Th2 Responses Induce Humorally Mediated Injury in Experimental Anti-Glomerular Basement Membrane Glomerulonephritis

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Abstract. Acute autologous phase anti-glomerular basement membrane glomerulonephritis was compared in Th1-prone (C57BL/6) and Th2-prone (BALB/c) mice. Sensitized BALB/c mice, given a subnephritogenic intravenous dose of anti-mouse glomerular basement membrane globulin, developed acute glomerulonephritis characterized by marked proteinuria and glomerular deposition of mouse immunoglobulin and complement. A significant glomerular neutrophil influx was observed, but few T cells and macrophages were present. C57BL/6 mice, given the same dose of disease-inducing globulin, also developed acute glomerulonephritis, although their proteinuria was significantly less. Glomerular deposition of mouse immunoglobulin and complement and the influx of neutrophils were also significantly less than in BALB/c mice. However, their glomerular accumulation of macrophages and T cells was significantly greater. Complement depletion attenuated neutrophil influx and proteinuria in BALB/c mice but did not affect T cell or macrophage accumulation or proteinuria in C57BL/6 mice. CD4+ T cell depletion significantly reduced glomerular macrophage, T cell influx, and proteinuria in C57BL/6 mice, but had no effect on proteinuria or neutrophil influx in BALB/c mice. Thus, immune responses to planted glomerular antigens in Th2-prone mice induce acute injury as a result of antibody deposition, complement activation, and neutrophil influx, whereas immune responses to the same antigen in Th1-prone mice induce delayed-type hypersensitivity-like lesions in affected glomeruli. (J Am Soc Nephrol 8: 1101–1108, 1997)

Human and experimental glomerulonephritis (GN) can be induced by host immune responses to planted glomerular antigens. The nature of these host immune responses may potentially determine the nature of the immune effector responses in glomeruli and the resultant pattern of glomerular injury (1–3). T helper cells play an important role in both humoral and cellular immune responses to protein antigens. Evidence for functional dichotomy among mouse CD4+ T cell clones suggests the existence of two subsets among fully differentiated helper T cells (4–7). Th1 cells produce interleukin (IL)-2 and interneron (IFN)-γ, as well as several other lymphokines, after antigen stimulation. Th2 cells produce IL-4, IL-5, and probably IL-6. This difference in pattern of lymphokine secretion appears to result in different functional behavior of T cells. Th1 cells, but not Th2 cells, are reported to mediate delayed-type hypersensitivity (DTH) in mice (8). IFN-γ produced by Th1 cells activates macrophages (9,10) and induces increased expression of MHC antigens (11). IL-4, which is secreted by Th2 cells, has been demonstrated to be a cofactor for B cell proliferation (12,13) and to enhance IgG1 and IgE production (14).

These observations suggest two classes of immune response: one involving Th1 cells and macrophages and the other involving principally Th2 cells and B lymphocytes. The functional differences between Th1 and Th2 cells correlate with what has traditionally been described as cellular and humoral immunity. Mice with different genetic backgrounds have been found to demonstrate different propensities to humoral or cellular responses to a variety of antigens (15,16). In particular, C57BL/6 mice develop Th1-predominant immune responses and BALB/c mice develop Th2-predominant immune responses. Th1-prone C57BL/6 mice develop crescentic GN in response to a planted glomerular antigen (sheep anti-mouse glomerular basement membrane [GBM] globulin). This lesion is characterized by prominent accumulation of T cells and macrophages and can be inhibited by treatment with functionally inhibitory antibodies to IFN-γ (17). We now report a distinctly different pattern of glomerular injury and effector pathways induced by the immune response to the same planted antigen in Th2-prone BALB/c mice. Comparison of the features of GN in the two strains suggests a role for both Th1 and Th2 cells in inducing GN by distinctly different effector pathways.

Materials and Methods

Animals

Male BALB/c and C57BL/6 mice 8 to 10 wk of age were obtained from Monash University, Central Animal Services, Clayton, Victoria, Australia. All experiments were conducted in accordance with Na-
tional Institutes of Health guidelines for the care and use of laboratory animals.

