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J Immunol 2007; 179:172-178; ;
doi: 10.4049/jimmunol.179.1.172
<http://www.jimmunol.org/content/179/1/172>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Induction of Passive Heymann Nephritis in Complement Component 6-Deficient PVG Rats¹

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Passive Heymann nephritis (PHN), a model of human membranous nephritis, is induced in susceptible rat strains by injection of heterologous antisera to rat renal tubular Ag extract. PHN is currently considered the archetypal complement-dependent form of nephritis, with the proteinuria resulting from sublytic glomerular epithelial cell injury induced by the complement membrane attack complex (MAC) of C5b-9. This study examined whether C6 and MAC are essential to the development of proteinuria in PHN by comparing the effect of injection of anti-Fx1A antisera into PVG rats deficient in C6 (PVG/C6⁻) and normal PVG rats (PVG/c). PVG/c and PVG/C6⁻ rats developed similar levels of proteinuria at 3, 7, 14, and 28 days following injection of antisera. Isolated whole glomeruli showed similar deposition of rat Ig and C3 staining in PVG/c and PVG/C6⁻ rats. C9 deposition was abundant in PVG/c but was not detected in PVG/C6⁻ glomeruli, indicating C5b-9/MAC had not formed in PVG/C6⁻ rats. There was also no difference in the glomerular cellular infiltrate of T cells and macrophages nor the size of glomerular basement membrane deposits measured on electron micrographs. To examine whether T cells effect injury, rats were depleted of CD8⁺ T cells which did not affect proteinuria in the early heterologous phase but prevented the increase in proteinuria associated with the later autologous phase. These studies showed proteinuria in PHN occurs without MAC and that other mechanisms, such as immune complex size, early complement components, CD4⁺ and CD8⁺ T cells, disrupt glomerular integrity and lead to proteinuria. *The Journal of Immunology*, 2007, 179: 172–178.

Both the active (HN)³ (1) and passive forms of Heymann nephritis (PHN) (2) are animal models of human membranous nephropathy (3). PHN is induced in susceptible rat strains by injection of heterologous antiserum from sheep or rabbits that have been immunized with a crude extract of rat renal tubular Ag known as Fx1A (2) which includes the main target Ag, megalin (4, 5). Treatment with anti-Fx1A leads to the rapid development of subepithelial glomerular basement membrane (GBM) deposits of IgG and complement (C). Activation of the complement cascade by IgG binding to Fx1A on glomerular epithelial cells (GEC) results in insertion of the membrane attack complex (MAC) of C5b-9 into GEC which is thought to mediate sublytic injury (6, 7). Proteinuria in PHN, and by consequence human membranous nephropathy, has been suggested to be entirely dependent on C5b-9/MAC-mediated injury, especially to GEC (8).

In HN, it has been shown that C5b-9/MAC is not an obligatory effector pathway, as HN can be induced in PVG C6-deficient (PVG/C6⁻) rats (9). This study correlated the degree of proteinuria with the immune complex size in the glomeruli and suggested that large complexes lead to proteinuria. Our group has demonstrated that CD8⁺ T cells are critical in the development of proteinuria in HN (10), in that depletion of CD8⁺ T cells totally eliminated the development of proteinuria.

These findings in HN, a disease model that is closely related to PHN, raised the question as to whether C6/MAC was essential for the rapid onset of proteinuria observed in PHN and whether there were fundamentally different mechanisms of immunological injury in the two models. This study induced PHN with sheep anti-Fx1A in both PVG/C6⁻ and PVG/c rats to ascertain whether there is a difference in glomerular injury, and thereby whether C6 and thereby C5b-9/MAC deposition in glomeruli are mandatory for the induction of PHN. As both strains developed proteinuria, we also examined whether PVG/C6⁻ rats had reduced immune complex size and whether CD8⁺ T cell depletion affected proteinuria.

Materials and Methods

Experimental animals

Inbred male PVG/c and PVG/C6⁻ were bred in the animal facility at Liverpool Hospital. The characteristics of the PVG/C6⁻ rats has been described elsewhere (11) and the strain backcrossed from PVG/C6⁻, provided by Dr. P. Leenaerts (Catholic University, Leuven, Belgium), onto the PVG/c strain at Liverpool has been described (12). Both strains have an identical PVG/c background and accept cross-skin grafts. Weight-matched male PVG/c and PVG/C6⁻ rats 6–8 wk of age were used. All animal care and operative procedures were as described (13). The Animal Care and Ethics Committee of University of New South Wales approved all protocols.

Hemolytic complement assay

The hemolytic complement assay described by Kent (14, 15) was modified as described (16). Briefly, anti-RBC serum was produced in SD rats immunized with human RBCs and serum was prepared by inactivation of

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Received for publication August 21, 2006. Accepted for publication April 24, 2007.

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¹ This work was supported by the National Health and Medical Research Council (NH&MRC) of Australia, Australian Kidney Foundation, the South Western Sydney Area Health Service, and Ingham Research Foundation. S.T.S. was an NH&MRC PhD Scholar.

