

# Inhibition of Complement Regulation Is Key to the Pathogenesis of Active Heymann Nephritis

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## Summary

Crry (complement receptor 1-related protein/gene y) is a key cellular complement regulator in rodents. It is also present in Fx1A, the renal tubular preparation used to immunize rats to induce active Heymann nephritis (HN), a model of membranous nephropathy. We hypothesized that rats immunized with anti-Fx1A develop autoantibodies (auto-Abs) to Crry as well as to the megalin-containing HN antigenic complex, and that anti-Crry Abs promote the development of injury in HN by neutralizing the complement regulatory activity of Crry. Rats immunized with Fx1A lacking Crry remained free of proteinuria and glomerular deposits of C3 during a 10-wk follow-up despite typical granular immunoglobulin (Ig)G deposits in glomeruli. Anti-Fx1A auto-Abs were present in their sera at levels that were not different from sera pooled from proteinuric rats with HN induced with nephritogenic Fx1A. Passive administration of sheep anti-Crry Abs to rats immunized with Crry-deficient Fx1A led to proteinuria and glomerular C3 deposition, which were not seen in such rats injected with preimmune IgG, nor in rats with collagen-induced arthritis injected with anti-Crry IgG. To directly examine the role of Crry in HN, rats were immunized with Crry-deficient Fx1A reconstituted with rCrry. This led to typical HN, with 8 out of 15 rats developing proteinuria within 14 wk. Moreover, the extent of glomerular C3 deposition correlated with proteinuria, and anti-Crry Abs were present in glomerular eluates. Thus, Crry is a key nephritogenic immunogen in Fx1A. Formation of neutralizing auto-Abs to Crry impairs its function, leading to unrestricted complement activation by Abs reactive with the HN antigenic complex on the epithelial cell surface.

**Key words:** complement regulation • Heymann nephritis • Crry • autoantibodies • glomerulus

Heymann nephritis (HN)<sup>1</sup> is a widely studied model of membranous nephropathy with many similarities to the human disease (1, 2). Active immunization of susceptible rat strains with fraction 1A (Fx1A), a crude renal tubular preparation, induces the production of IgG auto-Abs that accumulate in glomeruli. Within 8 wk of immunization with Fx1A, ~60% of animals develop proteinuria, which is associated with glomerular deposition of C3 and C5b-9 (3, 4). As shown in passive HN, heterologous anti-Fx1A, when injected into rats, reacts with antigens on the glomerular epithelial cell surface and activates complement. In this model, the assembly of C5b-9 on glomerular epi-

thelial cell membranes is directly pathogenic (5–7). Similar mechanisms are presumed to be operative in active HN.

Much effort has gone into identifying the antigen(s) contained within Fx1A that confer pathogenic potential to this immunogen. One well-described antigen was originally termed gp330 and is now known as megalin (8–10). Active immunization with megalin, or passive administration of anti-megalin Ab, leads to glomerular deposition of immune complexes. Yet despite the accumulation of IgG Ab in immune deposits, there is no glomerular C3 or C5b-9 deposition and animals do not develop abnormal proteinuria (2, 11).

Crry (complement receptor 1-related protein/gene y) is a rodent complement inhibitor that combines the functions of human decay-accelerating factor (CD55) and membrane cofactor protein (CD46). Although not a true genetic homologue, Crry is considered a functional analogue of these two proteins, and is widely expressed (12–17). Not surpris-

<sup>1</sup>Abbreviations used in this paper: CIA, collagen-induced arthritis; Crry, complement receptor 1-related protein/gene y; HN, Heymann nephritis; IF, immunofluorescence.

ingly, given that Fx1A is a crude extract of renal tubules which contain Crry (18, 19), heterologous anti-Fx1A Abs contain reactivity towards Crry. These Abs inhibit the function of Crry in cultured glomerular epithelial cells leading to unrestricted complement activation (20, 21).

Since Crry is contained in Fx1A, we were interested in determining if autoAbs were generated to Crry and whether these were of pathogenic importance in HN. In this study, animals immunized with Fx1A lacking Crry did not develop abnormal proteinuria or glomerular C3 deposition despite the formation of anti-Fx1A Abs, which formed typical glomerular subepithelial deposits. Nephritogenicity of this preparation could be reconstituted by passive transfer of anti-Crry Abs or by inclusion of rCrry in the immunogen.

