Fc receptor-mediated accumulation of macrophages in crescentic glomerulonephritis induced by anti-glomerular basement membrane antibody administration in WKY rats

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Abstract

Anti-glomerular basement membrane (GBM) glomerulonephritis induced in WKY rats is characterized by glomerular accumulation of CD8⁺ T cells and monocytes/macrophages, followed by crescent formation. The mechanism of leukocyte accumulation after antibody binding to GBM is still unclear. To unveil an involvement of FcγR receptors (FcγR) in leukocytes recruitment we examined the expression of FcγR in glomeruli and the effects of the administration of F(ab')² fragment of anti-GBM antibody or FcγR blocking on the initiation and progression of this model. A gradual increase of FcγR mRNA expression in glomeruli during the course of disease suggested their significance in the development of glomerulonephritis. Glomerular lesions and proteinuria were induced only in rats injected with intact IgG of anti-GBM antibody, but not with the F(ab')² fragment. In vivo blocking of FcγR by administering heat-aggregated IgG led to the decrease of mRNA expression for all types of FcγR (types 1, 2 and 3) and a significant amelioration of glomerulonephritis manifestations. By flow cytometry and immunohistochemistry FcγR²-expressing cells in glomeruli were identified as macrophages, but not CD8⁺ T cells. The expression of FcγR1 and 3 was significantly decreased, and that of FcγR2 became undetectable in CD8⁺ T cell-depleted rats. Thus, CD8⁺ T cells may stimulate FcγR expression on macrophages, contributing to their glomerular accumulation and injury. These studies provide direct evidence for a crucial involvement of IgG Fc–FcγR interaction in glomerular recruitment of macrophages and following induction of anti-GBM glomerulonephritis in WKY rats.

Introduction

Anti-glomerular basement membrane (GBM) glomerulonephritis induced by the administration of a small dose (25 µl/100 g body wt) of anti-GBM antibody is characterized by glomerular accumulation of CD8⁺ T cells and macrophages, followed by crescentic formation (1). This model is comparable with anti-GBM antibody-mediated crescentic glomerulonephritis in humans, which progresses to renal failure rapidly. To develop therapeutic methods for the crescentic glomerulonephritis, its pathogenesis needs to be understood. Our previous studies provided evidence for crucial roles of CD8⁺ T
cells in the glomerular accumulation of macrophages, which were then presumed to injure glomerular structure and functions (1,2). Later on, roles of leukocyte adhesion molecules and chemokines in the development of this model were demonstrated (2–10). Although the glomerulonephritis is initiated by the binding of anti-GBM antibody to GBM, it is unclear how the infiltrates are accumulated in the glomeruli after anti-GBM antibody binding. One of the possible mechanisms is immune adherence of leukocytes through interaction between the Fc portion of anti-GBM antibody and Fc receptors (FcγR) expressed on the surface of the infiltrating cells.

Three distinct types of receptors for IgG have been defined: FcγR1 (CD64) is the high-affinity receptor for IgG and FcγR2 (CD32)/FcγR3 (CD16) are the low-affinity receptors. These FcγR bind to the Fc portion of IgG molecule in the form of immune complexes, leading to the activation of various FcγR-expressing cells such as granulocytes, monocytes, macrophages, NK cells and subtypes of T cells (11–15). These cells express each FcγR subtype at different intensities and change the expression levels under pathological conditions (16–21). The roles of FcR in various inflammatory diseases have been verified by using FcγR-deficient mice. FcγR chain knockout mice, which exhibited no expression of FcγR1, 2 and 3, failed to induce IgG-mediated endocytosis by macrophages. FcγR were also shown to play crucial roles in antibody-mediated hypersensitivity and immune complex-mediated hypersensitivity (16–19,22,23). Recent works demonstrated that FcγR-deficient mice were resistant to nephrotoxic glomerulonephritis, indicating a critical role for FcγR in the induction of murine glomerulonephritis (24,25). On the other hand, autoimmune glomerulonephritis occurred in FcγR-deficient MRL/lpr mice as in wild-type MRL/lpr mice, indicating that the nephritis was FcγR independent (26).