**Induction of Anti-GBM GN**

Sheep anti-mouse GBM globulin was prepared from the serum of a sheep immunized against a particulate fraction of mouse GBM by absorption with mouse red blood cells and ammonium sulfate precipitation, as described previously (18). Mice were sensitized by subcutaneous injection of 2 mg of sheep globulin in 200 μl of Freund’s complete adjuvant (Sigma, St. Louis, MO) in each flank. GN was initiated by intravenous administration of 5 mg of sheep anti-mouse GBM globulin 10 d later. This dose of antibody resulted in the binding of 124 μg of globulin per gram wet weight of kidney (determined by trace-labeling studies) and did not induce proteinuria in nonimmunized mice.

**Assessment of Proteinuria**

Mice were housed individually in cages to collect urine over a 24-h period. Urinary protein concentrations were determined by a modified Bradford method adapted to a microtiter plate assay as described previously (19,20). The sensitivity of this assay was less than 125 μg/ml. Urine samples were diluted 1 in 10 in phosphate-buffered saline to obtain protein concentrations in the linear-response range of the assay. Bovine serum albumin (Sigma Chemical-Aldrich, Sydney, Australia) was used to provide a reference standard for this assay. The 24-h urinary protein excretion was calculated from the 24-h urine volume and the urine protein concentration.

**Histological Assessment of Glomerular Injury**

Kidney tissue was fixed in Bouin’s fixative and embedded in paraffin, and 3-μm tissue sections were cut and stained with periodic acid-Schiff’s reagent. Neutrophils, identified by their typical polymorphonuclear appearance, were counted in glomeruli cut in equatorial sections. A minimum of 20 glomeruli from each animal were counted using a blinded protocol, and the results are expressed as cells per glomerular cross-section (c/gcs).

**Assessment of Immunological Effectors in Glomeruli**

**Glomerular T Cell and Macrophage Accumulation.** Spleen and kidney tissue was fixed in periodate lysine paraformaldehyde for

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**Figure 1.** Histological features of anti-glomerular basement membrane (GBM) glomerulonephritis (GN) in Th2- and Th1-prone strains of mice 24 h after initiation of injury. In sections stained with periodic acid-Schiff’s reagent (PAS), a proliferative GN was observed in both strains; however, polymorphonuclear neutrophils (PMN) were prominent in glomerular capillary loops in BALB/c mice (A), whereas mononuclear cells were prominent in C57BL/6 mice (B). Immunoperoxidase staining of sections from C57BL/6 mice demonstrated that the infiltrating mononuclear cells were CD4⁺ T cells (arrowheads, panel C) and macrophages (arrowheads, panel D) (magnification, ×400).
4 h, washed in 7% sucrose solution, then frozen in liquid nitrogen. Tissue sections (5 μm) were stained to demonstrate macrophages and T cells, using a three-layer immunoperoxidase technique as described previously (17). The primary antibodies were GK1.5 (monoclonal anti-mouse CD4, American Type Culture Collection, Rockville, MD) and FA-11 (monoclonal anti-macrophage, a gift of Dr. Michael Smith, Laboratory for Molecular Biology, Cambridge, United Kingdom). Sections of spleen provided a positive control for each animal, and protein G-purified rat immunoglobulin was substituted for primary monoclonal antibody to provide a negative control. A minimum of 20 equatorially sectioned glomeruli per animal were assessed per animal in a blinded protocol, and the results were expressed as c/gcs.

**Immunoglobulin and Complement.** Tissue sections (4 μm) were cut from snap-frozen kidney and stained by direct immunofluorescence with FITC-conjugated donkey anti-sheep IgG, FITC-conjugated sheep anti-mouse IgG (both from Silenus, Hawthorn, Victoria, Australia), and FITC-conjugated goat anti-mouse C3 (Cappel Laboratories, Durham, NC) antibodies. Semiquantitative assessment of the glomerular deposition of these inflammatory mediators was performed by determining the end-point positive titer for detection of staining of these antigens using serial dilutions of each antibody.

