Suppression by Cyclosporin A of Anti-GBM Nephritis in Rats

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ABSTRACT — The suppressive effect of cyclosporin A (CyA) on the development of glomerulonephritis was evaluated in rats with either original- or crescentic-type anti-glomerular basement membrane (GBM) nephritis. CyA (2.5, 10 or 20 mg/kg) was given p.o. daily to original-type anti-GBM nephritic rats for 10 days from the day after the injection of anti-GBM serum. The development of the nephritis was dose-dependently suppressed by CyA before the production of specific antibody against rabbit γ-globulin (the heterologous phase). In addition, CyA suppressed glomerular infiltration of leukocyte subsets (leukocyte with common antigen, T cell, helper T cell, suppressor/cytotoxic T cell, macrophage/monocyte). CyA was given p.o. daily to crescentic-type anti-GBM nephritic rats for 10 days from the 10th day after the injection of anti-GBM serum. CyA-administration caused a distinct suppression of the deterioration of nephritis during the autologous phase. In addition, CyA markedly suppressed the antibody production. The above data indicate that CyA has a beneficial effect on anti-GBM nephritis, and the antinephritic action of this agent may be due to the inhibition of glomerular infiltration of leukocyte subsets as well as the suppression of the antibody production.

For the last two decades, it has been understood that immunological events have been involved in the pathogenesis of a certain type of human glomerulonephritis through the use of experimental models of nephritis. Anti-glomerular basement membrane (GBM) nephritis is considered to be one of models for proliferative glomerulonephritis (1). The development of anti-GBM nephritis consists of a heterologous phase and an autologous phase (2, 3). The heterologous phase results from the binding of anti-GBM antibody to the antigen(s) on the GBM, followed by the activation of complement. The autologous phase progresses through the immunological reaction of fixed antibody (anti-GBM antibody) with the antibody produced against the fixed rabbit IgG on the GBM. Immunosuppressive drugs have been clinically utilized for the therapy of some types of nephritis (4). However, the effects of these drugs on glomerulonephritis have been controversial until now. In 1970, cyclosporin were extracted from Clyndro-carpon luidam and Trichoderma polysorum Rifai as a metabolite that had anti-fungi effects. Then in 1976, Borel et al. (5) found that cyclosporin A, C and G out of the series of cyclosporines exhibited a marvelous immunosuppressive effect (5). Cyclosporin A (CyA) was initially used for cardiac transplantation in rats and the cardiac allograft proved to be viable (6). Thereafter, many in-
vestigations have been performed to determine the best route of CyA administration in order to reduce the death from infection. Recently, CyA has been found to be a strong immunosuppressive drug for organ transplantation (7–9). Moreover, CyA is effective for the treatment of experimental autoimmune diseases (10–13) besides being effective in murine glomerulonephritis (14).

In the present study we designed experiments to evaluate the effect of CyA on anti-GBM nephritis in rats.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (Japan SLC), weighing 160–170 g, were used in all experiments.

Experimental nephritis
Rats were intravenously injected with 0.75 ml/rat of anti-GBM serum, which was produced in rabbits as previously reported (15). Original-type anti-GBM nephritis was induced by the injection of anti-GBM serum alone. Crescentic-type anti-GBM nephritis was induced by the injection of anti-GBM serum, followed by the immunization with an intradermal injection of 6.5 mg of rabbit γ-globulin in 0.25 ml of Freund's complete adjuvant into the hind footpads on the next day (16).

Drug administration
CyA (Sandoz Co., Ltd.) was dissolved in 5% ethanol in olive oil. Animals orally received CyA in a volume of 0.5 ml/200 g of body weight. The rats with original-type anti-GBM nephritis were daily administered CyA in a dose of 2.5, 10 or 20 mg/kg for 10 days after the injection of anti-GBM serum alone. In addition, to evaluate the effect of CyA on infiltration of leukocyte subsets (OX-1, OX-19, W3/25, CD8 and ED1) in the glomeruli of the rats with original-type anti-GBM nephritis, this agent (10 mg/kg, p.o.) was given daily for 5 or 10 days after the injection of anti-GBM serum. In the subsequent experiment, the rats with crescentic-type anti-GBM nephritis received CyA (2.5, 10 or 20 mg/kg) for 10 days from the 10th day after the challenge of anti-GBM serum (effect of CyA given during the autologous phase). In addition to the drug-treated groups, non-treated nephritic (control) and normal groups were used in both experiments. Each group consisted of 9 or 10 rats.