Since roles for FcγR in the initiation of the crescent glomerulonephritis of WKY rats had not been elucidated, we examined the involvement of FcγR in this glomerulonephritis model and found a pivotal role for these receptors in the glomerular recruitment of macrophages, crescent formation and glomerular injury.

Methods

Animals

Animals used in this work were inbred male WKY rats (Charles River, Kanagawa, Japan), 200–250 g body wt, aged 12–16 weeks old.

Preparation of rabbit anti-GBM IgG and F(ab')2

Partially purified IgG was prepared from rabbit anti-GBM serum by 50% ammonium sulfate fractionation, followed by DEAE–cellulose column chromatography. IgG was further purified by gel filtration on a Superdex 200 pg column using an AKTAexplorer 10S protein purification system (Amersham Pharmacia Biotech, Piscataway, NJ). F(ab')2 fragment was generated from the partially purified IgG fraction by incubation with immobilized pepsin (Immunopure F(ab')2 preparation kit; Pierce, Rockford, IL) as described in the manufacturer's instructions. The F(ab')2 fragments were separated from undigested IgG and other proteolytic fragments by gel filtration on Superdex 200 pg. The purified IgG and F(ab')2 fragment showed apparent homogeneity on SDS–PAGE gel stained with Coomassie brilliant blue R-250.

Induction of anti-GBM glomerulonephritis

Rats were injected with the intact IgG at different doses (3.0, 1.5 and 0.75 mg/kg body wt, five rats each). As we usually inject with 250 μl of anti-GBM antisera (containing 3.25 mg IgG/kg body wt (4), the injected IgG doses in the present study were roughly equivalent to the amount of anti-GBM antisera used for the induction of anti-GBM glomerulonephritis in WKY rats. The ability to induce the glomerulonephritis was compared between intact IgG (1.5 mg/kg body wt, n = 5) and the F(ab')2 fragment (0.75 mg/kg; the same molar dose as 1.5 mg intact IgG/kg, n = 5). The animals were sacrificed at day 10 after the antibody injection. Urine specimens were collected on days 1, 4, 6 and 10 by housing animals in metabolic cages for 24 h. Urinary protein was estimated by using a protein assay kit (Nippon Bio-Rad, Tokyo, Japan).

Binding efficiency of intact IgG or F(ab')2 of the anti-GBM antibody to GBM was examined by immunofluorescence microscopy. Rats were sacrificed at 1 h, and days 4, 6 and 10 after intact IgG or F(ab')2 antibody injection. Kidney slices were snap-frozen in n-hexane at −70°C and sectioned in a cryostat. The cryostat sections (3 μm thick) were stained with fluorescein-conjugated affinity-purified anti-rabbit IgG F(ab')2 (Rockland Immunocchemicals, Gilbertsville, PA).

Histological examination by light microscopy and immunohistochemistry

Kidney specimens were fixed with methyl Carnoy’s fixative, and embedded in paraffin for light microscopic examination and immunohistochemistry. The sections for light microscopy were stained with periodic acid–Schiff. mAb against rat CD8 and ED1 (Dainippon Seiyaku, Tokyo, Japan) were used for staining of infiltrating CD8⁺ cells and macrophages respectively. To examine any involvement of platelets in the pathogenesis, they were immunolocalized in the kidneys using mouse monoclonal IgG antibody against rat platelet, PL-1 (kindly provided by Dr E. de Heer, Leiden University, The Netherlands). The numbers of immunostained cells were counted on >100 glomeruli in each kidney and expressed per glomerular cross-section. Rat FcγR2-bearing cells were immunostained with a mouse anti-rat CD32 mAb (BD PharMingen, San Diego, CA) in cryostat sections of frozen kidney samples.