**In Vivo Complement Depletion Protocol**

Purified cobra venom factor (CVF) was prepared from cobra venom (Naja, naja, Sigma Chemical-Aldrich) by ion exchange chromatography as described by Ballow and Cochrane (21). Mice received three intraperitoneal injections of a total of 2 ml of purified CVF over 24 h before initiation of GN. This treatment reduced serum C3 levels to less than 5% of normal as measured by radial immunodiffusion (18).

**In Vivo T Helper Cell Depletion Protocol**

T helper cell depletion was induced by a single intravenous injection of 2 mg per mouse of protein G-purified rat anti-mouse CD4 monoclonal antibody (GK 1.5) administered 24 h before injection of anti-GBM globulin. This antibody has previously been established to produce prolonged T helper cell depletion in vivo (22,23). The extent of T cell depletion in blood was assessed by flow cytometry at the end of each experiment using anti-CD3 monoclonal antibody (30H/12, American Type Culture Collection) as a pan T cell marker and GK1.5 to detect CD4+ T cells.

**In Vivo Assessment of Neutrophil and Monocyte Chemotaxis**

The in vivo chemotactic response of inflammatory cells to thioglycolate was compared in BALB/c and C57BL/6 mice. Mice of either strain were injected intraperitoneally with 1 ml of 3.8% thioglycolate solution (Becton Dickinson, Cockeysville, MD). Mice were killed after 3 or 72 h, and peritoneal cells were collected by lavage with 3 vol of 3 ml of phosphate-buffered saline. Total cell numbers were determined by counting in a hemocytometer after dilution in 3% glacial acetic acid to lyse any red blood cells. A differential white cell count on each lavage was obtained by counting nucleated cells on a cell smear-stained with Wright/Giemsa stain.

**Experimental Design and Statistical Analysis**

GN was induced as described above in BALB/c and C57BL/6 mice, and glomerular injury was assessed 24 h and 3, 5, and 7 d after administration of anti-GBM antibody. The effect of in vivo complement or CD4+ T cell depletion on the development of anti-GBM GN was studied in both strains 24 h after initiation of disease. Control mice were the same sex and age as experimental mice.

Experimental groups consisted of 6 to 8 mice (including for in vivo chemotaxis), and results were expressed as the mean ± SEM. The statistical significance of differences between groups was determined by the Mann-Whitney U test.

**Results**

**Characterization of Anti-GBM GN**

BALB/c mice developed a proliferative GN 24 h after anti-GBM globulin, with prominent accumulation of neutrophils in the glomerular lumina (4.5 ± 0.25 c/gcs; normal, 0.1 ± 0.01 c/gcs; Figure 1A). On subsequent days, this neutrophil accumulation declined slowly (Figure 2). In contrast, CD4+ T cells (CD4+, 0.5 ± 0.01 c/gcs; normal, 0.05 ± 0.01 c/gcs) and macrophages (1.0 ± 0.1 c/gcs, normal, 0.1 ± 0.01 c/gcs) were only sparsely observed in glomeruli. This histological evidence...
of injury was associated with abnormal proteinuria over the first 24 h (7.2 ± 1.5 mg/24 h; normal, 1.3 ± 0.25 mg/24 h), which was maintained on subsequent days (Figure 3).

C57BL/6 mice also developed an endocapillary proliferative GN 24 h after anti-GBM globulin (Figure 1B). In contrast to BALB/c mice, the inflammatory cells accumulating in their capillary lumina were mainly mononuclear and comprised predominantly CD4⁺ T cells (2.2 ± 0.1 c/gcs; normal, 0.05 ± 0.01 c/gcs; Figure 1C) and macrophages (4.5 ± 0.5 c/gcs; normal, 0.1 ± 0.01 c/gcs; Figure 1D). Neutrophils were seen only occasionally in glomeruli at 24 h (0.5 ± 0.01 c/gcs; normal, 0.1 ± 0.01 c/gcs), and their numbers did not change significantly on subsequent days (Figure 2). Abnormal proteinuria in C57BL/6 mice (3.5 ± 0.5 mg/24 h; normal, 0.5 ± 0.1 mg/24 h; P < 0.05) was significantly less than in BALB/c mice (P < 0.05) over the first 24 h of disease, but increased progressively on subsequent days (Figure 3).