Urine, blood and kidney
Urine samples were obtained from rats that were loaded with 8 ml of tap water, and then each rat was placed in a separate metabolic cage for 24 hr without any access to food and water. The urine was then centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was used for urinalysis. From the tail vein of conscious rats, blood was drawn with a syringe and put into a tube with 1.1 μmole of EDTA • 2Na. The blood was centrifuged at 3,000 rpm for 20 min at 4°C, and the plasma was utilized for the analysis. On the last day of the experiments, kidneys were isolated under anesthesia with sodium pentobarbital (30 mg/kg, i.p.) and then used in histological preparations.

Determination of parameters in urine and plasma
The protein content in urine was determined by the method of Kingsbury et al. (17) and expressed as mg per day. N-Acetyl-β-glucosaminidase (NAG) activity in the urine was determined as described by Hasebe (18) using 4-nitrophenyl-N-acetyl-β-glucosaminidase (Sigma) as the substrate and expressed as mU per day (24 hr urine). Cholesterol content in plasma was determined with a commercial assay kit (Determina TC-5, Kyouwa Medix Co., Ltd.) (19). Urea nitrogen content in plasma was determined by the urease-indophenol spectrophotometry assay (20). Both cholesterol and urea nitrogen contents were reported as mg per dl of plasma. Anti-rabbit γ-globulin antibody titer in plasma was determined by the passive hemagglutination method using sensitized sheep red blood cells (21). The antibody titer was expressed as the log of the
highest dilution that caused no visible agglutination of sensitized sheep blood cells. White blood cells were counted by a particle counter, PC-602A (Erma Co., Ltd.) and expressed as \( \times 10^3 \) cells/mm\(^3\).

**Light microscopic studies**

The kidneys isolated on the last day of the experiments were dehydrated and fixed by progressively higher concentrations of chilled alcohol diluted with Tris • HCl buffer at pH 7.5. The tissue was then embedded in paraffin and cut into 3-\( \mu \)m thick sections. The sections were stained with hematoxylin and eosin (HE), periodic acid Schiff, and Masson's trichrome. Ten glomeruli with vascular pole per section were counted in the number of cells to determine the degree of hypercellularity and represented as cells/glomerular cross section (GCS). Fifty glomeruli per section were observed under a light microscope for evaluating crescent formation, adhesion of capillary wall to Bowman's capsule and fibrinoid necrosis, respectively. The respective histological parameters were calculated as indexes for crescent formation, adhesion of capillary wall to Bowman's capsule and fibrinoid necrosis as reported previously (22). Each histological parameter was graded as normal (0 point), mild (1 point), moderate (2 points) or severe (3 points), according to the extent of the alterations. The number of glomeruli corresponding to each score was represented as \( n_0 \), \( n_1 \), \( n_2 \) and \( n_3 \). Indexes for crescent formation (CI), adhesion of capillary wall to Bowman's capsule (AI) and fibrinoid degeneration (FI) were calculated as follows:

\[
CI, AI \text{ or } FI = (1 \times n_1) + (2 \times n_2) + (3 \times n_3)
\]

Moreover, the index for glomerular lesions (IGHL) was calculated as follows:

\[
IGHL = \frac{(3 \times CI) + (2 \times AI) + (1 \times FI)}{(3 + 2 + 1) \times 50}
\]

We gave 3, 2 and 1 points to DI, AI and FI, respectively, because we consider that CI, AI and FI are associated with the glomerular lesion in this order; “50” shows the number of glomeruli.

**Immunoperoxidase studies**

An indirect immunoperoxidase technique employing avidin-biotin peroxidase kits (Becta stain) was used throughout the study (23). In brief, cryostat sections of snap-frozen material were dried for 30 min. Sections were subsequently incubated with 1 to 5 diluted normal rabbit serum, an appropriate dilution of the individual mouse monoclonal antibody (OX-1, OX-19, W3/25, CD-8, ED-1), affinity-purified rabbit anti-mouse peroxidase conjugated immunoglobulin also at the appropriate dilution, and finally with DAB (0.5 mg/ml in PBS plus 0.01% \( \text{H}_2\text{O}_2 \)) for three to five minutes. Only cells with a clear identifiable nucleus were counted.

