Marked Antinephritic Action and Less Adverse Effects of Methylprednisolone Suleptanate by Intermittent Administration in Rats

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ABSTRACT—To establish the regimen for beneficial prolonged treatment with glucocorticoids on nephritis, we investigated the antinephritic effect of methylprednisolone suleptanate (MPS) and its influence on adrenal function by intermittent administration (IA) in comparison with daily administration (DA) in crescentic-type anti-GBM nephritic rats. In IA, MPS (0.25, 1.0 and 3.0 mg/kg) was injected for 3 successive days followed by a 3-day withdrawal during a 40-day period. MPS inhibited the elevation of urinary protein and serum cholesterol and glomerular alterations by both IA and DA. The effect of MPS on these parameters was more potent by IA than by DA. MPS significantly suppressed the increment of the number of ED-1(+) cells and TH-1(+) cells in nephritic glomeruli. DA, but not IA, caused atrophy of the adrenal glands. IA prevented the remarkable decrease in corticosterone level provoked in nephritic rats. In conclusion, for the treatment of nephritis, IA seems to be a better regimen for the administration of MPS. MPS may exert an antinephritic action by inhibiting mesangial cell proliferation and infiltration of monocytes/macrophages into glomeruli in addition inhibiting antibody production.

Keywords: Anti-GBM nephritis, Methylprednisolone suleptanate, Intermittent administration, Daily administration

Adrenocortical steroids have been used in renal transplantation to prevent the rejection caused by effector cells (1). Additionally, the steroids are the treatment of first choice for nephrosis and glomerulonephritis in the clinical stage. However, their adverse effects, the rebound phenomenon and withdrawal syndrome after long-term administration, have been serious issues for steroid therapy (2). Although rare, death from an unknown cause in connection with the administration of steroids was also reported (3). Therefore, considerable efforts have been made to create synthetic steroids that have potent yet less adverse effects in order to prevent the side effects and rebound phenomenon due to long-term administration (4–6). The regimen of steroid treatment, including pulse therapy and cocktail therapy, has also been studied to increase the remission rate of renal disease in the clinical stage (7–10). There have been no reports about long-term effects and side effects using an experimental model of glomerulonephritis, although a few reports demonstrated the short-term effect of glucocorticoids (11, 12). Methylprednisolone suleptanate is one of the methylprednisolone derivatives, in which a suleptanate group substitution to the C-21 hydroxyl of methylprednisolone is made to improve stability in water and bioavailability (13, 14). The present study explored beneficial methods for long-term steroid treatment by using crescentic-type anti-GBM nephritic rats. We also investigated the putative mechanisms by which methylprednisolone suleptanate suppresses nephritis.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley strain SPF rats (Nihon SLC, Shizuoka), weighing approx. 160 g, were used in the experiments. The animals were housed in an air-conditioned room at 23 ± 1°C during the experimental periods.

Induction of crescentic-type anti-GBM nephritis
Crescentic-type anti-GBM nephritis was induced in rats by i.v. injection of 0.8 ml of rabbit anti-GBM serum into the tail vein followed by injection of 6.5 mg rabbit γ-globu-
lin (γ-G) in 0.25 ml of Freund complete adjuvant (FCA) into their hind foot pads as described previously (15). A urine sample from each rat was collected for 24 hr immediately after anti-GBM serum injection to determine protein excretion into the urine. The animals were then divided into 8 groups (n=8), which had a similar urinary protein content as an average in individual groups. After grouping, the animals were immunized with rabbit γ-G in FCA. A non-treated (normal) group (n = 8) was added to the experiment for comparison with the nephritic group.

**Fig. 1.** Chemical structure of methylprednisolone sulptanate.

Drugs

Methylprednisolone sulptanate (Fig. 1) (Upjohn Pharmaceuticals Co., Tokyo) and dipyridamole (Sigma, St. Louis, MO, USA) were used. Methylprednisolone sulptanate (MPS) was dissolved in saline and administered i.v. at 0.25, 1.0 or 3.0 mg/kg/day. Dipyridamole was suspened in 1% gum arabic solution and administered p.o. at 400 mg/kg/day. MPS was given to animals by daily or intermittent (i.v. treatment for 3 days followed by withdrawal for 3 days) administration, and dipyridamole was administered daily from the day following anti-GBM serum injection to the 40th day. The remaining group received i.v. the vehicle (saline) instead of test drugs and served as the control.