## Materials and Methods

**Fx1A and rCrry.** Fx1A was prepared from normal Sprague-Dawley rat kidneys by standard techniques (1). One batch was made from freshly isolated Sprague-Dawley rat kidneys, which led to typical HN upon active immunization of Lewis rats. The second Fx1A preparation was isolated from commercially obtained Sprague-Dawley rat kidneys (Pel-Freez Biologicals, Rogers, AR). This failed to induce classic disease manifestations of HN, the reasons for which are studied here.

The five NH<sub>2</sub>-terminal short consensus repeats of Crry (22, 23) containing the active sites of Crry were produced as a recombinant soluble protein in *Pichia pastoris*. rCrry was purified by sequential Mono Q and Mono P chromatography (Amersham Pharmacia Biotech, Piscataway, NJ; reference 24).

**Anti-Crry Abs.** Serum was obtained from a single sheep hyperimmunized with rCrry. IgG was isolated by protein G affinity chromatography (Amersham Pharmacia Biotech). Preimmune IgG from the same sheep was used as a control in these studies. Anti-Crry F(ab')<sub>2</sub> was generated by pepsin treatment followed by size exclusion chromatography on a Sephacryl S-100HR column. No intact heavy chains were present by SDS-PAGE under reducing conditions.

**Animal Studies.** HN was induced by immunizing 150-g female Lewis rats with Fx1A. 10 mg of Fx1A was mixed with 4 mg of *Mycobacterium butyricum* in 0.1 ml of PBS and emulsified with an equivalent volume of complete Freund's adjuvant (Difco, Detroit, MI), which was injected in equally divided doses into both hind footpads. Starting 6 wk after immunization, animals were housed biweekly in metabolic cages for urine collection. Sera were obtained at death by cardiac puncture. Renal tissue was obtained by survival renal biopsy and at death.

To have controls with an actively produced autoimmune disease, collagen-induced arthritis (CIA) was induced contemporaneously in Lewis rats. These animals were immunized with 0.2 mg of bovine type II collagen (Sigma Chemical Co., St. Louis, MO) in incomplete Freund's adjuvant twice over 7 d (25). This resulted in a polyarticular arthritis, which peaked in severity by 4 wk after immunization and then resolved.

Passive administration of anti-Crry Abs was performed by injecting animals with 10 mg of anti-Crry IgG or F(ab')<sub>2</sub> intravenously given in divided doses over 2 d. Control animals received 10 mg of preimmune IgG intravenously over 2 d.

**Measures of Disease.** Urinary protein excretion was measured by a sulfosalicylic acid precipitation method (26). Proteinuria

was considered abnormal when excretion was  $\geq 6$  mg/d (1). Direct immunofluorescence (IF) was performed on cryostat sections for rat IgG and C3 (26). Semiquantitative scores for IF staining intensity were assigned to coded sections (27).

To measure glomerular anti-Fx1A and anti-Crry Abs, glomeruli were isolated from one kidney by sieving (6), after which Abs were eluted by sonication in 20 mM of citric acid, pH 3.2, in the presence of protease inhibitors (11). Glomerular protein content was determined by a bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

**ELISA.** ELISAs for anti-Fx1A and anti-Crry Abs were performed by coating polystyrene plates with 10  $\mu$ g/ml detergent-solubilized Fx1A or rCrry. Diluted sera or glomerular eluates were added, which were detected with peroxidase-conjugated anti-rat IgG (Sigma Chemical Co.), followed by development with *o*-phenylene-diamine. Measurements were made at OD<sub>450</sub>, and data are presented as OD<sub>450</sub> U/ml. When comparisons were made, samples were run in duplicates on a single ELISA plate. For measurements of Crry, polystyrene plates were coated with 20  $\mu$ g/ml of anti-Crry IgG, after which serial dilutions of rCrry or detergent-solubilized Fx1A were added. Bound Crry was detected with biotinylated anti-Crry, followed by streptavidin-horseradish peroxidase. The ELISA was sensitive to 10 ng/ml Crry.

