after the induction of disease (n = 6/group; data not shown). On day 4 (n = 10–11/group) there was significantly more PAI-1, Egr1, TF, and PAR-1 mRNA in C1qa−/− mice than wild-type mice, and there was significantly less VEGF RNA in C1qa−/− mice compared with wild-type mice. There was no difference in the expression of either uPA or TFPI. Results (expressed as the median) for C1qa−/− and wild-type mice were: PAI-1, 84 arbitrary units (range, 45–100) compared with 28 arbitrary units (range, 18–47; p < 0.0005); Egr-1, 95 arbitrary units (range, 48–100) compared with 28.5 arbitrary units (range, 5–62; p < 0.0005); TF, 79 arbitrary units (range, 25–65) compared with 49.5 arbitrary units (range, 25–100; p < 0.05); PAR-1, 59 arbitrary units (range, 22–100) compared with 13.5 arbitrary units (range, 12–37; p < 0.001); VEGF, 44 arbitrary units (range, 14–83) compared with 82.5 arbitrary units (range, 23–100; p < 0.001); uPA, 62 arbitrary units (range, 52.5–72) compared with 67 arbitrary units (range, 63.5–73.5); and TFPI, 76 arbitrary units (range, 66–85) compared with 80 arbitrary units (range, 66–85), respectively. These results, shown graphically in Fig. 6, showed that differences in the expression of genes encoding in procoagulant and antifibrinolytic genes were observed at the same time as, but not before, thrombosis was evident histologically.

Discussion

In this study we have shown that in a murine model of accelerated autologous nephrotoxic nephritis, C1qa−/− and C1qa/H2-Bf/C2−/− mice developed severe glomerular injury within 4 days of injection of nephrotoxic Ab, whereas wild-type and H2-Bf/C2−/− mice showed only mild glomerular hypercellularity. These results suggest that complement proteins proximal to C2 in the classical pathway of complement activation have a protective role against the development of immune complex-mediated inflammatory injury in the kidney. In the context of the previous observations that C1q- and C4-deficient mice develop autoimmunity and spontaneous glomerulonephritis (15, 22) and the epidemiologic evidence in humans that genetically determined deficiencies of C1q and C4
strongly predispose to severe SLE, frequently complicated by renal disease, the most likely candidate proteins for this protective role are C1q and C4.

Our study also provides evidence against a major role for the complement system as an effector of inflammatory tissue damage in this model. Although C3 deposition was seen in the glomeruli of C1qa−/− mice, C3 was not deposited in the glomeruli of C1qa/H2-Bf/C2−/− mice. Because severe glomerular thrombosis and hypoalbuminemia developed in both groups of mice, this showed that C3 activation was not required for the development of disease, although C1qa/H2-Bf/C2−/− mice appeared to be protected from the development of renal failure. In this respect, the findings of the current study corroborate our previous observation that spontaneous proliferative glomerulonephritis developed in 64% of C1qa/H2-Bf/C2−/− mice by 8 mo of age (19), indicating that the autoimmune glomerulonephritis in these animals also develops largely independently of either classical or alternative pathway complement activation. However, previous comparable studies of experimental nephritis indicate that the genetic background of the mice studied may be critical in determining the complement dependence of the phenotype observed. For example, a study using CVF to induce complement depletion in a murine model of accelerated nephrotoxic nephritis suggested that complement played a role in mediating tissue injury in BALB/c, but not C57BL/6, mice (17).

The role of complement has been examined previously in other animal models of immune-mediated glomerular inflammation. A number of studies have been performed in complement-deficient mice using the model of heterologous nephrotoxic nephritis (23–25). This is a model of neutrophil-mediated acute inflammation caused by a single injection of heterologous nephrotoxic Ab. These previous studies have also yielded conflicting data for the role of complement in mediating the neutrophil influx that is the hallmark of this model. Two studies suggested a decrease in neutrophil numbers in mice with C3 or C4 deficiency (23, 25), but a third found no difference, although C3 deficiency did protect from proteinuria (24). Earlier studies in mice, using CVF to cause complement depletion, also suggested that complement did not play a major role in mediating the ingress of neutrophils (26, 27).