**Urine, blood and adrenal gland collections**

The urine sample was obtained by keeping each animal in an individual metabolic cage for 24 hr at various intervals after the induction of nephritis. At the beginning of the urine collection, each animal received p.o. 8 ml of distilled water without feeding. The collected urine was then centrifuged at 5,800 × g for 15 min at 4°C, and the supernatant was used for the determination of protein. A blood sample was also obtained at various intervals after the induction of nephritis. In this case, each 0.4 ml of blood was drawn from the tail vein of conscious animals with a disposable syringe. The blood was centrifuged at 7,500 × g at 4°C to obtain the plasma for the determination of cholesterol, creatinine and antibody titer against rabbit γ-G. Immediately after the last collection of the urine samples, blood was also taken from the carotid artery to determine the corticosterone level, under pentobarbital anesthesia. Then the adrenal glands were isolated to determine their weight.

**Measurements of protein, cholesterol, creatinine, corticosterone and antibody titer**

Urinary protein was determined by the method of Kingsbury et al. (16) and expressed as mg/24 hr urine. Serum cholesterol and creatinine were determined by commercial assay kits (Determina TC-5, Kyouwa Medix Co., Ltd., Tokyo and CRE-EN Kainos, Kainos Laboratories, Inc., Tokyo), respectively, and expressed as mg/dl. The serum antibody titer against rabbit γ-G was determined by indirect hemagglutination using sensitized sheep red blood cells (17). Serum corticosterone was determined by a spectrophotofluorometer, model FP-770 (Nihon Bunkou, Tokyo), in accordance with the method of Zenker and Bernstein (18). The excitation wavelength was set at 470 nm, and emission intensity was recorded continuously at a wavelength of 400 nm.

**Assessment of histopathological parameters in glomeruli**

For light microscopic study, the kidney was isolated under pentobarbital anesthesia, then fixed in ethyl alcohol. The tissues embedded in paraffin were cut into 2- to 3-μm-thick sections. The sections were stained with Masson’s trichome. Crescent formation, adhesion of capillary walls to Bowman’s capsule (adhesion) and fibrinoid necrosis in glomeruli were observed under a light microscope. For assessing each histopathological parameter, fifty glomeruli per section were observed under a light microscope, and the appearance rate for each alteration was calculated. The observations were performed on the sections, which were identified by a number only.
Immunohistochemistry
An indirect immunoperoxidase technique employing specific binding of avidin to biotin (Becta Stain ABC Kit; Vector Laboratories, Burlingame, CA, USA) was used (19). Briefly, tissues were fixed with paraformaldehyde-lysine-periodate. Cryostat sections of snap-frozen material were dried and preincubated with 1–2% normal rabbit serum for 20 min. Sections were then incubated with mouse anti-rat monoclonal antibodies (Serotec Co., Oxford, England): Antibody ED-1 to detect monocytes and macrophages infiltrated into the glomeruli, antibody MRC OX-7 to detect Thy-1.1 antigen on the surface of the mesangial cell, followed by incubation with biotinated affinity purified anti-mouse IgG and avidinated horseradish peroxidase. The complexes on the section were visualized with diaminobenzidine (0.5 mg/ml in PBS plus 0.01% H₂O₂). ED-1(+) cells (monocyte and macrophage) with a clear identifiable nucleus were counted under a light microscope. Thy-1(+) cells, the so-called mesangial cells, were analyzed by an image analyzer, Image Analyzer V1 (Toyobo Co., Tokyo) and expressed as pixels.

Statistical analysis
The data were analyzed by one-way analysis of variance and the Duncan multiple range test or non-parametric statistics. All values are reported as the mean±S.D. Differences were considered significant if the P value was less than 0.05.

RESULTS

Body weight (data not shown)
Throughout the experimental period, untreated nephritic rats (nephritic control) gained less weight than normal
rats (normal). On the 37th day the normal group had a body weight of 318 ± 24 g and the control group, 285 ± 15 g. The group treated with MPS at 3.0 mg/kg, daily weighed 265 ± 13 g on the 37th day, which was significantly less than the body weight of the nephritic control group. On the other hand, the rats in the intermittent treatment group exhibited better growth than the rats of the nephritic control group, namely, the mean body weight of the intermittent group treated with MPS at 3 mg/kg was 295 ± 19 g on the 37th day.