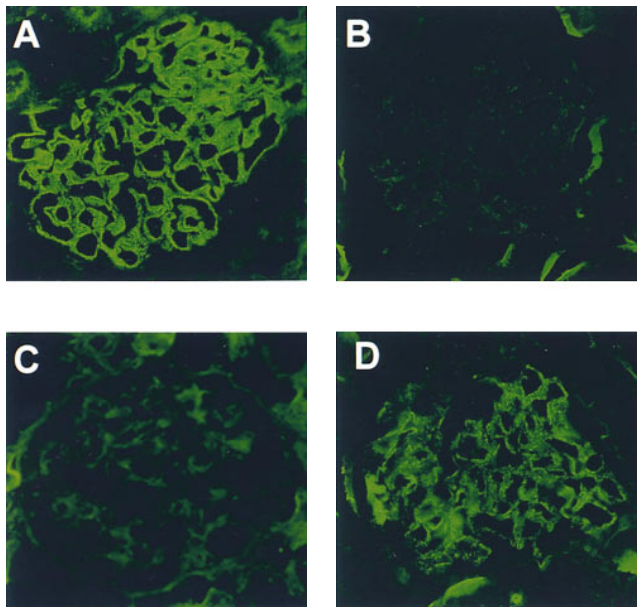
**Statistics.** Statistical analyses were performed with Minitab software (College Park, MD). Data are expressed as mean  $\pm$  SEM and were analyzed by Student's *t* test when two groups were compared or by analysis of variance for multiple groups.

## Results

Two preparations of Fx1A were compared in our studies. These preparations differed in their capacity to induce classic HN, as defined by the development of abnormal proteinuria in the majority of animals starting 6–8 wk after immunization. The nephritogenic preparation of Fx1A isolated from freshly obtained kidneys contained 3.1% Crry (wt/wt), whereas the Fx1A that was prepared from freeze-thawed kidneys failed to induce disease manifestations of HN and lacked Crry by a sensitive ELISA. These results suggested that Crry was an important component in nephritogenic Fx1A.

The Fx1A preparation lacking Crry was further studied through the active immunization of 12 female Lewis rats. Animals were followed for 10 wk, during which time no significant proteinuria occurred ( $0.98 \pm 0.12$  and  $2.14 \pm 0.53$  mg/d at 6 and 10 wk, respectively, after immunization). At this time, all animals underwent renal biopsy. By IF microscopy there was strongly positive granular staining for IgG outlining glomerular capillary walls in 10 out of 12 animals (Fig. 1 A), whereas the remaining 2 animals had only faintly apparent IgG deposits. In all animals, characteristic deposits of C3 were absent (Fig. 1 B).

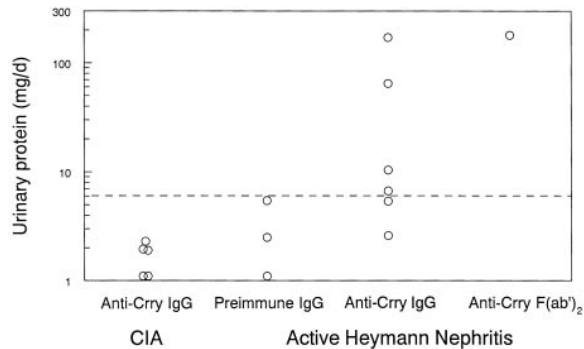
To test if the absence of C3 deposits and proteinuria in HN rats immunized with Crry-deficient Fx1A was due to preservation of glomerular Crry complement regulatory activity, function-neutralizing anti-Crry Abs were administered passively. HN animals received either anti-Crry IgG or preimmune IgG as a control 10 wk after immunization with Crry-deficient Fx1A. For these studies, the 10 animals



**Figure 1.** Representative IF micrographs in animals immunized with Fx1A lacking Crry. 10 wk after immunization, renal biopsies were performed and tissue was stained for IgG (A) and C3 (B). Animals were then injected with preimmune IgG (C) or anti-Crry IgG (D). 6 d later, animals were killed and renal tissue was stained for C3 (C and D). The IF micrographs shown in A, B, and D are from the same animal, and show typical granular staining for IgG in HN (A) and the appearance of a similar staining pattern for C3 only after injection of anti-Crry IgG (D).

with strongly positive glomerular IgG staining were used. To exclude an effect of anti-Crry IgG to activate the classical pathway, anti-Crry F(ab')<sub>2</sub> was also used in these studies. As further controls, Lewis rats in which CIA had been induced 10 wk earlier were injected with anti-Crry IgG. HN animals injected with anti-Crry had a progressive rise in urinary protein which peaked 5 d after Ab injection, with five out of seven animals developing abnormal proteinuria (Fig. 2). In contrast, there was no significant proteinuria in either HN rats injected with preimmune IgG or CIA rats injected with anti-Crry IgG. In the three animals with heavy proteinuria (>60 mg/d), there was strong staining for C3 by IF microscopy in glomeruli (Fig. 1 D), whereas the remaining animals with <60 mg/d proteinuria continued to have absent C3 staining (Fig. 1 C).

