Antinephritic Effect of Prostaglandin E₁ on Serum Sickness Nephritis in Rats (3)

Suppression of Leukocytes by Prostaglandin E₁ as a Mechanism for Preventing Immune Complex Glomerulonephritis

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Abstract—Serum sickness nephritis was produced in rats by repeated i.v. injections of rabbit serum albumin. After 12 weeks of antigen injection, the rats with proteinuria were subjected to a renal biopsy. Then half of the group was continuously given 300 μg/rat/day of PGE₁-α-cyclodextrin (PGE₁-CD) with mini osmotic pumps for 3 weeks. PGE₁-CD inhibited the development of glomerulonephritis and the deposition of immune complexes in the glomeruli, although the control group showed developed glomerular alteration and increased immune deposits in the glomeruli in the autopsy specimens. PGE₁-CD particularly improved the intraluminal hypercellularity of the histological findings. In the early stage of this model, 0.5 and 1.0 mg/kg of PGE₁ and 6.0, 12.5 and 25.0 mg/kg of azathioprine were administered s.c. and p.o., respectively, for 3 weeks. There was a significant suppression of about 20% in the 1.0 mg/kg PGE₁ group on the antibody synthesis as compared with the control group throughout the experimental period, although the inhibition was less than that of azathioprine. In the same experimental protocol, PGE₁ significantly suppressed the increase in leukocyte counts; at the third week after PGE₁, the number of leukocytes was 25.6±4.2×10³ in the control group and 14.1±3.0×10³ in the 1.0 mg/kg PGE₁ group. It is considered that PGE₁ could exert the antinephritic effect in this mode through decreasing the leukocyte counts in the circulation and the glomeruli independently of immune deposits in the glomeruli.

Some investigators (1–6) have demonstrated the beneficial effect of prostaglandin E₁ (PGE₁) on experimental nephritis since Zurier et al. (7, 8) reported that a pharmacological dose of PGE₁ could protect New Zealand Black/New Zealand White hybrid F₁ mice against the development of nephritis and could prolong their survival. Nagamatsu et al. (9, 10) reported previously that both PGE₁ and PGE₁-α-cyclodextrin host molecule (PGE₁-CD), a water-soluble PGE₁ derivative, reduced the proteinuria more rapidly than the control when the treatment was started after the withdrawal of rabbit serum albumin (RSA) i.v. injection in serum sickness nephritis. They also demonstrated that the rats treated with PGE₁-CD had less glomerular alteration than the control rats in their model. Although attempts have been made to clarify the correlation of the suppressive effect of PGE₁ on the antibody synthesis with the inhibitory effect on the development of glomerulonephritis, the results on the inhibitory effect of PGE₁ on antibody synthesis were not necessarily consistent (1–7). The purpose of this paper is to histologically confirm the antinephritic effect of PGE₁ by a renal biopsy technique and to clarify whether PGE₁ could suppress the antibody synthesis in this model. Furthermore, we
investigated the effect of PGE₁ on leukocyte counts in the circulation. An attempt is made to explain the possible mechanisms by which PGE₁ depresses the glomerulonephritis.

Materials and Methods

1. Induction of serum sickness nephritis (Fig. 1): Serum sickness nephritis was induced as reported previously (10). Briefly, thirty male Sprague-Dawley rats (Nihon Seibutsu Kagaku Center Co., Ltd.), weighing about 180 g, were s.c. immunized with 3 mg/rat of rabbit serum albumin (RSA) in complete Freund’s adjuvant (Difco). Animals received an i.v. injection of RSA in 0.25 ml of saline at 0.5 mg/rat every day for the first week period from 2 weeks after the first immunization. Then, they were injected with RSA at 0.75 mg/rat for the second one week period and injected with 1.0 mg/rat the following week every day. The rats were reimmunized in the same manner as the first immunization at the fourth week after the first immunization.

To obtain the day course for proteinuria, urine was collected every week until 5 weeks and during the 8th and 11th weeks after the immunization. Each animal was given 8 ml of distilled water orally through a stomach tube. Then, urine samples were collected from animals kept in separate metabolic cages for 24 hr without feeding and water. The collected urine was centrifuged at 3,000 rpm for 15 min. The protein content in the supernatant was determined by the sulfosalicylic acid method (11) and expressed as mg per 24 hr urine.