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³ Abbreviations used in this paper: HN, active Heymann nephritis; PHN, passive Heymann nephritis; GBM, glomerular basement membrane; GEC, glomerular epithelial cell; MAC, membrane attack complex; ATx, adult thymectomy; EAE, experimental autoimmune encephalomyelitis; EM, electron microscopy.

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complement at 56°C for 60 min, before storage at -20°C. On the day of the assay, a rat Ab labeled human RBC solution was made as described (16) and rat serum for assay, added in 96-well round-bottom microtiter plate wells (Bio-Rad), were then incubated for 60 min at 37°C. Each plate was run with control specimens in triplicate, which included normal and complement-deficient serum samples, as well as 5% Triton X-100 which lysed 100% RBC. Red discoloration of the sample fluid indicated complement-dependent RBC lysis, while clear sample fluid with RBC sediment indicated lack of complement activity. A total of 150 ml of supernatant from each well was transferred to a flat-bottom 96-well microtiter plate and absorbance was read at 540 nm in a Bio-Rad plate reader. Results were expressed as a mean percentage absorbance compared with the Triton X-100 wells which were considered to represent 100% lysis. To confirm that PVG/C6⁻ rats were specifically deficient of C6, human C6 (Sigma-Aldrich) was added and restored hemolytic activity.

Induction and monitoring of PHN

PHN was induced according to methods described by Salant and Cybulsky (17). Fx1A was prepared from outbred Sprague-Dawley rat kidneys as described (18, 19). Anti-Fx1A was produced by immunization of sheep with the rat Fx1A preparation and purified as described (20). Following dose-finding studies and using a protocol supplied by Dr. D. Salant (Boston University Medical Center, Boston, MA), 0.6 ml of anti-Fx1A (20 mg/ml) was injected i.v. on 2 consecutive days to induce PHN. Controls were given a similar volume of normal sheep serum.

Assessment of disease activity

Twenty-four-hour urine samples were collected in metabolic cages (Nalgene) at 0, 3, 7, 14, and 28 days following anti-Fx1A injection. The rats had access to water ad libitum during the 24-h urine collections. Urine protein concentrations were determined by colorimetric assay (Bio-Rad) and expressed as mg/100 g/24 h.

When rats were sacrificed at day 28, kidneys were biopsied to prepare frozen sections and sections were fixed for ultrastructure studies. The remainder of the kidney was used to prepare isolated glomeruli to >95% purity by graded sieving, as described (21, 22).

Abs and immunostaining

Abs used were GARa, a polyclonal sheep anti-rat C3c (Nordic Immunology), rabbit anti-rat C9 (a gift of S. Piddleston, Alfred Hospital, Parhan, Victoria, Australia), goat anti-mouse Ig FITC labeled (Caltag Laboratories), mAb used were ED-1 (macrophages), R73 (TCR- $\alpha\beta$), MRC Ox-35 (CD4), MRC Ox8 (CD8), 3.2.3 (NK Cells), MRC Ox6 (class II MHC), MRC Ox12 (B cells, Ig κ -L chain) and were purchased from BD Pharmingen. PBL subset staining used the previously described methods (10). Renal cortex sections and isolated glomeruli were prepared from animals kidneys at day 28 after disease induction and were stained with Abs using an immunoperoxidase method, as described (22).

Ig and complement deposition was scored on a semiquantitative score: 0, no staining; 1+, sparse staining; 2+, intermediate diffuse staining; and 3+, heavy diffuse staining. Mononuclear cells were counted in multiple glomeruli or sections and expressed as mean \pm SD (number of cells per glomeruli or per high power field).

Electron microscopy (EM)

Segments of day 14 renal cortex biopsies were preserved in formalin, washed in sodium cacodylate buffer, then placed in EM fixative for 2 h. EM fixation of samples used 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 4 h, before rinsing in 2% sodium acetate, then staining with 2% uranyl acetate for 1 h. This was followed by dehydration in a graded alcohol series over 2 h, dehydration in dry acetone for 40 min, infiltration with Spurr resin/acetone solutions of 1:1 then 9:1, and then finally polymerization in 100% Spurr resin at 70°C. Ultra-thin sections were cut at 120 nm before examination on a Zeiss EM 109T transmission electron microscope (Carl Zeiss) at 80 kV.

EM images of whole capillary loops (magnification \times 20,000) were obtained and subepithelial deposit area as a percentage of total GBM area was quantified using image analysis software (NIH Image version 1.6.1; National Institutes of Health) according to the method of Danilewicz (23). In regions of interest, lines were drawn around GBM to calculate total GBM area. Then lines were drawn around each deposit to calculate a total deposit area for each capillary loop segment. To calculate a deposit index for each experimental animal, the total deposit area was divided by the total GBM area and was expressed as a percentage. A minimum of 10 capillary loop segments was assessed from each of four rats from each experimental group.