To determine whether absence of Crry affected Ab production to Fx1A, anti-Fx1A Abs were measured in sera of rats immunized with Fx1A lacking Crry. These were present before anti-Crry injection ( $4.12 \pm 0.55$  OD<sub>450</sub> U/ml) and were not different from anti-Fx1A levels in pooled sera from animals with active HN induced with nephritogenic Fx1A ( $4.72$  OD<sub>450</sub> U/ml). Anti-Crry Abs were also detectable ( $2.22$  OD<sub>450</sub> U/ml) in these latter sera, whereas in animals immunized with anti-Fx1A lacking Crry, anti-Crry levels were not significantly different from background ( $0.18 \pm 0.12$  OD<sub>450</sub> U/ml). Thus, immunization of animals with an anti-Fx1A preparation lacking Crry leads to the production of anti-Fx1A Abs that deposit in glomeruli.

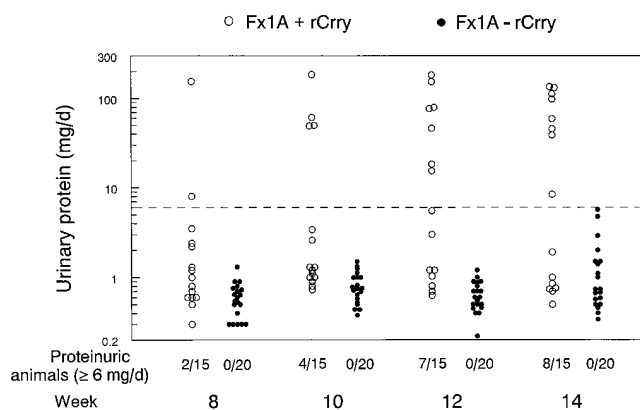


**Figure 2.** Effect of anti-Crry on urinary protein excretion in active HN. Animals were immunized with Fx1A lacking Crry (*Active Heymann Nephritis*) or with bovine type II collagen (*CIA*). 10 wk later, 5 mg of anti-Crry IgG or F(ab')<sub>2</sub>, or preimmune IgG, were injected intravenously twice over a period of 24 h. 5 d after intravenous injection, urine was collected and urinary protein was measured by the sulfosalicylic acid precipitation method (1). Proteinuria was considered abnormal when  $\geq 6$  mg/d (horizontal dashed line). Data for individual animals are shown.  $P = 0.022$  by one-way analysis of variance.

Yet complement activation and proteinuria do not occur. Neutralization of Crry function via the passive administration of anti-Crry Abs can lead to complement activation and proteinuria in the presence of these glomerular-bound IgG Abs.

These results suggested that generation of an immune response to Crry is necessary for the development of proteinuria in HN. The next set of studies directly tested whether reconstituting Fx1A lacking Crry with rCrry could restore nephritogenicity. Therefore, female Lewis rats were immunized with Fx1A lacking Crry ( $n = 20$ ) or with this same Fx1A preparation to which 0.5 mg of rCrry was added ( $n = 15$ ). Animals were observed for 14 wk to ensure that the potential development of proteinuria was not missed. As in the previous studies, animals immunized with Fx1A lacking Crry did not develop proteinuria (Fig. 3). In contrast, in animals immunized with Fx1A reconstituted with rCrry there was progressive development of proteinuria, such that by 14 wk after immunization, 8 out of 15 animals had significant proteinuria. This proportion of proteinuric rats is very similar to that typically seen in active HN (1). At death, anti-Fx1A Ab levels were comparable in both groups ( $2.17 \pm 0.23$  and  $2.17 \pm 0.28$  OD<sub>450</sub> U/ml).

In both groups of animals there was granular glomerular IgG staining by IF microscopy, yet in the animals immunized with Fx1A lacking Crry there was no glomerular C3 deposition. In contrast, animals immunized with Fx1A plus rCrry had glomerular C3 deposition. Furthermore, the semiquantitative score for C3 immunostaining correlated with proteinuria ( $r = 0.80$ ,  $P < 0.001$ ), suggesting that complement activation within glomeruli was pathogenic. Anti-Crry Abs were present in glomerular eluates of rats immunized with Fx1A plus rCrry ( $0.71 \pm 0.19$  OD<sub>450</sub> U/ $\mu$ g glomerular protein). As expected, anti-Crry Abs were not present in glomeruli of rats immunized with the Fx1A