Humoral Immune Effectors in Glomeruli
The nephritogenic antigen (sheep globulin) was evident in glomerular capillary loops in both strains of mice (Table 1).

There was no difference in the glomerular binding of this antigen in the two strains of mice as assessed by the mean immunofluorescence end-point titer required for its detection (both 1:5000). However, the deposition of autologous mouse antibody was markedly different between the two strains. Intense linear deposition of mouse IgG was seen in glomerular capillary loops in BALB/c mice (mean end-point titer for detection by immunofluorescence, 1:500), whereas only weak and sparse antibody deposition was seen in C57BL/6 mice (mean end-point titer for detection by immunofluorescence, 1:250). A similar difference between the two strains was noted for glomerular C3 deposition. Intense linear deposition of C3 was observed in glomerular capillary loops of BALB/c mice (end-point titer for detection by immunofluorescence, 1:500 dilution), and sparse glomerular deposition was detected in C57BL/6 mice (end-point titer for detection by immunofluorescence, 1:150).

Effect of Complement Depletion on Anti-GBM GN
After treatment with CVF, C3 was not detectable in glomeruli by immunofluorescence in either strain of mice (Table 1).

Table 1. Comparison of the humoral immune effectors of glomerulonephritis (GN) in glomeruli of BALB/c and C57BL/6 mice

<table>
<thead>
<tr>
<th>Category</th>
<th>BALB/c</th>
<th>C57BL/6</th>
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<tbody>
<tr>
<td></td>
<td>GN Intact</td>
<td>C3 Deplete</td>
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<tr>
<td>Sheep Ig (1:100)</td>
<td>+++</td>
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<tr>
<td>End-point titer</td>
<td>1:5000</td>
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<td>Mouse Ig (1:100)</td>
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<td>Mouse C3 (1:100)</td>
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Figure 4. Effect of complement depletion on the histological appearance of anti-GBM GN in BALB/c mice. PMN accumulation in glomeruli was prominent in untreated mice with anti-GBM GN (panel A), but was prevented by complement depletion (panel B) (PAS stain, magnification X400).
In complement-depleted BALB/c mice (Figure 4B), the histologic appearances of GN, 24 h after anti-GBM globulin, were markedly attenuated compared with untreated mice (Figure 4A). Complement depletion was associated with a significant reduction in proteinuria (2.1 ± 0.2 mg/24 h; P < 0.01) and neutrophil accumulation in glomeruli (0.5 ± 0.1 c/gcs; P < 0.01) compared with untreated BALB/c mice at the same time (Figure 5).

In C57BL/6 mice, complement depletion did not affect the histological appearances of GN. Proteinuria was not significantly attenuated (3.4 ± 0.2 mg/24 h), and glomerular CD4⁺ T cell (2.0 ± 0.1 c/gcs) and macrophage (4.1 ± 0.1 c/gcs) accumulation was also unaffected compared with untreated C57BL/6 mice 24 h after initiation of GN. Although the minor neutrophil infiltration in glomeruli of complement-depleted C57BL/6 mice (0.3 ± 0.1 c/gcs) was less than in untreated mice with GN (0.5 ± 0.1 c/gcs), this difference was not significantly different (Figure 5).