Table I. Proteinuria (mg/100 g/day \pm SEM) following injection of anti-Fx1: comparison in PVG and PVG/C6⁻ rats

Treatment Group	Sheep Serum <i>n</i> = 6		Anti-Fx1A <i>n</i> = 9	
	PVG	PVG/c6 ⁻	PHN-PVG	PHN-PVG/c6 ⁻
Day 0	4.9 \pm 2.8	5.0 \pm 2.6	5.2 \pm 1.1	3.7 \pm 1.0
Day 3	3.9 \pm 1.8	3.9 \pm 1.0	10.3 \pm 3.2 ^a	9.3 \pm 2.8 ^a
Day 7	3.6 \pm 1.5	8.6 \pm 1.6	16.5 \pm 4.5 ^a	13.8 \pm 3.7 ^a
Day 14	6.1 \pm 1.4	4.4 \pm 1.2	22.0 \pm 5.4 ^a	20.3 \pm 5.2 ^a
Day 28	7.5 \pm 3.8	7.4 \pm 3.3	57.2 \pm 10.2 ^a	66.4 \pm 27.6 ^a

^a Value of *p* < 0.02 when compared with controls treated with sheep serum.

Method of CD8⁺ T cell depletion

Groups of PVG/C6⁻ rats were permanently depleted of CD8⁺ T cells by adult thymectomy (ATx) 1 mo before disease induction, then treatment with the anti-CD8 mAb MRC Ox8, as described (10, 24). MRC Ox8 cell supernatant, prepared as described (10), was administered daily for 10 days concluding ~1 wk before disease induction. CD8⁺ T cell depletion was confirmed by FACS analysis of peripheral blood taken at days -1 and 28 after disease induction. Control thymectomized PVG/C6⁻ rats were treated with A6, an isotype control mAb, which has no reactivity with rat cells (25).

Statistics

Data were expressed as means and SEs on the mean. Comparisons between groups used ANOVA and the Bonferroni-Dunn multiple comparisons post-hoc test using Statview for Macintosh 5.0 (Abacus Concepts). A value of *p* \leq 0.05 was considered significant unless reduced by Bonferroni-Dunn corrections.

Results

PHN induced in both PVG/C6⁻ and PVG/c rats

All PVG/C6⁻ rats tested were confirmed to be C6 deficient by screening their serum in the hemolytic complement activity. This serum did not lyse the RBC. Following i.v. injection of anti-Fx1A antiserum, both PVG/c and PVG/C6⁻ rats developed similar amounts of proteinuria at all time points (Table I). As early as day 3, urinary protein was increased, being 10.3 \pm 3.2 mg/100 g/24 h in PVG/c and 9.3 \pm 2.8 mg/100 g/24 h in PVG/C6⁻. Proteinuria increased over time in both anti-Fx1A-treated PVG/c and PVG/C6⁻ rats, with no significant differences at 3, 7, 14, or 28 days (see Table I). At day 28, the PVG/c rats had 57.2 \pm 10.2 and PVG/C6⁻ had 66.4 \pm 27.6 mg/100 g/day of proteinuria. All rats in both groups developed significant proteinuria and at no time did the PVG/C6⁻ group of rats have less proteinuria. Control PVG/c and PVG/C6⁻ rats treated with sheep serum had normal levels of protein in urine and were always <10 mg/100 g/24 h. Thus, PVG/C6⁻ rats had the same degree of glomerular injury as PVG/c rats with both a heterologous phase in the first week and an autologous phase in the second to fourth week after injection of anti-Fx1A antiserum.

PVG/C6⁻ rats with PHN lack C9 deposition, but have levels of Ig and C3 similar to PHN in PVG rats

Isolated glomeruli obtained at day 28 revealed no difference in the deposition of sheep Ig, rat Ig, or rat C3 in either PVG/c or PVG/C6⁻ PHN groups. There was no C9 staining seen in the PVG/C6⁻ group, but C9 was abundant in glomeruli from PVG/c rats (Fig. 1A). This confirmed that MAC was not assembled in the PVG/C6⁻ rats but was in PVG/c rats.

PVG/C6⁻ and PVG/c rats have a similar T cell and macrophage infiltrate into glomeruli

Staining for mononuclear cell subsets infiltration into glomeruli identified that there was a significant increase in CD4⁺, CD8⁺,