To obtain the day course for anti-RSA antibody, blood was drawn from the tail vein with a heparinized (Shimizu Seiyaku Co., Ltd.) disposal microsyringe (Japan Medical Supply Co., Ltd.) every other week from the immunization as reported previously (12). The plasma sample was obtained by routine methods. The anti-RSA antibody titer was determined by the passive hemagglutination method (13) using sensitized sheep red blood cells. The antibody titer was expressed as log₂ of the highest dilution with no button of sensitized sheep red blood cells.

2. Evaluation of renal tissue by using biopsy: After confirming the induction of nephritis by means of the level of their protein excretions into urine, 16 of 30 rats were selected at 12 weeks after the first immunization with RSA. All of them had more than 40 mg/day of proteinuria. Protein contents in the urine were 437.7±180.5 (S.D.) mg/day in the PGE₁-CD group and 376.3±165.5 (S.D.) mg/day in the control group. They were subjected to a renal biopsy as reported previously (12), and a half of them then continuously received PGE₁-CD (Ono Pharmaceutical Co., Ltd.) corresponding to 300 μg/rat/day of PGE₁ with a mini osmotic pump (Alzet Model 2MLI, Alza) planted beneath the buck skin for 3 weeks as reported previously (10). The mini osmotic pump was exchanged to a new mini osmotic pump that was filled with PGE₁-CD every week. The renal tissue was fixed in alcohol and embedded in paraffin. The paraffin sections were stained with hematoxylin and eosin, periodic acid-Schiff, or fluorescein-conjugated antisera for rat IgG or RSA. The histological findings were evaluated in regards to the capillary and the mesangium in the glomerulus as reported previously (9). The extent of fluorescence staining for IgG or RSA was scored from 0 (-) to 4 (++++).

3. Effects of PGE₁ and azathioprine on production of anti-RSA antibody: The drugs used in this experiment were PGE₁ (Funa-koshi Yakuhin Co., Ltd.) and azathioprine (aza.) (Imuran®, Tanabe Seiyaku Co., Ltd.). Ten mg of PGE₁ was dissolved in 1 ml of absolute ethanol and stored at -20°C. Just prior to administration, PGE₁ was diluted with phosphate-buffered saline (PBS). The final
solution consisted of PGEₑ in 10% ethanol-PBS at pH 7.2. Aza. was suspended in 5% gum arabic.

Animals had been given PGE₁, s.c. or aza., p.o. every day for 3 weeks after the immunization with RSA. PGE₁ was continuously injected with the mini osmotic pump at 0.5 and 1.0 mg/kg/day. Aza. as a comparative drug was administered at 6.0, 12.5 and 25.0 mg/kg. The animals had been i.v. injected with 0.5 mg/rat of RSA every day from 2 weeks after the immunization. The control rats were given only the solvent for PGE₁ in the same manner as the administration of PGE₁. Blood was drawn every week, and the antibody titer was measured as described above.

4. Effect of PGE₁ on leukocyte counts in peripheral blood: In both the PGE₁ and control groups just mentioned above, the blood for the leukocyte counts was drawn prior to obtaining blood for the antibody every week until 4 weeks. Additionally, blood samples were obtained before the injection of antigen. Although PGE₁ had been given until 3 weeks, the determination was also performed on the next week when the animals had been injected with RSA at 0.75 mg/rat every day. The leukocyte counts were determined by the routine method.

5. Statistical analysis: All data presented are the means±S.D. and were analyzed by the F-test, unpaired t-test, and Mann-Whitney U-test. Differences at the levels of P<0.05, P<0.01 and P<0.001 were all considered significant.

Results

1. Time course of anti-RSA antibody titer (Fig. 2): The anti-RSA antibody titer began to rise 2 weeks after the immunization with RSA. Thereafter, it rapidly augmented and reached a plateau at 4 weeks. In the normal group, the antibody titer was never detected throughout the experimental period. Results were obtained from 30 rats until 5 weeks, and then they were obtained from the rats showing proteinuria.