Detection of glomerular mRNA for FcγR1, 2 and 3

Glomeruli were isolated from the renal cortex by a standard sieving method (27). Total cellular RNA was extracted from the isolated glomeruli homogenized using a modified guanidine thiocyanate method (TRIZol; Gibco/BRL, Grand Island, NY).

Ribonuclease protection assay was performed for the detection of FcγR expression in the glomeruli. FcγR1 (178 bp), 2 (249 bp) and 3 (289 bp) cDNA were amplified from the glomerular RNA obtained from rats 10 days after anti-GBM antibody injection by PCR with specific primers according to the sequences available in the GenBank database.
(AF143186, X73371 and M64369). Sense primers used for amplification of FcγR1, 2 and 3 fragments were as follows: 5′-CGGATCCCTGACGCCCAATGCTG-3′, 5′-CGGGATCCTGACGCTTCT-3′ and 5′-GGAAATTCATGGTCGACCTGACC-3′ respectively; and antisense primers were 5′-CTCTACCGGATTTGCTACACSGTCC-3′, 5′-GAGGGTGGAGTTGAGGATCAGCG-3′ and 5′-CGAAGAAAGTCCGTTGCGCTAAGGG-3′ respectively. As the sequence for rat FcγR1 was not available, the primers were designed to the regions of mouse FcγR1 sequence with the highest homology to human FcγR1 sequence. The amplified PCR product was 87% homologous to the mouse FcγR1 DNA sequence. The FcγR1, 2 and 3 cDNA fragments were subcloned in pBluescript SK+ vectors and then isolated at the 5′ ends to use as templates for preparation of 32P-labeled antisense cRNA probes. Ten micrograms of total glomerular RNA was hybridized with a mixture of 32P-labeled antisense cRNA probes (1 × 10^6 c.p.m. for each FcγR mRNA and GAPDH overnight at 45°C. Unhybridized cRNA probes were digested with ribonuclease T1 (120 U/ml; Gibco/BRL, Gaithersburg, MD) and ribonuclease A (4 μg/ml; Boehringer Mannheim, Tokyo, Japan) for 1 h at 30°C. The ribonuclease A were then digested with proteinase K (500 μg/ml; Promega, Madison, WI) for 30 min at 37°C. After phenol:chloroform extraction and sodium acetate:ethanol precipitation the hybridized RNA probes were denatured at 95°C for 5 min and electrophoresed on a 6% polyacrylamide gel. Detection and analysis of bands were performed by phosphor-imaging techniques using the Molecular Imager FX (Bio-Rad, Hercules, CA). The data was represented as a ratio of specific mRNA:GAPDH mRNA to verify the constant quantity of mRNA in each sample.

**Blocking of FcγR by heat-aggregated IgG (HA IgG)**

HA IgG was prepared as described previously (28,29). Briefly, rat IgG was extracted from normal rat serum by 33% ammonium sulfate precipitation and heated at 63°C for 30 min. Resulting aggregates were ultracentrifuged at 10,000 g for 90 min to obtain the soluble fraction of HA IgG. Then, the soluble HA IgG was diluted in PBS and used in the experiment. Rats were injected with HA IgG (n = 5) and unaggregated rat IgG (n = 5) at a dose of 50 mg/100 g body wt i.v. 30 min before the injection with anti-GBM antibody. Boost injections of HA IgG and unaggregated rat IgG were made on days 2 and 4 after the administration of anti-GBM antibody, and animals were sacrificed on day 5.

**Depletion of CD8+ cells**

CD8+ cells were depleted from the circulation by administrating mAb against rat CD8 (MRC-OX8, 28 mg of γ-globulin/kg body wt i.p. and 6 mg/kg i.v.) at two different time points of this glomerulonephritis model. One group of rats (pre-treated group, n = 20) was given the anti-CD8 antibody 2 days before the administration of 25 μl/100 g body wt of anti-GBM antibody as described in our previous studies (1,2), where we demonstrated that CD8+ cells in the circulation, spleen, cervical lymph node and thymus were completely depleted by this MRC-OX8 administration protocol, and the depletion was maintained for nearly 10 days. The other group of rats (post-treated group, n = 10) was given the antibody 3 days after anti-GBM antibody administration. As a control, rats were injected with irrelevant mAb and anti-GBM antibody or untreated. The animals from the pre-treated and control group were sacrificed at days 1, 3, 7 and 14, and the post-treated group animals were sacrificed at days 7 and 14 (five rats at each time point) for histological examination and RNA isolation from the glomeruli.