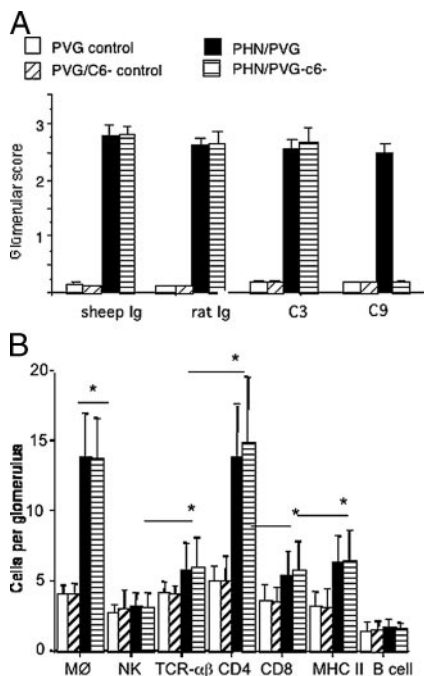


FIGURE 1. Quantitation of immune mediators and mononuclear cells in glomeruli of PVG/c and PVG/C6⁻ rats with PHN. *A*, Immune deposits of renal biopsy specimens identified by immunostaining for rat Ig, C3, and C9. The only difference was the lack of staining of C9 in PVG/C6⁻ rats. *B*, Cellular infiltrates in glomeruli identified by immunostaining of isolated glomeruli with mAb to T cells including TCR-αβ, CD8, CD4, macrophages (Mφ), NK cells, and class II MHC and to B cells. Cells per glomerulus, expressed as mean ± SD, of counts of 30 glomeruli from five rats per group. *, Significance ($p < 0.05$) compared with control PHN.

and TCR-αβ⁺ cells ($p < 0.05$) as well as macrophages and class II MHC-expressing cells ($p < 0.05$) in glomeruli from both PVG/c and PVG/C6⁻ rats with PHN, compared with control PVG/c and PVG/C6⁻ rats treated with sheep serum (Fig. 1*B*). There was no difference in cellular infiltrate detected between PVG/c and PVG/C6⁻ groups with PHN. There was no increase in NK cells or B cells. This infiltrate was very similar to the T cell and macrophage infiltrate we have described in Lewis rats developing HN (10, 22). It was assumed that the majority of CD8⁺ cells are T cells, as there was no NK cell infiltrate, and CD4 is expressed on macrophages, which also accounts for the class II MHC-expressing cells. Thus, by the end of 4 wk, there was a significant T cell and macrophage infiltrate into the glomeruli of both strains with proteinuria.

Immune complex size is similar in PVG/c and PVG/C6⁻ rats with PHN

As in HN PVG/C6⁻ rats, the degree of proteinuria was related to immune complex size (9); we examined glomeruli by electron microscopy of kidney sections taken at day 28. These showed typical subepithelial deposits in PVG/c and PVG/C6⁻ groups treated with anti-Fx1A (Fig. 2*A*) but not in control rats. Quantitative analysis of the glomerular deposit size found no significant differences in the GBM deposit index between groups. These were $10.47 \pm 3.7\%$ in PVG/c and $10.21 \pm 3.9\%$ in PVG/C6⁻ ($p = \text{NS}$) (Fig. 2*B*).

CD8⁺ T cell depletion prevents increased proteinuria in the autologous phase of PHN

To examine whether CD8⁺T cells were required for induction of proteinuria, both PVG/C6⁻ rats and PVG rats were permanently depleted of these cells by a combination of ATx and anti-CD8

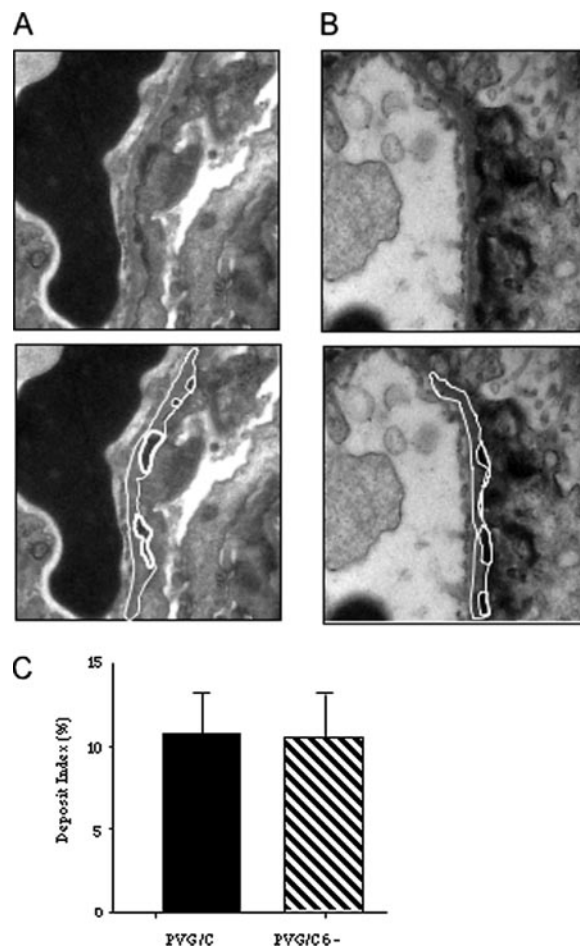


FIGURE 2. Estimation of immune complex size on electron micrographs of renal cortex biopsies that were taken 28 days after injection of anti-Fx1a Ab. The lower pictures show the white gating lines outlining the immune deposits seen in the subepithelial space and along the basement membrane to calculate deposit index. *A*, A representative sample from a PVG/c rat; *B*, from a PVG/C6⁻ rat. *C*, Comparison of deposit index between PVG/c and PVG/C6⁻ rats with PHN; $n = 4/\text{group}$. Deposit index is expressed as a percentage of the size of immune deposit to the total GBM area. There was no significant difference in PVG/c and PVG/C6⁻ rats with PHN.