**Statistical analysis**

All data were presented as the mean \( \pm \) S.D., and the results were statistically evaluated by Student's t-test or Mann-Whitney's U-test. In all comparisons, differences were considered significant at \( P < 0.05 \). Inhibitory percentage was calculated as follows:

\[
\text{Inhibitory percentage (\%)} = \frac{\text{Control} - \text{Test drug}}{\text{Control} - \text{Normal}} \times 100
\]

**RESULTS**

**Effect of CyA given p.o. daily during the heterologous phase on original-type anti-GBM nephritis**

At the onset of the experiment, the average body weight of all rats was 162 \( \pm \) 5.9 g (data not shown). By the 11th day, the body weight of the normal group increased by 44 g. However, the body weight gain of the nephritic control group during the same period was only 13 g. In groups given 2.5, 10 and 20 mg/kg of CyA, the gain in body weight was 13 g, 12 g and 9 g, respectively. No significant difference was observed between each CyA-treated group and the control group.

The suppression of proteinuria with CyA has been exhibited as early as the 5th day after the injection of anti-GBM serum (Fig. 1). The effect of CyA at doses of 2.5, 10 and 20
mg/kg on proteinuria was dose-dependent. Especially, at 20 mg/kg of CyA, the inhibitory percentage was 62% on the 10th day (CyA: 143 mg/day vs. control: 353 mg/day). In addition, urinary NAG activity, which implies that there is kidney injury, was at a significantly lower level in the CyA-treated groups than in the control group on the 5th day. In plasma, the administration of CyA resulted in a lower cholesterol level in comparison with that in the control rats.

In original-type anti-GBM nephritis, the histological features of the glomeruli were hypercellularity including leukocytes in addition to intrinsic glomerular cells and adhesion of capillary wall to Bowman’s capsule. On hypercellularity in the glomeruli, the administration of CyA, even at 2.5 mg/kg, reduced the increased cells in the control rats by about 30% (Fig. 2). Glomerular adhesion was observed in

![Fig. 1. Effect of cyclosporin A (CyA) given during the heterologous phase on urinary protein, urinary N-acetyl-β-glucosaminidase (NAG) and plasma cholesterol in original type anti-GBM nephritic rats. CyA was given daily p.o. for 10 days after the injection of anti-GBM serum. Each plot denotes the mean ± S.D. of 9 or 10 rats on each day. * and ** indicate significant differences from each control with P < 0.05 and 0.01, respectively. : Administration period of CyA. : Normal, : Control, : 2.5 mg/kg CyA, : 10 mg/kg CyA, : 20 mg/kg CyA.](image)

![Fig. 2. Effect of cyclosporin A (CyA) given during the heterologous phase on glomerular histological alteration in original-type anti-GBM nephritic rats. CyA was given daily p.o. for 10 days after the injection of anti-GBM serum, and glomerular histological evaluation was done on the 11th day. Each column denotes the mean ± S.D. of 9 or 10 rats. * and ** indicate significant differences from the normal rats at P < 0.05. * and ** indicate significant differences from the control with P < 0.05 and 0.01, respectively. : Normal, : Control, : 2.5 mg/kg CyA, : 10 mg/kg CyA, : 20 mg/kg CyA.](image)
about 16% of the glomeruli in the control rats, whereas CyA at 20 mg/kg markedly diminished the glomerular adhesion (inhibitory percentage 69% vs. the control, P < 0.05).

In the next experiment, we investigated the mechanism for the antinephritic action of CyA by examining the effect of CyA on antibody production, number of leukocytes in the blood and infiltration of leukocyte subsets in the glomeruli. The anti-rabbit γ-globulin antibody titer had already increased slightly in the control rats by the 11th day after the injection of anti-GBM serum (Fig. 3). However, the rats treated with CyA at 10 or 20 mg/kg showed a negligible antibody titer throughout the experimental period. The leukocytes consistently augmented the number in the control group with the progress of nephritis (Fig. 3). In the CyA groups, the number of leukocytes in the blood was transiently increased beyond that in the control group, but on the 8th day, the rats administered CyA at 20 mg/kg showed the basal number of leukocytes. Moreover, CyA at 20 mg/kg markedly inhibited the migrations of OX-1 (leukocyte with common antigen), OX-19 (T cell), W3/25 (helper T cell), CD-8 (suppressor/cytotoxic T cell), ED-1 (macrophage/monocyte)-positive cells into the glomeruli on the 5th and 10th days (Fig. 4).

