Accelerated Passive Heymann Nephritis in Rats as an Experimental Model for Membranous Glomerulonephritis and Effects of Azathioprine and Prednisolone on the Nephritis

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Abstract—Progressive passive Heymann nephritis (group II) was induced in rats by i.v. injections of rabbit antiserum against an antigen from the brush border of the proximal tubules of rat kidney following immunization with rabbit γ-globulin in Freund’s complete adjuvant, and the process of the disease was compared with that of rats that received the antiserum alone (group I). The rats of group I showed proteinuria (30–50 mg/day) and plasma cholesterol content (80–90 mg/dl) slightly higher than the normal level from the 17th or the 22nd day after antiserum injection to the 90th day. The rats of group II revealed a heavy proteinuria (300–400 mg/day) and hypercholesterolemia (approx. 200 mg/dl) during the same period. In group II, there were the thickening of glomerular basement membrane (GBM) and spike formation. Moreover, granular deposits of rat IgG, rabbit IgG and rat C3 were observed along the GBM. These changes were weaker in group I. When given orally, daily, azathioprine (20 mg/kg) and prednisolone (1 and 3 mg/kg) showed a beneficial effect on the nephritis in group II. The group II model closely resembles human glomerulonephropathy and may be useful for studying the effect of medication on glomerulonephropathy.

Human membranous glomerulonephropathy is characterized by a nephrotic syndrome and a slight impairment of renal function clinically and the thickening of glomerular capillary walls without the proliferation of mesangial cells (1). In 1959, Heymann and co-workers (2) first produced an experimental model for membranous glomerulonephropathy by immunizing rats with a mixture of rat kidney cortex homogenate and Freund’s complete adjuvant (FCA). Then, Edgington et al. (3) induced a similar membranous type of glomerulonephritis by using an antigen (Fx1A antigen) from the brush border of the proximal tubules of rat kidney instead of rat kidney cortex homogenate to clarify the pathogenic mechanism of the development of proteinuria. Thus, the actively induced model has been called active Heymann nephritis (HN). On the other hand, Feenstra et al. (4) reported that a chronic glomerulonephritis resembling active HN could be induced in rats by i.v. injection of heterologous antiserum prepared in rabbits against the Fx1A antigen. The passively induced model is known as passive HN. However, it takes a long period of 6 to 8 weeks to induce active HN in rats and the amount of urinary protein excretion in this model is variable from rat to rat. In the passive HN, a single injection of heterologous antibody directed against the Fx1A antigen induces little or no proteinuria in rats, unlike human membranous glomerulonephropathy which is characterized by massive proteinuria (4, 5). We have previously shown that the autologous phase of anti-glomerular basement membrane (anti-GBM) nephritis in rats is markedly enhanced by immunizing with rabbit γ-globulin in FCA following the injection of rabbit anti-rat glomerular basement membrane serum (anti-GBM serum), and a crescentic type nephritis can be induced (6). The
present study was undertaken to induce an accelerated passive HN closely resembling human membranous glomerulonephropathy by i.v. injections of heterologous antiserum produced in rabbits against the Fx1A antigen following immunization of rats with rabbit γ-globulin in FCA. Furthermore, the effects of azathioprine and prednisolone on the accelerated passive HN were investigated.

Materials and Methods

Animals: Male Sprague-Dawley strain SPF rats weighing approx. 180 g (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka) were used. For the preparation of antiserum against Fx1A antigen, male New Zealand white rabbits weighing approx. 2 kg (Chubu Kagaku Shizai) were used. These animals were housed in an air-conditioned room at 23±1°C.

Drugs: Drugs used were azathioprine (Tanabe Pharmaceutical Co., Ltd.) and prednisolone (Toyo Zyozo Co., Ltd.). Both drugs were suspended in 5% gum arabic.

Preparation of Fx1A antigen from the brush border of the proximal tubules of rat kidney: Fx1A antigen was prepared according to the method of Edgington et al. (7).

Production of rabbit antiserum against Fx1A antigen: Rabbit antiserum against Fx1A antigen was produced according to the method of Feenstra et al. (4). Namely, 20 mg of Fx1A antigen was suspended in 5 ml of 0.9% NaCl solution. To this suspension, an equal volume of FCA (Difco) was added, and the mixture was emulsified. The emulsion in a volume of 0.01 ml each was injected intracutaneously at 10 sites on the back of a rabbit. This procedure was repeated five times with a 7-day interval. Seven days after the last injection, blood was preliminarily taken from the ear vein and tested for antibody titer by indirect hemagglutination (8). After confirming a sufficient rise of titer, blood samples were taken, sera were separated and then inactivated by heating at 56°C for 30 min. The inactivated sera were preserved by freezing until use.