mAb treatment. CD8⁺ T cell depletion was confirmed on PBL subset analysis of all rats a day before giving anti-Fx1A and 28 days later (Fig. 3). The percentage of CD3⁺ cells that were CD8⁺ was $0.85 \pm 0.83\%$ at day -1 and $0.92 \pm 0.71\%$ at day 28 in ATx and anti-CD8 mAb treated—compared with $16.2 \pm 3.5\%$ at day -1 and $17.9 \pm 4.6\%$ at day 28 in ATx control A6 mAb-treated rats.

Thus, all rats that had received MRC Ox8 therapy had $<1\%$ CD8⁺CD3⁺ T cells in their blood before and at the end of the experiments, illustrated in Fig. 3. A smaller CD8⁺CD3⁻ population seen in controls (ATx and control mAb treated) as well as in normal PVG/C6⁻ rats was also depleted. These are most likely NK cells.

In PVG/C6⁻ rats permanently depleted of CD8⁺T cells, proteinuria developed 3–7 days after anti-Fx1A injection and was similar to untreated PVG/C6⁻ rats with PHN, as well as ATx PVG/C6⁻ rats with PHN that had been treated with an irrelevant control mAb, A6. However, at days 14 and 28 after anti-Fx1A treatment, the permanently CD8⁺ T cell-depleted PVG/C6⁻ rats had less proteinuria (Table II). In fact, there was stabilization of

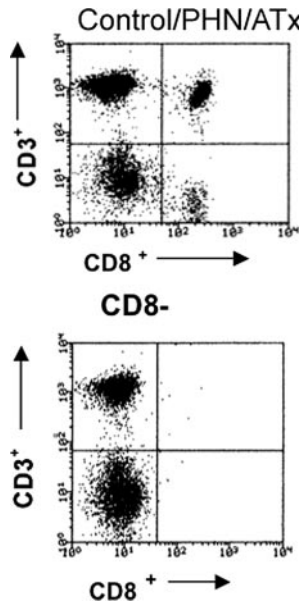


FIGURE 3. Flow cytometry analysis showed total depletion of CD3⁺CD8⁺ T cells in AT/MRC Ox8-treated PVG/C6⁻ rats. PBL from PVG/C6⁻ rats stained with mAb to CD3 and to CD8 and analyzed on a FACSscan. *Upper panel*, PVG/C6⁻ rat had been ATx and treated with control mAb A6 shows normal CD3⁺CD8⁺ T cells and some CD3⁺CD8⁻ cells that are probably NK cells. *Lower panel*, PVG/C6⁻ rat had been ATx and MRC Ox8 treated had virtually no CD8⁺ cells either CD3⁺ or CD3⁻.

the level of proteinuria in CD8⁺ T cell-depleted PVG/C6⁻ rats with mean levels remaining below 20 mg/100 g/day. Proteinuria had markedly increased by day 28 in the both the normal PVG/C6⁻ (78.8 ± 19.9 mg/100 g/day) and the ATx nondepleting mAb-treated PVG/C6⁻ (97.9 ± 23.2 mg/100 g/day) control groups that were treated with anti-Fx1A sera. The effect due to CD8⁺ T cell depletion was significant at day 14 (*p* < 0.05) and at day 28 (*p* < 0.002).

Examination of isolated glomeruli from permanently CD8⁺ T cell-depleted PVG/C6⁻ rats also did not reveal C9 deposition but there were deposits of sheep Ig, rat Ig, and C3 comparable to that in the other PHN groups that were not depleted of CD8⁺ T cells (Fig. 4A). Thus, there was deposition of the administered anti-Fx1A sera and development of a host Ig response that deposited in glomeruli and activated C3 but not C9/MAC. This deposition was

Table II. Proteinuria (mg/100 g/day ± SEM) following injection of anti-Fx1: comparison in CD8⁺ T cell-depleted and nondepleted PVG/C6⁻ rats

Group Treatment	PVG/c6 ⁻			
	Control	PHN	CD8 ⁰	ATx/A6
	Anti-Fx1A			
	Sheep serum (n = 3)	Anti-Fx1A (n = 5)	ATx/Ox-8 (n = 5)	ATx/A6 (n = 4)
0	8.4 ± 3.1	7.1 ± 3.2	8.2 ± 3.6	7.5 ± 4.4
3	5.6 ± 2.7	18.6 ± 3.1	16.9 ± 3.2	22.5 ± 13.7
7	3.2 ± 0.6	18.7 ± 5.7	17.7 ± 3.7	25.0 ± 12.5
14	4.9 ± 1.1	41.4 ± 12.5	17.8 ± 5.3 ^a	46.1 ± 27.2
28	7.4 ± 2.6	78.8 ± 19.9	15.4 ± 3.1 ^b	97.9 ± 23.2

^a Value of *p* < 0.05 compared to PHN and ATx/A6 group.