Urine specimens were collected on days 1, 3, 7 and 14 by housing animals in metabolic cages for 24 h. Urinary protein was estimated by using a protein assay kit (Nippon Bio-Rad).

**Flow cytometry analysis**

Flow cytometry was employed to examine FcγR2-bearing leukocytes obtained from the glomeruli and spleens of rats with anti-GBM glomerulonephritis. Glomeruli isolated from the renal cortices were disintegrated using a stainless mesh (30). Then, leukocytes were isolated by centrifugation on 45% Percoll solution and stained with different combinations of antibodies: FITC-conjugated mouse anti-rat CD32 (clone D34-485) mAb, R-phycocerythrin (PE)-conjugated mouse anti-rat CD8 mAb (clone OX-8), PE-conjugated mouse anti-CD11b/c mAb (clone OX-42) and PE-conjugated mouse anti-CD4 mAb (clone OX-35). All antibodies were obtained from BD PharMingen (San Diego, CA). Stained cells were analyzed by two-color flow cytometry using a FACSscan (Becton Dickinson Immunocytometry System, San Jose, CA).

**Statistical analysis**

Statistical evaluations were performed using the Mann–Whitney test (data meet the requirements for using the test). Analysis was performed with the use of Prism 3.0 for Windows (GraphPad, San Diego, CA). Data were considered statistically significant at P < 0.05. All statistical tests were two-sided.

**Results**

**Binding of anti-GBM antibody to the GBM**

Both intact IgG (1.5 mg/kg) and the F(ab')2 fraction (0.75 mg/kg) of rabbit anti-GBM antibody were localized by immunofluorescence microscopy along the GBM in a linear pattern at 1 h after injection (Fig. 1A and B). No significant difference in the intensity and distribution of rabbit IgG staining was observed between these groups throughout the experiment up to day 10 (Fig. 2A and B). No deposition of rat IgG was found in the glomeruli until day 5, and a faint but significant signal was observed at day 7 and later after the anti-GBM antibody injection (Fig. 2C and D). The intensity of immunofluorescence for rat IgG was much weaker than that for rabbit IgG.

**Induction of anti-GBM glomerulonephritis by intact IgG and F(ab')2 of anti-GBM antibody**

Although the binding of both IgG and F(ab')2 fractions of antibody to GBM was nearly identical, anti-GBM glomerulonephritis was induced only in animals injected with intact IgG at day 10, and was characterized by hypercellular glomeruli with proteinaceous material deposition and crescent formation (Fig. 1C). Immunohistochemical analysis revealed a significant number of CD8+ T cells (5.0 ± 0.8 cells/glomerular
cross-section, mean ± SD, \( P < 0.01 \) and ED1+ macrophages (27.4 ± 3.4 cells/glomerular cross-section, \( P < 0.01 \)) in the glomeruli of rats injected with anti-GBM antibody IgG (C) or F(ab\(^\prime\))\(_2\) (D). Glomerular accumulation of CD8\(^+\) T cells (E) and macrophages (G) was observed in the intact anti-GBM antibody IgG-injected rats, but no recruitment of CD8\(^+\) T cells (F) and macrophages (H) was detected in the glomeruli of rats injected with F(ab\(^\prime\))\(_2\) fragment of the anti-GBM antibody.