**Effect of Anti-CD4 Treatment on Circulating T Cells**

The role of CD4⁺ T helper cells as effectors of early glomerular injury was compared in both C57BL/6 and BALB/c mice by in vivo depletion using a monoclonal rat anti-mouse CD4 antibody. A single dose of antibody, given 24 h before administration of anti-GBM globulin, resulted in prolonged depletion of circulating CD4⁺ T cells in both strains of mice. Forty-eight hours after anti-CD4 antibody treatment (i.e., 24 h after anti-GBM globulin), CD3⁺ cells were significantly reduced to 10.2 ± 6.6% (normal, 56.5 ± 1.7%; P < 0.01) and CD4⁺ cells to 5.5 ± 0.5% (normal, 47.0 ± 2.0%; P < 0.01) of total circulating leukocytes in C57BL/6 mice. In BALB/c mice, CD3⁺ cells were reduced to 12.0 ± 8.8% (normal, 54.3 ± 1.9%; P < 0.01) and CD4⁺ cells to 9.0 ± 0.5% (normal, 45.5 ± 2.7%; P < 0.01).

**Effect of CD4⁺ T Cell Depletion on Anti-GBM GN**

CD4⁺ T cell depletion significantly attenuated the histological manifestations of GN in C57BL/6 mice (Figure 6B) compared with untreated mice with GN (Figure 6A). CD4⁺ T cells and macrophage infiltration were both significantly reduced by treatment with anti-CD4 antibody (CD4⁺ T cells, 0.1 ± 0.01 c/gcs; macrophages, 1.0 ± 0.01 c/gcs; P < 0.01 compared with untreated GN in both cases). Proteinuria was also significantly reduced (1.0 ± 0.2 mg/24 h; P < 0.01 compared with untreated GN). In BALB/c mice, CD4⁺ T cell depletion also abrogated the minor CD4⁺ T cell influx observed in control mice developing GN (0.1 ± 0.05 c/gcs; P < 0.01 compared with untreated GN). However, this was not associated with a significant reduction in proteinuria (6.2 ± 0.4 mg/24 h) or glomerular neutrophil influx (5.0 ± 0.05 c/gcs) (Figure 7).

**In Vivo Neutrophil and Macrophage Chemotaxis in BALB/c and C57BL/6 Mice**

The capacity of neutrophils and macrophages to respond in vivo to an intraperitoneal chemotactic stimulus was the same in BALB/c and C57BL/6 mice. Three hours after injection of thioglycollate, the total peritoneal leukocyte accumulation was similar in both strains (BALB/c mice, 7.23 ± 1.08 × 10⁵ leukocytes; C57BL/6 mice, 7.20 ± 0.83 × 10⁵ leukocytes). At this time, the majority of the peritoneal cells were neutrophils.
with similar proportions in both strains (BALB/c: 77.3 ± 3.2% neutrophils, 22.7 ± 3.2% macrophages; C57BL/6: 81.2 ± 2.0% neutrophils, 18.9 ± 2.0% macrophages). Three days after injection of thioglycollate, the total peritoneal leukocyte accumulation increased, with similar numbers in both strains (BALB/c mice, 2.60 ± 0.26 × 10^8 leukocytes; C57BL/6 mice, 2.20 ± 0.28 × 10^8 leukocytes). However, the majority of the peritoneal cells were macrophages at this time (BALB/c, 8.2 ± 1.3% neutrophils, 91.8 ± 1.1% macrophages; C57BL/6, 9.7 ± 1.1% neutrophils, 90.4 ± 1.4% macrophages).

**Discussion**

Studies of inbred mouse strains have established the concept of at least two patterns of T helper cell responses, associated with so-called Th1 and Th2 subsets. Th2 subsets facilitate antibody production and isotope switching (24–26), whereas Th1 subsets direct DTH responses (27). BALB/c mice have been shown to develop a predominant Th2 response, whereas C57BL/6 mice have been shown to have a predominant Th1 response to a variety of antigens.

The immunopathogenic mechanisms of GN have been characterized in various experimental models that emulate patterns of injury and outcomes in human GN. One of the best-characterized models of human proliferative GN is induced experimentally by the administration of anti-GBM globulin. Renal binding of heterologous anti-GBM antibody may directly induce acute injury if given at a large dose. However, if a small (subnephritogenic) dose is given, insufficient antibody is deposited in glomeruli to directly induce injury. The subsequent host immune response to this planted glomerular antigen results in severe crescentic GN 1 to 2 wk later. If animals are presensitized to the heterologous globulin before the injection of a subnephritogenic dose of heterologous anti-GBM globulin, immediate autologous injury results. This model has been developed in several species and may result in the development of crescentic GN and severe renal failure replicating the devastating outcome of crescentic anti-GBM GN in humans.