^b Value of *p* < 0.002 compared to PHN and ATx/A6 group.

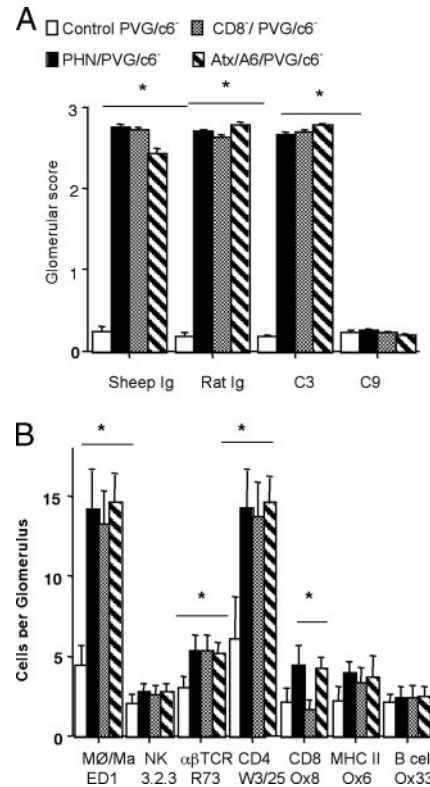


FIGURE 4. Isolated PVG/C6⁻ rats glomeruli with immunostaining for Ig, C3, C9, TCR, CD4, CD8, NK cells, and macrophages. Comparison of control given normal sheep serum, with PHN groups given sheep anti-Fx1A that had no other treatment (PHN), or had ATx and anti-CD8 mAb treatment or had ATx and control mAb (A6) treatment. *A*, All PHN had dense deposits of Ig, C3 but no C9. The absence of C9 in PVG/C6⁻ rats with PHN is clear as these were similar in appearance to controls, given normal sheep serum, with no PHN induced. *B*, In CD8-depleted rats, there was no CD8⁺ infiltrate into glomeruli and this was significantly less than PHN controls (*p* < 0.05). Other cellular infiltrates, including TCR-αβ, CD4⁺, class II MHC⁺, and macrophages, were similar to other PHN groups and all were significantly more than controls (*p* < 0.05). NK and B cells were at background levels in all groups as seen in controls.

large and indistinguishable from the other PHN groups that developed worsening proteinuria in the autologous phase.

The isolated glomerular mononuclear cell infiltrate studies showed that there was no CD8⁺ glomerular infiltrate in the CD8-depleted groups, but there was in the glomeruli of the two other PHN groups. The TCR-αβ⁺ and the CD4⁺ cell infiltration in the CD8⁺ T cell-depleted animals was similar to the nondepleted animals. Infiltration of macrophages and class II MHC-expressing cells in the CD8⁺ T cell-depleted hosts was comparable to that in the two other PHN groups (Fig. 4B). There was no infiltration of NK or B cells in any group. Thus, a selective absence of CD8⁺ cells in glomeruli of the CD8-depleted animals was associated with the reduced proteinuria. The macrophage and CD4⁺ and TCR-αβ⁺ infiltrate comparable to that in PHN with an autologous phase of proteinuria was by inference insufficient, even with the sheep anti-Fx1A Ig and rat Ig and C3 that had deposited in the glomeruli, to initiate the glomerular injury that increased proteinuria in the autologous phase of PHN.

Discussion

This study demonstrated that PHN was induced in PVG/C6⁻ rats that are deficient in complement component C6 and cannot form MAC. There was no difference in the severity of PHN induced in

PVG/C6⁻ rats as compared with PHN in normal PVG/c rats. Markers of disease activity such as degree of proteinuria, glomerular Ig, C3, and cellular infiltrate, and electron microscopy appearance of deposits were identical between groups. The only difference observed was glomerular deposition of complement component C9, a complement component only found in the terminal C5b-9 complex, in PVG/c but not in PVG/C6⁻ rats. This observation confirmed that C5b-9 complex, which is known as MAC, was not formed in PVG/C6⁻ rats. The fact that C6 deficiency does not protect from PHN is not consistent with the studies that describe C5b-9 as critical to the pathogenesis of PHN, summarized by Couser (8).

The PVG/C6⁻ rat is only deficient in C6 (11) and the ability to mediate complement-dependent lysis is restored with C6 alone. The PVG/C6⁻ rat strain has been shown to lack both antigenic and functional C6 (26) due to a 31-bp deletion in exon 10 of the C6 gene (27), but has an otherwise intact immune system (9). Complement component C6 plays an essential role in the formation of a stable terminal complement complex (28). The binding of C6 to C5b, to form C5b6, results in a complex that is capable of binding to membranes near the site of complement activation (28). Without C6, the terminal complex of the C5b-9 (MAC) complex required for cell lysis cannot be assembled. As there is no evidence that the pathogenesis of PHN in the PVG/C6⁻ rat strain is fundamentally different to PHN in PVG/c rats or other complement replete animals, our findings suggest that the mechanisms of injury in PHN must be independent of C6 and MAC.