**Figure 3.** Urinary protein excretion over time in animals immunized with Fx1A lacking Crry (Fx1A - rCrry, ●) or immunized with this identical preparation reconstituted with rCrry (Fx1A + rCrry, ○). Data for individual animals are shown. Proteinuria was considered abnormal when  $\geq 6$  mg/d (horizontal dashed line). At 14 wk after immunization, urinary protein excretion was  $42.4 \pm 13.5$  and  $1.4 \pm 0.3$  mg/d in animals immunized with Fx1A with and without rCrry, respectively (mean  $\pm$  SEM;  $P < 0.001$  by Student's *t* testing).

preparation lacking Crry ( $0.06 \pm 0.05$  OD<sub>450</sub> U/ $\mu$ g glomerular protein, which is not significantly different from background). Thus, in HN, active immunization with Fx1A containing Crry leads to formation of anti-Crry Abs that deposit in glomeruli.

## Discussion

In this study we show that rats immunized with Fx1A lacking Crry do not develop proteinuria or C3 deposition in glomeruli, despite the generation of anti-Fx1A Abs that deposit in glomeruli. If Abs to Crry are reconstituted either via the passive administration of anti-Crry Abs or by the inclusion of rCrry in the immunogen, animals develop proteinuria to the extent and level seen in active HN induced with Crry-containing Fx1A. These findings show that Crry is a critical immunogen within Fx1A and is essential for its nephritogenic effect. Formation of Abs to Crry impairs the function of this intrinsic complement regulator. Since Crry is the key regulator of complement at the C3/C5 convertase stage, inhibition of its function leads to unrestricted complement activation. As shown in the passive HN model of membranous nephropathy, complement activation on the glomerular epithelial cell results in injury of this cell and impairment of the glomerular permselectivity barrier to the passage of protein.

In our past studies, we found that heterologous anti-Fx1A Abs reacted with glomerular epithelial cell antigens

and could activate complement on the surface of these cells, leading to cellular injury (6). Subsequently, we observed that nephritogenic anti-Fx1A impaired intrinsic complement regulation in glomerular epithelial cells. Such an effect occurred at the level of complement C3/C5 convertases formed from either the classical or alternative pathways (20). At that time, the antigen responsible for these effects of anti-Fx1A was unknown. With the isolation of rat Crry as the antigen reactive with mAb 512 (28), it was possible to prove that anti-Fx1A reacted with rat Crry, consistent with its inhibiting intrinsic regulation of C3/C5 convertases (21). That heterologous anti-Fx1A Abs contains reactivity towards Crry is not surprising, given the widespread distribution of Crry, including in renal tubules (18, 19). Interestingly, we have also shown that heterologous anti-Fx1A has reactivity towards CD59, a regulator of C5b-9 formation, which can similarly be attributed to the presence of CD59 in renal tubular cells (18). The role for reactivity of heterologous anti-Fx1A with Crry or CD59 in passive HN is a subject of ongoing study.

Despite the identification of megalin as an unquestionably important immunogen within Fx1A, it is very clear that megalin is not the only nephritogenic component contained within Fx1A. Thus, either passive administration of antimegaline Ab or active immunization with megalin is insufficient to recapitulate the full disease of HN. This has stimulated a continuing search for additional antigenic component(s). A number of candidate antigens have been identified, including  $\beta 1$  integrins, dipeptidyl peptidase IV, aminopeptidase A, and an uncharacterized lipid antigen (29–31).

Although HN has many similarities to human membranous nephropathy, identifying target human antigens in this disease has proved elusive. For instance, as yet there is no identified analogue to megalin or the HN antigenic complex expressed in human glomeruli (8–10, 32). Human glomerular epithelial cells contain decay-accelerating factor and membrane cofactor protein (33, 34), which together contain the function of Crry. There are subepithelial IgG deposits in human membranous nephropathy, exactly as in HN. The evidence for the complement mediation of this disease in humans is strong. There is glomerular deposition of C3 and C5b-9, and the appearance of C5b-9 in the urine has prognostic importance (35–37). An intriguing possibility raised by these studies is that autoAbs to decay-accelerating factor and/or to membrane cofactor protein may form and accumulate in human membranous nephropathy. If so, it may be possible, with the use of recombinant complement regulators (38–42) to overcome the effects of these autoAbs and thereby prevent glomerular epithelial cell injury and proteinuria.

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