**Effect of CyA given p.o. daily during the heterologous phase on crescentic-type anti-GBM nephritis**

To evaluate the therapeutic effect of CyA on the nephritis, we utilized crescentic-type anti-GBM nephritis that maintains the nephritic state for a longer period and is more severe than the original-type anti-GBM nephritis in rats. On the 10th day, we confirmed nephritis by determining the protein content in the urine (370 ± 120 mg/day, P < 0.05 vs. normal). CyA at 20 mg/kg prominently decreased urinary protein as compared to the control rats on the 20th day (CyA: 84 ± 35 mg/day vs. control: 207 ± 23 mg/day; inhibitory percentage of 64%) (Fig. 5). Moreover, urinary NAG activity was diminished to the basal level by the administration of higher doses of CyA (CyA 10 and 20 mg/kg: 4.3 and 3.3 mU/day vs. control: 10.0 mU/day). However, we did not observe any notable effect of CyA on plasma cholesterol level. The histological characteristic of this model of nephritis was crescent formation and fibrinoid necrosis in addition to hypercellularity and adhesion in the glomeruli. Significant suppression by the ad-

![Fig. 3. Effect of cyclosporin A (CyA) given during the heterologous phase on antibody production and number of leukocytes in original-type anti-GBM nephritic rats. CyA was given daily p.o. for 10 days after the injection of anti-GBM serum. Each plot denotes the mean ± S.D. of 9 or 10 rats on each day. * and ** indicate significant differences from the control on the same day, with P < 0.05 and 0.01, respectively. △: Administration period of CyA. ○: Normal, ●: Control, □: 2.5 mg/kg CyA, ■: 10 mg/kg CyA, ▲: 20 mg/kg CyA.](image-url)
ministration of CyA at all doses was demonstrated on these histological parameters (Fig. 6). Especially, the administration of CyA at 20 mg/kg inhibited the adhesion and fibrinoid necrosis by more than 80%. The index of glomerular lesions calculated from these histological parameters was 0.43 for the control group. Those for the CyA groups were 0.18, 0.18 and 0.09 at 2.5, 10 and 20 mg/kg, respectively (P < 0.05 vs. control).

In the next experiment, to clarify the mechanisms for the antinephritic action of CyA on crescentic-type nephritis, we examined the effect of this agent on antibody production and the number of leukocytes in the blood. The antibody titer against rabbit γ-globulin was 2.5 on the 8th day prior to the administration of CyA. In the control group, the antibody titer increased over the course of the experiment (Fig. 7). Although 2.5 mg/kg of CyA failed to suppress the production of antibody, the higher doses of CyA affected it. On the 25th day, the antibody titer of rats that received 20 mg/kg of CyA was depressed to nearly the basal level. In this model, we detected a notable increase in the number of leukocytes as well as in original-type anti-GBM nephritis (Fig. 7). However, we did not find any significant effect of CyA on the number of leukocytes.

Fig. 4. Effect of cyclosporin A (CyA) given during the heterologous phase on the infiltration of leukocyte subsets in the glomeruli of original-type anti-GBM nephritic rats. CyA was given daily p.o. for 5 and 10 days after the injection of anti-GBM serum, and the evaluation of the infiltration of leukocytes was done on the 5th and 10th days. Each column denotes the mean ± S.D. of 5 rats on the 5th and 10th days. ** and *** indicates significant differences from the normal rats with P < 0.01 and 0.001, respectively. *, ** and *** indicate significant differences from the control with P < 0.05, 0.01 and 0.001, respectively. The number in parenthesis indicates inhibitory percentage. □: Normal, ■: Control, ◇: 20 mg/kg CyA.
Fig. 5. Effect of cyclosporin A (CyA) given during the autologous phase on urinary protein, urinary N-acetyl-β-glucosaminidase (NAG) and plasma cholesterol in crescentic-type anti-GBM nephritic rats. CyA was given daily p.o. for 10 days from the 10th day after the injection of anti-GBM serum. Each plot denotes the mean ± S.D. of 9 or 10 rats on each day. * and ** indicate significant differences from each control at P < 0.05 and 0.01. — Administration period of CyA. O: Normal, ●: Control, □: 2.5 mg/kg CyA, ■: 10 mg/kg CyA, ▲: 20 mg/kg CyA.