2. Time course of proteinuria (Fig. 2): The animals had normal protein contents in the urine until 6 weeks after the immunization. However, at 8 weeks, proteinuria developed in 12.8% of the treated rats. The mean protein content for all 30 rats was 79.6 mg/day, and then it increased gradually with the augmenting incidence. On the 12th week, in 52.5% of the rats, severe proteinuria developed.

3. Effect of PGE₁·CD on histological alteration in glomerulus (Table 1): In the renal biopsy before the experiment, the rats showed capillary wall thickening, hypercellularity, and mesangial proliferation. The score of each histological parameter in the control group steadily increased in the autopsy specimens as compared with the respective ones in the biopsy specimens. On the other hand, 3 weeks of PGE₁·CD inhibited the progress of glomerular alteration. In the autopsy for PGE₁·CD-treated rats, the scores of space narrowing and hypercellularity decreased, and the score of the mesangium was the same level as that of the biopsy

Fig. 2. Changes in anti-RSA antibody and proteinuria in serum sickness nephritis. Results are the mean±S.D. obtained from 30 rats. ○: normal, ●: nephritis.
specimens, although the score of wall thickening slightly increased.

4. Effect of PGE₁·CD on immune deposits in glomerulus (Table 2): In the immunofluorescence findings, granular staining of rat IgG or RSA were observed in the glomerular mesangium and on the glomerular capillary wall. The autopsy specimens of the control group revealed prominently increased deposits of IgG and RSA in the glomerulus as compared with the biopsy specimens. In contrast, 3 weeks of PGE₁·CD resulted in a slight increase of both deposits as compared with those before the treatment. Therefore, the PGE₁·CD group had less glomerular deposits than the control group after the treatment.

5. Effects of PGE₁ and azathioprine on the production of anti-RSA antibody (Fig. 3): In the control group, the antibody titer rose by 6.3±1.6 at the first week, 7.0±1.6 at the second week, and 10.1±0.2 at the third week. There was a significant suppression of about 20% in the 1.0 mg/kg PGE₁ group as compared with the level of the control group throughout the experimental period. The

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**Table 1. Effect of PGE₁·CD on histological alteration in the glomerulus**

| Groups       | Wall thickening | Space narrowing | Hypercellularity | Proliferation |
|--------------|-----------------|-----------------|------------------|--------------|
| Control      | biopsy          | −               | −                | −            |
|              | autopsy         | ++−++          | +−+              | +−+          |
| PGE₁·CD 300 µg | biopsy         | +−+            | +−+              | +            |
|              | autopsy         | +              | +                | ++           |

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**Table 2. Effect of PGE₁·CD on immune deposits in the glomerulus**

| Groups       | IgG       | RSA       |
|--------------|-----------|-----------|
| Control      | biopsy    | ++        | ++−++++     |
|              | autopsy   | +++−++++  | +++         |
| PGE₁·CD 300 µg | biopsy   | +−+       | +−+         |
|              | autopsy   | +         | +−++        |

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**Fig. 3. Effects of PGE₁ and azathioprine on antibody titer in plasma.** Results are the mean±S.D. obtained from 5 to 8 rats. Significant difference from the control: *P<0.05, **P<0.01 and ***P<0.001.

PGE₁: 0.5 mg/kg ■, 1.0 mg/kg □; azathioprine: 6.0 mg/kg △, 12.5 mg/kg ▲, 25.0 mg/kg ◆; normal: ○; control: ●; drug treatment ▼▼▼.
lower dose of PGE₁ showed significantly less antibody titer than the control at only the first week. The treatment with aza. resulted in a notable suppression of about 30 to 40% on the antibody titer except in the 6.0 mg/kg group.

6. Effect of PGE₁ on leukocyte counts (Table 3): At the 0 week, normal rats had $16.3\pm3.1 \times 10^3$ leukocytes. Leukocyte counts noticeably increased around 60% in both PGE₁ groups and 131.4% in the control group at the first week after the immunization. At the third week, PGE₁ caused around 50% less leukocyte counts than the control, and the leukocyte counts for both PGE₁ groups showed the normal level. After the cessation of PGE₁, however, leukocyte counts increased about 50% again, and there was no significant difference between both PGE₁ groups and the control group.