We have shown previously that Th2 predominance of BALB/c mice and Th1 predominance of C57BL/6 mice also hold true for their immune responses to the antigen (sheep globulin) used to initiate GN current experiments. Sensitized Th1-prone C57BL/6 mice developed strong skin DTH to sheep globulin and predominant IFN-γ production by splenic T cells, whereas BALB/c mice showed higher-circulating antibody titers but only minimal DTH responses to this antigen. Their splenic T cells produced higher levels of IL-4 and less IFN-γ in response to this antigen than C57BL/6 mice (17). The intraperitoneal accumulation of inflammatory cells in response to thioglycollate demonstrates that these strains do not show any differences in the chemotactic capacity of their neutrophils or macrophages. Therefore, differences in neutrophil and macrophage chemotaxis in these strains do not contribute to the observed differences in their patterns of immune injury.

In the present study, acute glomerular injury in the two strains of mice demonstrated quite different patterns. BALB/c mice had significantly greater deposition of mouse IgG, and consequently C3, in affected glomeruli. A prominent glomerular neutrophil infiltration was observed in association with significant proteinuria. C57BL/6 mice showed less deposition of mouse immunoglobulin and C3 in glomeruli and significantly less neutrophil influx, but exhibited a prominent CD4+ T cell and macrophage infiltrate in affected glomeruli.

Depletion of C3 with CVF prevented glomerular C3 deposition. Although similar amounts of mouse IgG deposition in glomeruli were seen in CVF-treated and CVF-untreated BALB/c mice, complement depletion significantly attenuated neutrophil influx and proteinuria. This is the first report of the abrogation of autologous anti-GBM injury by complement depletion. These studies confirm the potential for humoral induction of injury in response to planted glomerular antigens.
However, these results also suggest that this pattern of injury requires a Th2-predominant immune response to the nephritogenic glomerular antigen. The minor Th1 involvement in BALB/c mice was confirmed by the observation that CD4+ T cell depletion had no significant effect on injury.

The acute injury seen in Th1-prone C57BL/6 mice was similar to the pattern reported previously at later a time point in this model of GN (17). The predominant leukocyte infiltrate was mononuclear and consisted mainly of macrophages and T cells. The reduction of injury by CD4+ T cell depletion confirms Th1 dependence of the glomerular lesion in this strain. The minor contribution of humoral factors to the glomerular injury was demonstrated by significantly less glomerular mouse immunoglobulin and complement deposition and fewer glomerular neutrophils. This minor contribution was illustrated further by the failure of complement depletion to attenuate glomerular injury in Th1-prone mice.

Human studies suggest that individuals differ in their pattern of Th1 or Th2 responses. Atopy can be demonstrated in up to 30% of the population, suggesting predominant Th2 responses (28–30). The results presented here suggest that the predisposition of the immune response to Th1 or Th2 patterns may significantly alter the effector pathways involved in glomerular injury if GN develops. The recognition that different patterns of genetically determined immune responses to glomerular antigens result in quite different patterns of injury with different susceptibilities to therapeutic interventions has important implications for the understanding and treatment of human GN. Knowledge of the pattern of immune response (suggested by the cytokine profiles or the pattern of glomerular injury) may suggest different outcomes and responses to therapy. The increasing availability of biological intervention through specific cytokine inhibition or treatment with recombinant human cytokines and their antagonists may allow for selective and more specific diminution of pathological immune responses to glomerular antigens.

Acknowledgments
This work was supported by grants from the National Health and Medical Research Council of Australia and the Australia Kidney Foundation.

References

Figure 7. Effects of CD4+ T cell depletion on proteinuria, PMNs, macrophage, and CD4+ T cell accumulation in glomeruli in BALB/c and C57BL/6 mice 24 h after initiation of anti-GBM GN. *, significant differences from control (P < 0.01).


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