Fig. 6. Effect of cyclosporin A (CyA) given during the autologous phase on glomerular histological alteration in crescentic-type anti-GBM nephritic rats. CyA was given daily p.o. for 10 days from the 10th day after the injection of anti-GBM serum, and glomerular histological evaluation was done on the 25th day. Each column denotes the mean ± S.D. of 9 or 10 rats. # # indicates significant differences from the normal rats at P < 0.05. * and ** indicate significant differences from the control at P < 0.05 and 0.01, respectively. □: Normal, ■: Control, □□: 2.5 mg/kg CyA, □☐: 10 mg/kg CyA, □▲: 20 mg/kg CyA.
DISCUSSION

The present study demonstrated that CyA suppressed the development of anti-GBM nephritis in rats. As mentioned in the introduction, the pathogenesis of anti-GBM nephritis is associated with antibodies, anti-GBM antibody and anti-rabbit γ-globulin antibody. We observed that anti-rabbit γ-globulin antibody production was remarkably inhibited by the administration of CyA during the autologous phase in crescentic-type anti-GBM nephritis. Therefore, it seems reasonable to consider that CyA exerted antinephritic action through the suppression of antibody production in the nephritic rats. There have been reports concerning the mechanism of the suppressive action of CyA on antibody production. It has been generally believed that CyA inhibits the interaction of antigen-presenting cells with macrophages and T lymphocytes, i.e., the major histocompatibility complex on these macrophages and T cell receptors. Bunjes (24) reported that CyA inhibited the antibody production via the prevention of the release of interleukin I (IL-I) from Ia positive macrophages and interleukin II from helper T cells.

In the present study, treatment with CyA during the heterologous phase of original type anti-GBM nephritis suppressed proteinuria as early as the 8th day after the injection of anti-GBM serum, prior to the time when the antibody level would be expected to increase. This result indicates that the inhibition of antibody production is not the only mechanism for the antinephritic action of CyA. In this connection, Thaiss et al. (25) reported that CyA prevented the development of proteinuria in rats with membranous nephropathy without inhibiting the production of specific antibody and the deposition of immune complex in the glomeruli. Neild et al. (26) has also indicated that the antinephritic effect of CyA on rabbit acute serum sickness nephritis is independent of its suppressive action on antibody production. Recently, Boyce et al. (27) observed that macrophages and cytotoxic T cells markedly infiltrated in the glomeruli of rats with nephrotoxic nephritis corresponding to original-type anti-GBM nephritis used in the present study. Furthermore, Bahn et al. (28) reported that lymphocytes sensitized anti-rabbit γ-globulin induced glomerular damage in rats that were intravenously injected with a subnephrotoxic dose of anti-GBM serum. Namely, these findings suggest that cellular immunity may also be associated with the development of anti-
GBM nephritis in rats. In the present experiment on the effect of CyA given during the heterologous phase, this agent markedly inhibited the migration of leukocyte subsets such as macrophages and cytotoxic T cells into the glomeruli of original-type anti-GBM nephritic rats. The proliferation of cellular immunity-associated cells (i.e. macrophages and cytotoxic T cells) may be regulated by various cytokines. Camussi et al. (29) demonstrated that systemic injection of IL-1 and tumor necrosis factor (TNF) into nephritic rabbits markedly increased the number of macrophages and polymorphonuclear leukocytes (PML) that migrated into the glomeruli as compared with that of untreated nephritic rabbits. Furthermore, they found that the production of IL-1 and TNF in isolated glomeruli from nephritic rabbits was significantly enhanced in comparison with that from normal rabbits. Recently, CyA has been indicated to inhibit the secretion of TNFα from lipopolysaccharide-stimulated macrophages (30). In addition, CyA prevents the release of IL-1 from Ia positive macrophages as mentioned above. Therefore, it is considered from these reports that CyA may inhibit the migration of macrophages and PML into glomeruli, which may lead to glomerular injury, by suppressing the release of IL-1 and the secretion of TNF. Hess (31) reported that high doses of CyA (0.5 – 2.5 μg/ml) suppressed the cytotoxic T cell activity in the mixed lymphocyte response in the presence of IL-II. This finding suggests that CyA may inhibit the glomerular injury by cytotoxic T lymphocytes. Therefore, it is postulated from our results and the above-mentioned findings that the antinephritic effect of CyA given during the heterologous phase may be mainly due to the inhibitory action of this agent on the migration of leukocyte subsets such as macrophages and cytotoxic T cells into glomeruli or their proliferation via inhibition of the production of several kinds of cytokines in lymphocytes or macrophages. Furthermore, the effect of CyA given during the autologous phase may be due to the suppressive action of this agent on the proliferation or migration of cellular immunity-associated cells as well as suppression of the antibody production.

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