Table 1. Effects of methylprednisolone suleptanate by daily or intermittent administration on serum cholesterol and creatinine levels in crescentic-type anti-GBM nephritis in rats

| Groups               | Serum cholesterol (mg/dl) | Serum creatinine (mg/dl) |
|----------------------|---------------------------|--------------------------|
| Normal               | 34.3 ± 2.4*               | 0.27 ± 0.01*             |
| Control              | 162.0 ± 31.2              | 0.49 ± 0.05              |
| Daily administration |                           |                          |
| MPS 0.25 mg/kg, i.v. | 132.9 ± 39.0              | 0.42 ± 0.06*             |
| 1.0 mg/kg, i.v.      | 92.4 ± 16.5***            | 0.44 ± 0.07              |
| 3.0 mg/kg, i.v.      | 98.4 ± 42.3**             | 0.43 ± 5.7***            |
| Intermittent         |                           |                          |
| administration       |                           |                          |
| MPS 0.25 mg/kg, i.v. | 102.1 ± 32.2**            | 0.38 ± 0.04**            |
| 1.0 mg/kg, i.v.      | 96.7 ± 5.3***             | 0.41 ± 0.07*             |
| 3.0 mg/kg, i.v.      | 75.0 ± 12.7***            | 0.30 ± 0.07***           |
| Dipyridamole 400 mg/kg, p.o. | 86.7 ± 15.1*** | 0.43 ± 0.12 |

Numbers indicate the mean ± S.D. obtained from 7 rats on the 41st day after the injection of anti-GBM serum. *, ** and *** show significant differences from the control at P < 0.05, P < 0.01 and P < 0.001, respectively.

Urinary protein excretion (Fig. 2)

The nephritic control exhibited a typical biphasic increase in urinary protein excretion exceeding 200 mg/day. Both daily and intermittent administrations of MPS significantly inhibited the development of urinary protein excretion in a dose-dependent manner from the 20th day after the injection of anti-GBM serum. The inhibitory effect with MPS was greater than that with 400 mg/kg dipyridamole. Additionally, the effect of MPS on proteinuria was more potent by intermittent administration.

Serum cholesterol and creatinine levels (Table 1)

On the 41st day, intermittent administration of MPS caused a diminished level of serum cholesterol in a dose-dependent manner when compared with the nephritic control. Furthermore, intermittent treatment with MPS remarkably suppressed elevation of serum creatinine level at all doses.

Histopathological parameters in glomeruli (Fig. 3 and Table 2)

In the histopathological observation of the glomeruli on the 41st day, the incidence of crescent, adhesion of capillary walls to Bowman's capsule and fibrinoid necrosis declined dose-dependently, both by daily and intermittent MPS administrations. Intermittent treatment had a more potent effect on histopathological parameters than by daily treatment. The incidence of fibrinoid necrosis was apparently suppressed only by intermittent treatment with MPS.

Table 2. Effects of methylprednisolone suleptanate by daily or intermittent administration on histopathological parameters in crescentic-type anti-GBM nephritis in rats

| Groups               | Incident rate (%) |
|----------------------|-------------------|
|                     | crescent formation| adhesion| fibrinoid necrosis |
| Control              | 62.9 ± 7.2        | 50.9 ± 7.9 | 9.7 ± 4.5         |
| Daily administration |                   |          |                   |
| MPS 0.25 mg/kg, i.v. | 24.0 ± 5.2***     | 40.6 ± 7.8* | 8.6 ± 3.6         |
| 1.0 mg/kg, i.v.      | 17.1 ± 3.0***     | 28.0 ± 5.2*** | 8.0 ± 3.3         |
| 3.0 mg/kg, i.v.      | 12.6 ± 4.9***     | 24.0 ± 5.7*** | 7.4 ± 2.8         |
| Intermittent         |                   |          |                   |
| administration       |                   |          |                   |
| MPS 0.25 mg/kg, i.v. | 20.0 ± 5.2***     | 22.9 ± 6.4*** | 6.3 ± 2.1         |
| 1.0 mg/kg, i.v.      | 13.1 ± 7.9***     | 17.1 ± 6.4*** | 5.1 ± 2.0*        |
| 3.0 mg/kg, i.v.      | 4.7 ± 3.9***      | 9.3 ± 3.3*** | 2.0 ± 2.2**       |
| Dipyridamole 400 mg/kg, p.o. | 25.3 ± 4.1***     | 24.0 ± 9.8*** | 9.3 ± 4.1         |