PHN has come to be regarded as the archetypal complement-dependent nephritis model for a number of reasons. Pretreatment of rats with cobra venom factor that prevents deposition of C3 and subsequent activation of the complement cascade prevents proteinuria in PHN (29, 30). Glomerular deposition of C5b-9 is also prevented by treatment with cobra venom factor (29). The terminal components of complement have been shown to increase the albumin permeability of isolated glomeruli (31) and urinary C5b-9 excretion correlates with the development PHN (32). In 1989, Baker et al. (33) were unable to induce PHN in rats depleted of C6 with an anti-C6 Ab, a finding directly contrary to our findings. They found C3, C7, C8, and C9 levels were the same in anti-C6-treated rats (33). Depletion of C6 by means of anti-C6 Ab has also been used to inhibit disease in an in situ subepithelial immune complex nephritis in rats (34) and in a passive transfer model of autoimmune myasthenia gravis (35).

A number of experimental disease models have been studied in the PVG/C6⁻ strain. In the Thy1 nephritis model, PVG/C6⁻ rats developed less proteinuria and less apoptotic cells within glomeruli compared with normal PVG/c rats (36). In two other models of inflammatory glomerulonephritis, PVG/C6⁻ rats had similar reduction in proteinuria and protection from apoptosis (37). In puromycin aminonucleoside nephrosis, PVG/C6⁻ rats also have less tubulointerstitial inflammation than PVG/c control rats (38). PVG/C6⁻ rats are also protected from thrombotic microangiopathic glomerulonephritis (39). PVG/C6⁻ rats do not develop hyperacute rejection of xenografts (40), and have delayed rejection of allografts, but normal T cell responses (12). In experimental autoimmune encephalomyelitis (EAE), PVG/C6⁻ rats had less paralysis and less T cell and macrophage infiltrate into the brain (16). EAE is a disease mediated by CD4⁺ T cells and macrophages and is not thought to be Ab and complement mediated. In EAE, there was reduced expression of P-selectin and ICAM on vascular endothelium (16), which was considered to be due to loss of the sublytic actions of MAC which includes activation of vascular endothelium to promote mononuclear cell infiltration into sites of immune inflammation (16). These studies in PVG/C6⁻ rats established the

role of the C5b-9 terminal complement complex in these disease models, whether it be lytic or sublytic actions of MAC.

In both phases of PHN, lytic or nonlytic effects of the C5b-9 complex have been thought to injure glomeruli and lead to proteinuria. The insertion of the C5b-9 complex into cell membranes results in lysis of the cell if molecules that protect against the formation and action of MAC are overcome. For nucleated cells including GECs, the response to C5b-9 insertion is usually activation rather than lysis (41). Sublytic GEC injury/activation by MAC leads to abnormal GBM matrix formation and the typical thickening of the GBM that is seen in both HN and PHN (8). Podocytes produce collagen in response to injury by C5b8 and C5b-9 (42). The C5b6 complex in concert with C7, 8, or 9 also leads to release of eicosanoids, oxygen radicals, and IL-1 from a number of cell types (41, 43).

Not all studies support a central role for C5b-9 in the development of proteinuria in PHN. Infiltration of inflammatory mononuclear cells does occur early in both glomeruli and interstitium during PHN (30, 44) and other mediators of proteinuria have been noted in PHN, including leukotriene B4 (45) and reactive oxygen species (46). Monocytes and earlier complement components have also been shown to be important in the mediation of injury in PHN (30, 47). Anaphylotoxin, C5a, reduces renal blood flow when infused into rat kidneys (48). As C5a is intact in the C6-deficient rats, its role in mediation of injury in PHN has not been excluded.

The findings in this study were similar to those in HN which can be induced in PVG/C6⁻ rats (9) and showed that C5b-9 was not essential in this model. In this HN study, immune complex size was similar in PVG/c and PVG/C6⁻ rats and was correlated with the degree of proteinuria. We also found GBM deposit size was also similar in PVG/c and PVG/C6⁻ rats with PHN. The deposition of Ab at the base of the GEC and the in situ formation of immune complexes in the GBM has been shown to cause charge and hemodynamic effects capable of causing proteinuria (49). The structural effect on the GBM of enlarging sub-GEC deposits may cause proteinuria (9, 50, 51). The mechanical effects of these sub-GEC deposits such as retraction of GEC and reduced density of anionic sites in the lamina rara externa may be critical to the development of proteinuria (50). A number of other local mediators have been postulated to damage GBM and GEC, such as reactive oxygen species (52) and PGs (53). Increased expression of the transmembrane form of heparin-binding epidermal growth factor in the GBM has also been associated with the development of proteinuria in PHN and is postulated to contribute to proteinuria through abortive GEC mitogenesis (20). Activation of GEC NF- κ B also occurs and may contribute to autologous phase proteinuria (54). Heparanase, an enzyme responsible for loss of anionic sites on the GBM, is up-regulated during the heterologous and autologous phases of PHN. Inhibition of the activity of heparanase reduces proteinuria during the autologous phase (55).