What is the mechanism leading to the development of severe, complement-independent glomerular thrombosis in C1qa−/− mice after the induction of accelerated nephrotoxic nephritis in our present study? We considered a number of possibilities. Firstly, we investigated whether there was a greater initial deposition of nephrotoxic (rabbit) Ab in the kidney. Immunofluorescence did not show any difference in glomerular binding of nephrotoxic Ab in C1qa−/− compared with wild-type mice. A second possibility was that an increased amount of autologous mouse Ab bound to the rabbit Ab deposited in glomeruli of C1qa−/− mice. Quantitative immunofluorescence studies performed on day 1 showed that this was indeed the case (Fig. 4). We considered two possible explanations for this observation. It could have occurred as a result of a heightened murine immune response to the rabbit Ig, leading to greater deposition due to elevated circulating Ab levels in the complement-deficient animals. However, previous work has shown a defective humoral immune response in C1qa−/− mice (28). In the present study we did not detect significantly increased circulating levels of mouse anti-rabbit IgG in C1qa−/− mice compared with wild-type mice.

The second possible explanation for the increased glomerular IgG in C1qa−/− mice was a defect in the clearance of murine Ab bound to the target Ag (rabbit nephrotoxic immunoglobulin) in the kidney. Complement has been shown to play a role in the solubilization of aggregated immune complexes, but the alternative pathway was thought to play a dominant role (29). However, solubilization is delayed in C2-deficient serum (30), and there is evidence that some solubilization occurs in the absence of the alternative pathway (31). Moreover, these in vitro studies used immune aggregates in the fluid phase, and different mechanisms may operate when immune complexes are deposited in tissues, as in this model. The presence of increased murine IgG in the glomeruli of C1q-deficient mice may contribute to the increased renal injury by ligation of Fc receptors on inflammatory cells. This is supported by the observation of an increase in glomerular neutrophil numbers in C1qa−/− mice compared with wild-type mice.

Several recent reports have supported a role for Fc receptors in mediating glomerular inflammation. Accelerated nephrotoxic nephritis has been studied previously in Fcγ-chain deficient mice, lacking the functional activating receptors FcγRI and FcγRIII. Animals lacking the Fcγ-chain were shown to be protected from disease (32). This followed work with lupus-prone New Zealand Black/White mice (33), showing that Fcγ deficiency also prevented the development of spontaneous glomerulonephritis.

Results in other models of immune complex-mediated inflammation, such as the reverse passive Arthus reaction, have suggested that Fc receptors play a greater role than complement in
certain contexts in causing tissue injury (34, 35). In the present study the observations support the hypothesis that C1q deficiency results in impaired processing of immune complexes, with persistence of a proinflammatory stimulus within the glomerulus, inducing Fc-mediated influx of neutrophils. However, there is also clear evidence that complements may contribute to inflammatory disease caused by immune complexes in other experimental situations (36–38). Other genetic factors may be important in determining the role of Fc receptors vs complement in the induction of inflammatory renal injury.

A role for complement in the clearance of apoptotic cells was suggested by the observation that C1q binds to apoptotic human keratinocytes in vitro (14). Further supportive evidence includes the presence of increased numbers of apoptotic cells in the glomeruli of C1q-deficient mice with spontaneous glomerulonephritis (15), and the defective clearance of apoptotic cells in the absence of complement components both in vitro (39) and in vivo (16). In the present study 1 day after induction of disease, we saw increased numbers of apoptotic cells in the glomeruli of C1qa−/− mice compared with wild-type mice. Abnormal clearance of apoptotic cells could lead to either autoimmunity or dysregulated inflammation. In addition, a procoagulant role for apoptotic cells has been demonstrated (40), and this may be relevant to the glomerular thrombosis demonstrated in the present study.

We explored the possibility that there was increased expression of genes involved in the coagulation and fibrinolytic pathways in C1qa−/− compared with wild-type animals. Abnormal clearance of apoptotic cells might explain the development of glomerular thrombosis. Taken together, our results show that the exacerbated glomerular thrombosis in C1qa−/− mice can be accounted for by coordinated up-modulation of the procoagulant and the anti-fibrinolytic proteins. There was increased PAI-1, TF, PAR-1, and Egr-1 mRNA expression at 4 of the procoagulant and the anti-fibrinolytic proteins. There was increased PAI-1, TF, PAR-1, and Egr-1 mRNA expression at 4.