Induction of accelerated passive HN: Rats were divided into 3 groups, each consisting of 10 animals as follows: Normal group: untreated rats, Group I (original type passive HN): the rats were injected with 0.5 ml of antiserum into the tail vein once a day for 5 days. Group II (accelerated passive HN): the rats were injected with 0.5 ml of antiserum into the tail vein daily for 5 days from the next day of injection of 5 mg of rabbit γ-globulin in FCA into the hind foot pads. The process of accelerated passive HN in comparison to that of original type passive HN was followed for 90 days.

Effects of azathioprine and prednisolone on accelerated passive HN: In the first experiment, rats were divided into 3 groups, each consisting of 8 animals. Accelerated passive HN was induced in the rats of 2 groups. One group was given p.o. daily 20 mg/kg of azathioprine from the first day of antiserum injection to the 49th day. Another group was given 5% gum arabic p.o. instead of test drug as the control. The remaining one group without nephritis was used in the experiment as the normal group. In the second experiment, rats were divided into 4 groups, each consisting of 8 animals. Accelerated passive HN was induced in the animals of 3 groups. Two groups were given p.o. daily 1 and 3 mg/kg of prednisolone, respectively, for the same period as that of the administration of azathioprine. The remaining nephritic group was given the vehicle (5% gum arabic, p.o.) as the control. In addition, one group in which nephritis was not induced was used as the normal group.

Determination of biochemical parameters in urine: The protein content and N-acetyl-β-D-glucosaminidase (NA-β-G), γ-glutamyl transpeptidase (γ-GTP) and plasmin-like (PL) activities were periodically determined as biochemical parameters in the urine. The protein content was determined by the method of Kingsbury et al. (9). NA-β-G activity was determined according to the method of Hasebe (10) by using p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) as substrate, and γ-GTP activity was determined by a γ-GTP Test Pack Sankyo (Sankyo) (11). Moreover, PL activity was determined by a slight modification of the fibrin plate method described by Astrup and M üllertz (12). The fibrin plate was prepared by using plasminogen-free bovine fibrinogen (fibrinogen type II, Daiichi Kagaku).
Determination of biochemical parameters in plasma: Cholesterol (CL) content was determined by using Determiner TC 5 (Kyowa Medex) (13), and urea nitrogen content was determined by the method of Searcy and Cox (14). The antibody titer against rabbit r-globulin was determined by indirect hemagglutination using sensitized sheep red blood cells (8). Moreover, recalcification time (sec) to assess blood coagulation and fibrinogen content were measured by a fibrometer (BBL).

Measurement of blood pressure: Blood pressure was measured by tail plethysmography [KN-209] (Natsume).

Light microscopy: For light microscopic study, kidneys were dehydrated and fixed by immersing the tissues stepwise into low to high concentrations of alcohol. Paraffin sections (2–3 μm thick) were stained with hematoxylin and eosin for observing hypercellularity in glomeruli and stained with periodic acid-methenamine silver for observing the thickening of the glomerular basement membrane (GBM) and the spike formation in the GBM.

Fluorescent microscopy: For fluorescent study, paraffin sections (3–4 μm thick) of the kidney were stained with fluorescein isothiocyanate (FITC)-labeled antibodies such as goat anti-rat IgG (Cappel), goat anti-rat C₃ (Cappel), goat anti-rat fibrinogen (Cappel) and goat anti-rabbit IgG (Cappel).

Statistical analysis: The data represent the mean±S.E. or S.D. and the results were statistically evaluated by Student’s t-test, the Aspin t-test or the Mann-Whitney’s U-test.

Results
1. Changes in biochemical parameters in urine and plasma, blood pressure, histopathological parameters and immunopathological parameters after induction of nephritis in groups I and II

Biochemical parameters in urine (Fig. 1): The urinary protein content of the normal group was 10–15 mg/24 hr urine through the 90 day observation period. The urinary protein excretion of group I began to increase slightly from the 17th day after the antiserum injection and was 30–50 mg/24 hr urine from the 17th to the 90th day. On the other hand, the protein excretion of group II remarkably increased from the 17th day onwards and maintained 300–400 mg/24 hr urine up to the 90th day. The urinary NA-β-G activity of group I was approx. 60% higher than that of the normal group on the 17th day and then returned to nearly the normal level by the 30th day. In group II, this enzyme activity showed the maximum (approx. 140% higher than in the normal group) on the 17th day. The NA-
β-G activity was significantly higher in group II than in group I on the 7th, 50th and 60th days. The urinary τ-GTP activity also elevated due to nephritis and was significantly higher in group II than in group I from the 30th day onwards (data not shown). Although PL activity was not detectable in the urine from the normal group, the apparent lysis of the fibrin plate was observed in urine from groups I and II from 17th day. The PL activity was also significantly higher in group II than in group I from the 17th day onwards.