Numbers indicate the mean ± S.D. obtained from 7 rats. Fifty glomeruli per section were observed, and the appearance rate for the glomeruli with each alteration was calculated. *, ** and *** show significant differences from the control at P < 0.05, P < 0.01 and P < 0.001, respectively.
Serum antibody titer (Fig. 4)

The elevation of antibody titer observed in the nephritic control group was dose-dependently inhibited by daily and intermittent administrations of MPS. However, no apparent difference was seen between either regimen of MPS treatment. Dipyridamole failed to inhibit the elevation of antibody titer.

ED-1(+) cells (monocytes/macrophages) and Thy-1.1(+) cells (mesangial cells) in glomeruli (Figs. 5 and 6)

The glomeruli from untreated nephritic rats showed up to three-fold more ED-1(+) cells on the 10th day after the anti-GBM serum injection. Daily and intermittent treatments with MPS (3 mg/kg/day, i.v.) inhibited the cell increment in the nephritic control by about a half. On the 41st day, the number of Thy-1(+) cells in the glomeruli...
of the nephritic control group markedly increased. Both daily and intermittent treatments attenuated the increment of Thy-1(+) cells by 30% as compared to the nephritic control.

**Fig. 4.** Effects of methylprednisolone sulenpate by daily or intermittent administration on serum antibody titer against rabbit γ-globulin in crescentic-type anti-GBM nephritis in rats on the 41st day after anti-GBM serum injection. Each column denotes the mean ± S.D. of 8 rats. Abbreviations: Con., nephritic control group; MPS, methylprednisolone sulenpate group; Dip., dipyridamole group. a and b indicate significant differences from the control at P < 0.05 and P < 0.01, respectively.

Weight of adrenal gland (Fig. 7A)

No significant difference was observed in the weight of adrenal glands between the nephritic control and the normal groups. The weight of the adrenal glands of rats in the daily treatment group decreased to less than 60% of those in the nephritic control or the normal groups in a dose-dependent manner, whereas the intermittent treatment group did not show any reduction in the weight of the adrenal glands at any doses of MPS.

Serum corticosterone level (Fig. 7B)

The serum corticosterone level of the nephritic control group fell to about 20% of the normal group (Normal: 0.46 ± 0.05 μg/ml, Control: 0.08 ± 0.02 μg/ml). Daily treatment with MPS did not exert any effects on the serum corticosterone level that was markedly reduced in the nephritic control. In contrast, the intermittent administration of MPS elicited a marked elevation of the serum corticosterone level as compared to the nephritic control, although it was significantly less than the normal level. The serum corticosterone level in the dipyridamole group ranged between those of the daily and the intermittent treatment groups.

**DISCUSSION**

Glucocorticoid therapy works dramatically well on various immunological disorders (20). Glucocorticoids are the first choice drug for minimal-change nephropathy and glomerulonephritis (8). Glucocorticoid therapy, however, carries the risk of severe and even life-threatening complications (2, 3). To establish a prolonged regimen for glucocorticoids, methylprednisolone sulenpate was evaluated for its antinephritic effects and for the adverse influence on the adrenal glands by daily and intermittent administrations using crescentic-type anti-GBM nephritic rats. In the present studies, we demonstrated that MPS exerted antinephritic effects by the intermittent treatment, which were equivalent to or more potent than those by the daily treatment. The daily administration of MPS resulted in atrophy of the adrenal glands and remarkable attenuation of the serum corticosterone level during long-term treatment. It is noteworthy that the intermittent administration did not affect weight gain in the nephritic control. The present study thus provides experimental evidence that intermittent therapy is a better regimen than daily therapy with respect to reducing the adverse action of glucocorticoids.