Fig. 1. Glomerular binding of intact IgG (A) or F(ab\(^\prime\))\(_2\) (B) of anti-GBM antibody at 1 h after injection. Light microscopic findings in glomeruli of rats injected with anti-GBM antibody IgG (C) or F(ab\(^\prime\))\(_2\) (D). Glomerular accumulation of CD8\(^+\) T cells (E) and macrophages (G) was observed in the intact anti-GBM antibody IgG-injected rats, but no recruitment of CD8\(^+\) T cells (F) and macrophages (H) was detected in the glomeruli of rats injected with F(ab\(^\prime\))\(_2\) fragment of the anti-GBM antibody.

Fig. 2. Immunofluorescence microscopy shows the deposition of rabbit IgG (A and B) and rat IgG (C and D) in the glomeruli of WKY rats at day 1 (A and C) and day 7 (B and D) after the administration of anti-GBM antibody. The immunofluorescence intensity for rabbit IgG along the GBM at day 5 (E) is not affected by HAlgG administration (F).

Fig. 3. Urinary protein excretion in WKY rats injected with intact IgG (filled circles) or F(ab\(^\prime\))\(_2\) (open circles) anti-GBM antibody. Proteinuria was completely absent in the group injected with F(ab\(^\prime\))\(_2\) fragment of the anti-GBM antibody.

and density of platelets in the glomeruli were almost the same as those detected in the interstitial capillaries (data not shown).

Expression of mRNA for FcγR in the glomeruli of rats from two experimental groups

Expression of FcγR1, 2 and 3 mRNA was detected in the glomeruli of rats injected with intact anti-GBM IgG, but not in
the glomeruli of F(ab')2-injected animals (Fig. 4). FcγR1, 2 and 3 mRNA was intense in the glomeruli of rats injected with 3.0 mg of anti-GBM IgG/kg; the mRNA:GAPDH ratio was 0.142 ± 0.002 (FcγR1), 0.352 ± 0.003 (FcγR2) and 0.434 ± 0.003 (FcγR3) respectively; or in 1.5 mg/kg-injected rats, 0.121 ± 0.001 (FcγR1), 0.302 ± 0.002 (FcγR2) and 0.473 ± 0.003 (FcγR3). The expression was a little less in animals given a smaller dose of the antibody; 0.103 ± 0.001 (FcγR1), 0.161 ± 0.001 (FcγR2) and 0.189 ± 0.002 (FcγR3) respectively in 0.75 mg/kg-injected rats. Rats injected with 1.5 or 3.0 mg of anti-GBM antibody IgG/kg and 250 ml of anti-GBM antisera/kg body wt expressed a similar intensity of mRNA for all three types of FcγR in the glomeruli. The mRNA:GAPDH ratios were 0.113 ± 0.001 (FcγR1), 0.282 ± 0.002 (FcγR2) and 0.394 ± 0.00 (FcγR3) respectively in the group injected with the anti-GBM sera. The histological glomerular changes and the amounts of protein excreted in the urine were also almost identical in rats given anti-GBM antibody IgG at 1.5 or 3.0 mg/kg and in rats given anti-GBM antisera of 250 μl/kg (data not shown).

Blocking of FcγR by HAIgG

Control animals given unaggregated rat IgG + anti-GBM antibody exhibited high levels of proteinuria at day 5. In contrast, animals given HAIgG + anti-GBM antibody demonstrated a significant reduction in protein excretion in the urine (Fig. 5).

Rabbit anti-GBM antibody was localized by immunofluorescence microscopy along the GBM in a linear pattern in both groups at day 5 after injection. No significant difference in the intensity of staining was observed between the two groups (Fig. 2E and F). The marked endcapillary hypercellularity and crescent formation induced in the control group rats were reduced in rats treated with HAIgG. The frequency of glomeruli with crescent formation was decreased from 76.8 ± 3.5% in the control rats to 30 ± 9.7% (P < 0.01) in the HAIgG-treated rats. Accumulation of CD8+ T cells and ED1+ macrophages was observed in the glomeruli of the control rats (Fig. 6A and B). The numbers of macrophages detected in the glomeruli were significantly decreased by the HAIgG treatment from 26.4 ± 3.4 to 15.8 ± 3.0 cells/glomerular cross-section (P < 0.01), while that of CD8+ T cells was unaffected (4.7 ± 1.1 versus 3.9 ± 0.8 cells/glomerular cross-section, P = 0.24) (Fig. 6C and D).