Discussion

In the present studies, PGE₁·CD inhibited the increase of immune complexes in the glomerulus as shown in the immunofluorescence findings for IgG and RSA as compared with the control group. These results on autopsy specimens is consistent with our previous study (10) and the reports of others (1, 2, 5, 6, 8). Many authors have suggested that the antinephritic effect of PGE₁ could due to a decrease of immune complexes in the glomeruli (1, 2, 5, 6, 8). This can result from inhibiting the formation of immune complexes in the circulation, diminishing the deposition of immune complexes in the glomeruli, or by enhancing the ability to expel the immune complexes from the glomeruli.

It seems likely that PGE₁ could inhibit the antibody synthesis, leading to a decrease of immune complexes in the circulation. McLeish and his colleagues (5) demonstrated that PGE₁ and PGE₂ caused the reduction of specific antibody synthesis in murine immune complex glomerulonephritis induced with apoferritin. Izui et al. (3) reported the selective suppression of antibody directed to retroviral gp70 by PGE₁ in NZB/W F1 mice with lupus-like nephritis. On the other hand, Zurier et al. (8) and Kelley et al. (1) demonstrated that PGE₁ could fail to inhibit the production of anti-DNA antibody in NZB/W F1 mice. Both anti-DNA (14) and anti-gp70 antibodies (15, 16) are thought to be factors involved in the development of nephritis in mice. Thus, although the results reported have been in disagreement on the inhibitory effect of PGE₁ on antibody synthesis, this is thought to be due to the difference in subclass of IgG antibody. In the present study, we investigated the effect of PGE₁ on the antibody synthesis using the rats that developed no proteinuria, since we have reported that anti-RSA antibody was excreted into the urine together with other proteins in the nephritic stage (12). If PGE₁ could exert the antinephritic effect apart from the inhibition of antibody synthesis, it would be difficult to interpret the results on the inhibitory effect on antibody synthesis. That is, when PGE₁ can inhibit the development of nephritis, but fails to decrease the antibody titer in the circulation, we can not exclude the possibility that the antibody could increase due to PGE₁ inhibition of the excretion of the antibody into the urine. In addition to this, we consider that it is

### Table 3. Effect of PGE₁ on leukocyte counts

| Groups   | Dose (mg/kg) | N  | 0     | 1    | 2    | 3    | 4 weeks |
|----------|--------------|----|------|------|------|------|---------|
| Normal   | 6            | 16.3±3.1 | 15.6±3.6 | 15.8±3.6 | 17.5±2.3 | 21.8±1.9 |
| Control  | solvent      | 5   | 14.0±4.5 | 32.4±7.0* | 23.4±3.3* | 25.6±4.2* | 21.9±4.0* |
| PGE₁     | 0.5          | 8   | 18.5±5.5 | 30.2±7.0* | 23.9±3.4* | 14.1±3.0* | 26.8±5.9* |
| PGE₁     | 1.0          | 8   | 16.4±5.1 | 27.6±6.6* | 20.4±6.7* | 13.2±5.4* | 24.6±5.1* |

Results are the mean±S.D. PGE₁ was administered for 3 weeks. After 3 weeks, PGE₁ was withdrawn. Significant difference from the normal: *P<0.05, from the control: **P<0.05. RSA was intravenously injected 2 weeks after.
necessary to determine whether PGE\textsubscript{1} can inhibit the production of antibody to RSA. Therefore, we believe that the time course of the experiment in this study would be reasonable. In this study, PGE\textsubscript{1} could suppress the synthesis of antibody to RSA, although the suppression was one half of that withaza. Since PGE\textsubscript{1} suppressed the level of IgG\textsubscript{1}, but not IgG\textsubscript{2}, subclass in MRL/1 female mice (3), anti-RSA antibody would belong to the IgG\textsubscript{1} subclass in part. Using the present higher dose, aza could inhibit the development of experimental nephritis (17, 18). Furthermore, if PGE\textsubscript{1} could remarkably inhibit the antibody synthesis, the amount of antigen could exceed that of antibody in the circulation. Under this circumstance, the glomerular deposits should be more decreased than that in this study, since the excess antigen in the circulation leads to release of complexes deposited in the glomeruli (19, 20). In conclusion, this inhibitory effect on antibody synthesis seems advantageous, although slightly, in the inhibition of development of nephritis.