Biochemical parameters in plasma (Fig. 2): The plasma CL level in the normal group was 60–70 mg/dl during the experimental periods. The CL level of group I showed a significant increase (80–90 mg/dl) from the 22nd to the 78th day. On the other hand, the CL level of group II was approx. 200 mg/dl during the period of the 35th to the 78th day and was much higher than that of group I. The plasma urea nitrogen levels in groups I and II remained at the normal levels during the observation periods (data not shown). The serum antibody titer against rabbit τ-globulin was not recognized in the normal group. The antibody titer of group I began to elevate from the 15th day and then reached a peak on the 60th day. The antibody titer of group II was approx. two times higher than that of group I from the 15th through the 90th day. When the plasma recalcification time and fibrinogen content were measured on the 40th, the results indicated an apparent enhancement of blood coagulation in group II.

Blood pressure (data not shown): The blood pressure of groups I and II showed normal levels (110–115 mmHg) through the experimental periods.

Histopathological parameters in glomeruli (Figs. 3 and 4): In the glomeruli from rats of groups I and II, hypercellularity was seen in glomeruli through the experimental periods. The GBM of the normal group was thin and smooth (Fig. 3a). In group II, on the 15th day, occasional spikes could be seen on the outer border of the GBM. On the 30th day, the GBM was thickened and moderate spikes were observed on the outer border of the GBM. Thereafter, on the 60th (Fig. 3b) and 90th days, severe thickening of the GBM was seen and numerous silver-positive spikes diffusely appeared in the glomeruli. On the 90th, the index of glomerular lesions (IGL) was significantly greater in group II than in group I (Fig. 4).

Immunopathological parameters in glomeruli (Table 1 and Fig. 5): In the sections of the normal rats, no fluorescent staining for rat IgG, rabbit IgG, rat C3 and rat fibrinogen could be detected. In group I, a weak but unmistakable (mild, +) granular deposition of rat IgG and rabbit IgG was observed along the GBM on the 90th day (Table 1). In group II, mild (+) deposits of rat IgG appeared on the
15th day and then the intensity of the fluorescence for the rat IgG reached the maximum (severe, ++++) on the 30th day (Table 1). Later, the deposition of the IgG decreased and became moderate (+++) on the 90th day (Table 1 and Fig. 5a). A moderate (++) deposition of rabbit IgG was recognized in group II on the 15th day (Table 1). Thereafter, from the 30th through the 90th day, mild (+) deposition of the rabbit IgG was maintained (Table 1 and Fig. 5b). In addition, in group II,

Table 1. Changes in the intensity of the fluorescence for immunoglobulin after the induction of nephritis in groups I and II

| Days | Rat IgG (Granular) | Rabbit IgG (Granular) | Rat C3 (Granular) | Rat fibrinogen |
|------|------------------|----------------------|------------------|---------------|
| Group I |                  |                      |                  |               |
| 90    | +                | +                    |                 |               |
| Group II |                 |                      |                  |               |
| 0     |                  |                      |                 |               |
| 15    | +                | ++                   |                 |               |
| 30    | ++               | +                    |                 |               |
| 60    | ++               | +                    |                 |               |
| 90    | ++               | +                    |                 |               |

The intensity of fluorescence: −, negative; +, mild; ++, moderate; and ++++, severe. See Fig. 1 for the explanation of groups I and II.
mild (+) deposition of rat C3 was seen along the GBM through the observation period from the 15th day, although the deposition was not detectable in group I (Table 1 and Fig. 5c). However, no fluorescent staining for rat fibrinogen was seen on any day in groups I and II (Table 1).

2. Effects of azathioprine and prednisolone on accelerated passive HN (group II)

Urinary protein excretion (Fig. 6): Azathioprine (20 mg/kg/day, p.o.) showed a marked inhibition of approx. 60% on the urinary protein excretion during the period of the 30th to the 46th day. Prednisolone (1 and 3 mg/kg/day, p.o.) had only a tendency to inhibit the protein excretion on the 30th and 38th days, although a significant inhibition (32%) was recognized by a low dose on the 46th day.

Plasma CL content (Fig. 6): Azathioprine (20 mg/kg/day, p.o.) inhibited the elevation of the plasma CL level by approx. 40% from the 25th to the 50th day. Prednisolone (3 mg/kg/day, p.o.) also showed a significant inhibition of approx. 30% from the 15th to the 35th day. The low dose of this drug significantly inhibited only on the 25th day.