The expression of mRNA for all three types of FcγR in the glomeruli was significantly decreased by the HAIgG treatment: FcγR1:GAPDH mRNA ratio from 0.163 ± 0.002 to 0.081 ± 0.001 (P < 0.01), FcγR2:GAPDH mRNA ratio from 0.604 ± 0.004 to 0.261 ± 0.002 (P < 0.01) and FcγR3:GAPDH mRNA ratio from 0.402 ± 0.003 to 0.214 ± 0.002 (P < 0.01).
The decrease was also demonstrated in the cortices: FcγRI:GAPDH mRNA ratio from 0.065 ± 0.006 to 0.046 ± 0.002 (P < 0.01), FcγRII:GAPDH mRNA ratio from 0.085 ± 0.003 to 0.064 ± 0.005 (P < 0.01) and FcγRIII:GAPDH mRNA ratio from 0.089 ± 0.009 to 0.054 ± 0.007 (P < 0.01) (Fig. 7).

Effect of CD8+ cell depletion on FcγR expression

Glomerular hypercellularity caused by the accumulation of CD8+ T cells (4.5 ± 1.7 cells/glomerular cross-section) and ED1+ macrophages (25.3 ± 6.0 cells/glomerular cross-section) was found in the rats 7 days after anti-GBM antibody administration (Fig. 8A and B). In contrast, glomerular accumulation of CD8+ T cells was negligible (0.0 ± 0.0 cells/glomerular cross-section, P < 0.01) and that of macrophages (14.4 ± 4.2 cells/glomerular cross-section, P < 0.01) was significantly reduced by anti-CD8 antibody administration before anti-GBM antibody injection (Fig. 8C and D). Through treatment with anti-CD8 antibody after anti-GBM antibody injection, the numbers of CD8+ T cells and ED1+ macrophages were also significantly reduced in the glomeruli (0.1 ± 0.0, P < 0.01 and 10.6 ± 3.0, P < 0.01 respectively) (Fig. 8E and F).

Excretion of urinary protein was almost completely suppressed in animals treated with anti-CD8 antibody before anti-GBM antibody injection (Fig. 9). In contrast, the amount of urinary protein was unaffected by day 7 in animals given anti-CD8 antibody after anti-GBM antibody injection. However, urinary protein excretion was significantly decreased at day 14 in these rats (208.5 ± 50.2 versus 109.5 ± 13.1 mg/day, P = 0.02) (Fig. 9).

Glomerular expression of mRNA for all three types of FcγR gradually increased during the time course of anti-GBM glomerulonephritis, reached a plateau at day 3 and noticeably decreased at day 14 (Fig. 10). By administration of anti-CD8 antibody before anti-GBM antibody injection, expression of mRNA for FcγRI and FcγRIII became faint, and FcγRII expression was undetectable in the glomeruli until day 7. However, expression of all FcγR greatly increased in the glomeruli at day 14, which corresponds with the influx of CD8+ T cells and ED1+ macrophages into the glomeruli at this time point as detected by immunohistochemical staining. While intense mRNA expression for all FcγR was demonstrated in the glomeruli at day 7 in animals treated with anti-CD8 antibody after anti-GBM antibody injection, expression of FcγRI and 3 was obviously decreased, and that of FcγRII was undetectable at day 14 (Fig. 10).

Flow cytometric analysis of leukocytes accumulating in the glomeruli

Immunostaining of frozen kidney cross-sections revealed that the number and distribution of FcγRII+ cells was comparable to those of ED1+ macrophages. In addition, FcγRII staining was clearly observed in giant cells, which were apparently transformed from macrophages since they were ED1+. These results bring us to the conclusion that FcγRII is expressed by infiltrating macrophages (Fig. 11A and B).