In the previous therapeutic trial, we demonstrated that PGE\textsubscript{1}-CD resulted in less alteration and immune complex deposits in the glomeruli than the control (10). Since in that trial, however, renal specimens were obtained only at the end of the experiment, it is considered that prior to the trial, there might have differences in the alteration of renal pathology between PGE\textsubscript{1}-CD and the control groups. Furthermore, there are few reports that compare the histology of the glomeruli before treatments with that after the treatments in other therapeutic trials in experimental nephritis. In the present study, first of all we attempted to clarify the glomerular alteration for the specimens obtained by renal biopsy before the trial. Three weeks later, in the control rats, the glomerular alteration steadily developed, whereas in the PGE\textsubscript{1}-CD-treated rats, the alterations were inhibited or did not develop. Before PGE\textsubscript{1}-CD treatment, rats had a large number of leukocytes in the glomerular capillary lumen, whereas the leukocyte counts in the capillary lumen were remarkably reduced in the autopsy specimens for PGE\textsubscript{1}-CD. Kelley et al. (1) also observed a striking decrease in the number of cells infiltrating the glomeruli in PGE\textsubscript{1}-treated NZB/W F\textsubscript{1} mice. Kunkel et al. (4) suggested that the ability of PGE\textsubscript{1} to prevent glomerulonephritis may occur through a direct effect on inflammatory cells in anti-GBM nephritis in rats. Further, Ham et al. (21) demonstrated that PGE\textsubscript{1} obviously inhibited LT\textsubscript{B}\textsubscript{2}, a potent polymorphonuclear leukocyte (PMN) chemoattractant, release from PMN by increased levels of cyclic AMP, supporting that PGE\textsubscript{1} could inhibit the accumulation of leukocytes in the glomeruli. Thus, PGE\textsubscript{1} could decrease the number of leukocytes in the glomeruli through the direct effect on leukocytes in the nephritic rats.

It has been well-known that leukocytes are associated with the inflammatory process (22, 23). Recent studies in a number of experimental models support that leukocytes may play an important role in the development of glomerulonephritis (24–28). In the previous papers, we observed the hypercellularity in our nephritic model (9), and the rats treated with PGE\textsubscript{1} or PGE\textsubscript{1}-CD had less leukocyte counts in the glomerulus than the control animals (9, 10), suggesting the possibility that PGE\textsubscript{1} might inhibit the migration and/or accumulation of leukocytes in the glomerulus. However, we could not exclude the possibility that PGE\textsubscript{1} might suppress the proliferation of leukocytes in nephritis. In the present study, the control rats showed increased leukocyte counts from the first week after the immunization. The increase at one week is thought to be due to inflammation by immunization with RSA and complete Freund’s adjuvant. However, the augmenting numbers of leukocytes could be attributed to the stimulation of circulating immune complexes at 3 and 4 weeks later, because animals had received i.v. injections of RSA since the blood collection for the determinations at the second week. Higher dose of PGE\textsubscript{1} slightly, although insignificantly, suppressed the increase of leukocyte counts at the first and second weeks. At the third week, PGE\textsubscript{1}-treated rats revealed significantly lower leukocyte counts than the control rats. At the fourth week when PGE\textsubscript{1} was withdrawn, leukocyte counts in the PGE\textsubscript{1} group was significantly augmented again as com-
pared with the normal rats; the level was the same as that of the control group. These results may agree with the report by Santoro et al. (29) who demonstrated that PGE₁ could suppress the proliferation of B-16 melanoma cells in vivo. In addition, PGE₁ and PGE₂ inhibited mitogen stimulation of lymphocytes (30–32). Therefore, PGE₁ could diminish leukocyte counts systemically also in nephritic rats.

It is considered that PGE₁ could exert the antinephritic effect in this model through not only the suppression of leukocytes accumulating in the glomeruli but also through a decrease of leukocyte counts in the circulation independently of the extent of immune deposits in the glomeruli.

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