Glucocorticoids are known to initiate the synthesis of antiinflammatory protein(s) (21), thereby suppressing vascular permeability in inflammatory conditions. Additionally, glucocorticoids elicit lipocortin (22), which inhibits the activity of phospholipase A2 in the plasma membrane, leading to diminished production of prostaglandins, which are mediators for local inflammation and to reduced production of thromboxane A2 (23), which induces platelet aggregation and vasoconstriction. Hence, it is likely that MPS promoted the synthesis of these proteins and then exerted antinephritic effects by means of them. In the present study, however, MPS revealed its antinephritic effect from the 20th day after the induction of crescentic-type anti-GBM nephritis. Therefore, there seems to be a time lag between the antinephritic effects and the above-described effects that could appear in a few hours after the administration of MPS. Therefore, there are additional implications of the present study with respect to the antinephritic effect of MPS. In our experimental protocol for MPS, we started administration of MPS one day after the injection of anti-GBM serum. It is well documented that anti-GBM nephritis develops in a biphasic process. The heterologous phase is induced in a few hours after
Fig. 5. Light micrographs of glomeruli from rats of the normal group (A); nephritic control group (B); group given methylprednisolone suleptanate, 3.0 mg/kg, i.v. by daily administration (C); group given methylprednisolone suleptanate, 3.0 mg/kg, i.v. by intermittent administration (D). The photographs for ED-1(+) cells indicate the glomerulus of the kidney isolated on the 10th day, and for Thy-1(+) on the 41st day after the injection of anti-GBM serum. Note that the group treated with methylprednisolone suleptanate showed far fewer ED-1(+) cells and markedly less numerous Thy-1(+) cells than the control group.
the injection, which is associated with severe proteinuria, complement activation, accumulation of polymorphonuclear leukocytes in the glomeruli and production of eicosanoid. Namely, very severe inflammation had been brought about in the heterologous phase before the treatment with MPS. Therefore, MPS could not exert its antiinflammatory effect nor recover damaged glomeruli in a week. About 1 week after the injection, the autologous phase begins in nephritic rats. Nephritic rats raise antibody against anti-GBM antibody fixed in the GBM, fol-
lowed by gradually amplifying inflammation in the glomeruli. In such a circumstance, MPS may elaborate its antiinflammatory effect, which involves inhibition of the antibody production, on crescentic-type anti-GBM nephritis from the 20th day.

The present studies indicated that MPS markedly suppressed the glomerular macrophage accumulation besides inhibiting antibody production. The pathogenic significance of the macrophage in glomerulonephritis is supported by considerable evidence (24–26). This result partly accounts for the antinephritic effect of MPS. Glucocorticoid treatment in vivo prevents the accumulation of leukocytes into local tissue sites induced by a variety of stimuli (27, 28). Because Schleimer et al. demonstrated that a relatively lower concentration of glucocorticoid failed to inhibit chemotaxis of leukocytes in response to formylmethionyl-leucyl-phenylalanine (29), MPS may not directly inhibit leukocyte chemotaxis. On the other hand, cultured mesangial cells produce the protein chemotactic factor(s) for macrophages/monocytes by stimulating cytokine, including interleukin-1 and tumor necrotizing factor (30). Rovin et al. demonstrated that nephritic glomeruli produced the lipid chemotactic factor(s) for macrophages/monocytes (31). Therefore, MPS may inhibit the production of some potent monocyte chemoattractant(s) in the nephritic glomeruli.

Furthermore, we demonstrated that the proliferation of mesangial cells was remarkably depressed in the MPS-treated group. Mesangial cells in culture vigorously produced interleukin-1 without any stimulant when they are in the proliferating stage (32). Additionally, interleukin-1 was reported to be a proliferation factor for mesangial cells under coexistence of a competence factor such as platelet activating factor (33). Tipping et al. reported that glomerular interleukin-1 production is dependent on macrophage infiltration in anti-GBM glomerulonephritis (34). On the other hand, glucocorticoids abrogate the gene expression for interleukin-1, interleukin-2, and interleukin-6 (35, 36). There are some lines of evidence that glucocorticoids suppressed the proliferation of T helper cells by involving the inhibition of a cytokine-signaling event (37). Together with the above description about chemoattractants, the possibility also can be considered that MPS could inhibit the production of chemoattractants by cytokine through suppressing the proliferation of mesangial cells in the nephritic glomeruli, although we can not elucidate it in the present study.

In summary, MPS showed antinephritic effects on crescentic-type anti-GBM nephritis in rats, but less effects on the adrenal glands by intermittent treatment. It was considered that MPS exerted its antinephritic effect partly through the diminished monocyte/macrophage infiltration into the nephritic glomeruli.

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