To identify the exact type of FcγRII-bearing immune cells in the glomeruli, leukocytes isolated from glomeruli were stained with different combinations of antibodies and examined by flow cytometry. The results showed that nearly equal proportions (~10%) of glomerular infiltrates were CD4+ T cells and
CD8+ T cells, and that no expression of FcγR2 was detected on both CD4+ T cells and CD8+ T cells. In contrast, macrophages detected as CD 11b/c-bearing cells expressed FcγR2 (Fig. 11C–E).

**Discussion**

Anti-GBM glomerulonephritis in WKY rats, initiated by a single injection with a small dose of anti-GBM antibody, is characterized by the predominant infiltration of monocytes/macrophages and considerable accumulation of CD8+ T cells, and by the high frequency of glomerular crescent formation. The respective involvement of cell-mediated and humoral immunity in the development of this model is still indistinct. The present study was designed to examine the role of FcγR for the anti-GBM IgG antibody in the development of the crescentic glomerulonephritis model, which has not been clearly established in previous studies on this model.

In the current study we demonstrated that crescentic glomerulonephritis was induced only in animals injected with intact IgG of anti-GBM antibody, but not in animals injected with F(ab')2 fractions of anti-GBM antibody, in spite of the equal binding of them to GBM. The gradual increase of expression of FcγR1, 2 and 3 mRNA during the time course of the disease was parallel to the progress of the disease. The observation suggested the significance of these molecules in this model. To corroborate these results we performed in vivo blocking of FcγR by administering HAlgG. In a previous study, macrophage accumulation and subsequent glomerular injury in an experimental anti-GBM glomerulonephritis model in rabbits were demonstrated to be dependent on the Fc portion of the disease-initiating IgG molecule (31). As HAlgG is a high-affinity ligand for FcγR (28,32), its injections were expected to block all FcγR types of leukocytes in experimental animals and to elucidate the roles of these receptors in anti-GBM glomerulonephritis. The study showed a significant amelioration of this model by FcγR blocking, suggesting that pathological changes and urinary protein excretion are dependent on the immune adherence of infiltrating cells to heterologous antibody planted along the GBM through FcγR. The immune adherence may be mediated by binding between the complement receptors of leukocytes and the complement components activated by the immune complex in this model. However, we recently demonstrated no effects of decomplexation by cobra venom administration to rats on the
development of this model (33), indicating that complement receptor-mediated immune adherence was not essential. However, these data leave undecided the following questions: which type of FcγR is important and which type of infiltrating cells express the FcγR? Previously, the macrophage was shown to be a major cell type accumulating in the glomeruli and crescentic lesions, and one of the most important mediators of glomerular injury and crescent formation (34,35). Furthermore, the crucial role of CD8+ T cells in the induction of the glomerulonephritis was demonstrated by almost complete suppression of the glomerular changes and injury in CD8+ T cell-depleted rats (1,2,36). The severity of glomerulonephritis, infiltration by T cells and macrophages, and, particularly, the formation of crescents was greatly reduced by the treatment of experimental animals with anti-CD8 mAb (1,2,36). We assumed a linkage between CD8+ T cells and FcγR expression, leading to the progression of glomerulonephritis. To clarify this linkage, we examined FcγR expression in the glomeruli of anti-GBM glomerulonephritis rats in a CD8+ T cell-depleted condition. This experiment demonstrated that expression of mRNA for FcγR1 and 3 was significantly decreased, but had not become null in the glomeruli, where no CD8+ T cells and a substantial number of macrophages were detected. The decrease of FcγR expression was associated with the decrease of macrophages accumulating to the glomeruli. These facts may indicate that these glomerular FcγR were expressed on the macrophages. On the other hand, the observation that FcγR2 expression was undetectable in the glomeruli of CD8+ T cell-depleted rats may indicate that FcγR2 are also expressed on the CD8+ T cells as well as macrophages. However, flow cytometry analysis of leukocytes isolated from glomeruli of anti-GBM glomerulonephritis rats showed that cells expressing FcγR2 were restricted to macrophages, but not to CD8+ T cells. Immuno-histochemistry also showed FcγR2 immunoreactivity in a close association with ED1+ staining. In addition, glomerular accumulation of CD8+ T cells was not affected by HAIgG treatment in the present study. It has been reported that the cell-surface expression of FcγR is up-regulated by various agents, including such cytokines as IFN-γ and IL-10 (37–40). CD8+ T cells could be the source of the cytokines which up-regulate the expression of FcγR on the surface of infiltrating macrophages. Thus, our results support the data that macrophages are the major population of FcγR-bearing cells in the glomeruli of this model and CD8+ T cells stimulate expression of FcγR, especially FcγR2 in macrophages in the glomeruli. The role of other FcγR-bearing cells, such as mast cells or platelets, in this model is considered to be minor since no glomerular accumulation of mast cells has been shown in this model. Although considerable numbers of platelets were detected by immunostaining in the glomerular capillaries, similar numbers of platelets were also observed in the glomeruli of normal rat kidneys or interstitial capillaries. Therefore, both mast cells and platelets may not essentially contribute to the induction of this model, although we could not neglect the possibility of their minor participation.