Plasma antibody titer against rabbit γ-globulin (Fig. 6): The elevation of the plasma antibody titer against rabbit γ-globulin was markedly inhibited by approx. 30%–50% by azathioprine (20 mg/kg/day, p.o.) from the 15th to the 50th day. Prednisolone (1 and 3 mg/kg/day, p.o.) was only prone to inhibit the elevation of the antibody titer on the 25th and 35th days.

Histopathological parameters (Fig. 7): On the 50th day, azathioprine (20 mg/kg/day, p.o.) and prednisolone (1 and 3 mg/kg/day, p.o.), though slightly, significantly reduced the IGL. Namely, both drug inhibited histopathological changes such as the GBM thickening and the spike formation.

Discussion

In the present study, we were able to induce an accelerated passive HN that closely resembles human membranous glomerulonephropathy by i.v. injections of rabbit antiserum against the Fx1A antigen once a day for 5 days following immunization of rats with rabbit γ-globulin in FCA. Namely, the rats of group II showed GBM thickening and spike formation on the outer border of the GBM histologically as well as massive proteinuria and hypercholesterolemia from the 15th day onwards. In addition, the immunopathological observations of the kidney indicated a granular fluorescent staining for rat IgG, rabbit IgG and rat C3 along the GBM. Changes in these biochemical and histopathological parameters were much greater in group II than in
Fig. 6. Effects of azathioprine and prednisolone on biochemical parameters in urine and plasma in accelerated passive HN (group II) in rats. Each plot denotes the mean value with S.D. obtained from 8 rats. *P<0.05, **P<0.01 and ***P<0.001 vs. the control.
There are a number of reports concerning the mechanism of the induction of passive HN in rats. For some years, it was believed that the induction of this disease is attributed to deposition of circulating antigen-antibody complexes in the GBM (15, 16). However, recent investigations by perfusion of isolated kidneys demonstrated that there is direct binding of circulating antibodies to a fixed glomerular antigen (17-20). In this connection, Kerjaschki and Farquhar (17) found that the pathogenic antigen of HN is a component of the glomerular epithelial cells. Therefore, currently, it is generally accepted that the injected heterologous antibody in the passive HN forms in situ immune complexes by fixing to preexisting antigen in glomerular epithelial cells. Recently, it has been also shown that approx. 10 days after the in situ immune complex formation in the heterologous phase, there occurs the second in situ immune complex formation by binding of the host’s antibody against the injected heterologous γ-globulin to prefixing heterologous γ-globulin in the GBM. In short, immune responses in the passive HN in rats may consist of heterologous and autologous phases, like what occurs in the anti-GBM nephritis in rats (6).

In the present experiment, the progression of the passive HN occurred by immunizing with rabbit γ-globulin in FCA, before i.v. injections of rabbit antiserum against the Fx1A antigen into rats. The plasma antibody titer against rabbit γ-globulin in group II rapidly elevated from the 15th day and was then maintained at a level approx. two times higher than that of group I up to the 90th day. In addition, on the 90th day, a granular deposition of rat IgG observed along the GBM was greater in group II than in group I. These results suggest that immunization with rabbit γ-globulin may enhance the immune response in the autologous phase via the persistent production of host’s antibody against rabbit γ-globulin. Therefore, the progression of passive HN by immunizing with rabbit γ-globulin in FCA may be related to increasing deposition of an autologous antibody directed against the heterologous protein.

In the present experiment, an apparent proteinuria in groups I and II was not still recognized even on the 7th day in contrast with anti-GBM nephritis in rats (6), which induces proteinuria from the next day of the anti-GBM serum injection and appeared from the 17th day, when the plasma antibody titer against rabbit γ-globulin began to elevate rapidly, that is, from the autologous phase. Thus, it takes about two weeks to induce nephrosis in the accelerated passive HN. In
the preliminary experiment, in order to induce nephrosis as soon as possible in this model, we immunized rats with rabbit γ-globulin in FCA two weeks prior to the injection of antiserum against the Fx1A antigen. However, all animals injected with the antiserum died of anaphylaxis. This result suggests that the injection of the antiserum after the elevation of plasma antibody titer against rabbit γ-globulin may induce anaphylaxis in rats. Therefore, in the present experiment, immunization with rabbit γ-globulin was done on the day before the antiserum injection.

In the next experiment, the effects of azathioprine and prednisolone on the accelerated passived HN (group II) were evaluated. Both drugs showed a clear effect on the morphology of glomeruli as well as on the proteinuria and hypercholesterolemia. The effect of azathioprine was superior to that of prednisolone. This experimental model closely resembles human glomerulo-nephropathy and may be useful for screening beneficial drugs on this type of nephritis.

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