The increased macrophage and CD8⁺ cell infiltrate in PHN was similar to that observed in HN in Lewis rats (10). In HN, not only do Tc1 lymphocytes and macrophage infiltrates parallel the development of proteinuria (22), but CD8⁺ T cell depletion prevents disease without affecting anti-Fx1a Ig formation or deposition (10). Administration of the Th2 cytokine IL-4 also blocks active HN by reducing CD8⁺ T cells in glomeruli, while stimulating Ab responses including anti-Crry Abs (13). In HN, there is restricted TCRV- β usage in cells infiltrating glomeruli and interstitium (56) and immunization with naked DNA for these TCRV- β reduced proteinuria (57), suggesting T cells directed at a selected epitope mediate injury. Collectively, these studies in HN found CD8⁺ T cells, particularly CTLs, mediate injury in HN. The presence of

CD8⁺ and T cells in the glomeruli of rats with PHN suggested that these cells also play a role in the pathogenesis. Rats lacking in CD8⁺ T cells did not develop the increased proteinuria observed in the autologous phase of PHN, which suggested these cells mediated this late injury. Permanent CD8⁺ T cell depletion did not affect the heterologous phase and showed that CD8⁺ cells are unlikely to be involved in early glomerular injury. In other experiments not detailed here, PVG/c6⁻ rats with PHN were treated with mAb that inhibited CD3 (all T cells) or CD4; both had no effect on the heterologous phase but reduced proteinuria in the autologous phase. In contrast to anti-CD8 mAb treatment, both anti-CD3 and CD4 mAb treatment reduced Ig and C3 deposition in glomeruli.

Our studies were consistent with the two separate immune mechanisms for the heterologous and autologous phases of PHN both of which were independent of MAC. The heterologous phase in the first week is characterized by deposition of the xeno (usually sheep or rabbit) anti-Fx1A Ab into the subepithelial GBM. Proteinuria develops by day 3, before the typical thickening of the GBM of PHN is established. This phase neither required MAC or CD8⁺ T cells. It appears that the early injury in the heterologous phase relates to deposition of sheep Ig and possibly activation of the early components of complement, as demonstrated by cobra venom factor treatment blocking proteinuria in PHN (7, 8). The large size of immune complexes may also lead to proteinuria in the heterologous phase.

After a week, the autologous phase develops when the rats develop Abs to Fx1A and the xenoantigen. In this study, MAC was found to be not required but depletion of CD8⁺ T cells did markedly slow progression of proteinuria, which remained at levels observed in the heterologous phase. There was reduced infiltration of CD8⁺ cells into glomeruli, but no reduction in macrophage and CD4⁺ cell infiltration and in Ig or C3 deposition. The CD8⁺ cells could be NK or T cells, but only CD8⁺ T cells are permanently depleted after ATx and anti-CD8 mAb treatment. There was no NK cell infiltration of glomeruli and the number of NK cells present was not affected by CD8⁺ T cell depletion. Thus, the autologous phase may be similar to the disease in active HN, which in Lewis rats is totally dependent upon CD8⁺ T cells. It is possible that in both PHN and HN, the deposition of anti-Fx1A Ab in glomeruli, attaching to the target molecule megalin, initiates a T cell-mediated response against GEC, especially by CD8⁺ T cells. One possibility is that megalin internalizes the attached Ab and complement molecules and presents peptides of these or other molecules on class I MHC. This would be consistent with the role of megalin in being able to internalize extracellular molecules and have them presented by the cells' class I MHC (58). GEC may act as APCs as they can express class I and II MHC as well as costimulatory molecules (59–61). Thus, the initial deposition of Ig and early complement components, especially C3, may activate a CD8⁺ T cell response against the GEC, leading to severe injury and greater proteinuria. This finding suggested that CTLs alone or in conjunction with macrophages mediate the glomerular injury in the autologous phase of PHN.

This study demonstrates that C6 and the full C5b-9 complex are not essential for the development of proteinuria in PHN. Other mechanisms, including immune complexes disrupting the structural integrity of the GBM, or other inflammatory mediators induced by complement components before C6 may contribute to injury of glomeruli in early PHN. CD8⁺ cells are required during the autologous phase of PHN, suggesting that these cells, as in HN, are involved in ongoing glomerular injury in this model.

Acknowledgments

The assistance of Drs. Peter Leenaerts and M. Penny in breeding the PVG/C6⁻ was appreciated. The expert help of M. Boutros in breeding and animal care is appreciated.

Disclosures

The authors have no financial conflict of interest.

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