Taken together, the present study proposes the following initiation mechanism of anti-GBM glomerulonephritis in WKY rats. At the beginning the heterologous anti-GBM antibody binds to GBM and attracts macrophages through the interaction between the IgG Fc portion of the antibody and FcγR on their surface. Macrophages begin to accumulate in glomeruli of this model from day 1 after the injection of anti-GBM antibody and their number increases during the time course of the disease (1). Rat antibody against rabbit IgG produced at the late phase binds to the rabbit anti-GBM antibody on GBM at day 5 after anti-GBM antibody injection, which was demonstrated by immunofluorescence microscopy. The rat anti-rabbit IgG antibody should also contribute to the macrophage accumulation at the late phase. The distribution of macrophages in glomeruli at the late phase better corresponded with the deposition of rat IgG rather than that of rabbit IgG, which also supports this suggestion.

The recognition of the Fc portion of anti-GBM antibody by FcγR should stimulate the macrophages to produce and release pro-inflammatory cytokines and chemokines, such as tumor necrosis factor, IL-1 and MCP-1 as described previously (1,2,4). Our previous study also showed that this model was CD8+ T cell dependent, and glomerular accumulation of CD8+ T cells was mediated through interaction between ICAM-1 and LFA-1 (1,3). Cytokines released from macrophages might stimulate induction of ICAM-1 expression on endothelial cells and activate LFA-1 on CD8+ T cells in the glomeruli. However, it is unclear how CD8+ T cells play a key role in this model. Up-regulation of IFN-γ in the glomeruli of this model was demonstrated in our previous study (2), suggesting that CD8+ T cells and CD4+ T cells might be stimulated by another cytokine, such as IL-12, released from macrophages in the glomeruli. IL-12 is a cytokine known to stimulate expression of CD4+ T cells to differentiate to T h1, and also stimulates CD8+ T cells and CD4+ T cells to produce IFN-γ (2,17). Then, IFN-γ could further activate macrophages to express FcγR and to promote macrophage-mediated glomerular injury as delayed-type hypersensitivity.

In conclusion, this is the first study providing direct evidence of the crucial participation and role of FcγR in the initiation of anti-GBM antibody-induced crescentic glomerulonephritis in WKY rats and the recruitment of macrophages to the glomeruli. Although the type of FcγR mostly contributing to the progression of this glomerulonephritis has not yet been identified, the important role of the FcγR-mediated immune adherence was clarified in the macrophage accumulation in this model.

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Abbreviations

FcR     Fc receptor
GBM     glomerular basement membrane
HAIgG   heat-aggregated IgG
PE      phycoerythrin

Fc receptor-mediated anti-GBM nephritis