Suppression of Experimental Crescentic-Type Anti-Glomerular Basement Membrane (GBM) Nephritis by FK506 (Tacrolimus Hydrate) in Rats

Kazumi Hayashi, Tadashi Nagamatsu, Mikio Ito and Yoshio Suzuki
Department of Pharmacology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Nagoya 468, Japan

Received August 28, 1995 Accepted October 24, 1995

ABSTRACT—The effect of FK506 (tacrolimus hydrate), an immunosuppressive agent produced by Streptomyces tsukubaensis, on crescentic-type anti-glomerular basement membrane (GBM) nephritis in rats was investigated. When rats were treated with FK506 from 1 or 20 days after the anti-GBM serum injection, FK506 inhibited the increase in urinary protein excretion. Histological observation demonstrated that FK506 suppressed glomerular alterations. In the FK506-treated rats, antibody production and rat-IgG and C3 deposits on the GBM were significantly less than those in the nephritic control group. FK506 treatment suppressed the accumulation of ED-1-positive cells, CD4-positive cells, CD8-positive cells, interleukin-2 (IL-2)-receptor-positive cells, leukocyte-function-associated antigen-1 (LFA-1)-positive cells and intercellular adhesion molecule-1 (ICAM-1)-expression in nephritic glomeruli. However, in the in vitro study, FK506 failed to inhibit the up-regulated ICAM-1 expression on endothelial cells in response to tumor necrosis factor (TNF)-α. On the other hand, IL-2 production from the spleen cells isolated from nephritic rats treated with FK506 was lower than that in the nephritic control rats. These results suggest that FK506 is effective against crescentic-type anti-GBM nephritis and that the antinephritic mechanisms of FK506 is due to the inhibition of intraglomerular accumulation and activation of leukocytes through the suppression of ICAM-1 expression and IL-2 production.

Keywords: Anti-GBM nephritis (crescentic type), FK506, Leukocyte, Interleukin-2, Intercellular adhesion molecule-1

FK506 is a macrocyclic lactone produced by Streptomyces tsukubaensis, a soil bacterium from the Tsukuba region of Japan (1).

Recent studies have shown that FK506 has an immunosuppressive action; namely, FK506 blocks T cell activation or proliferation (2–4) and suppresses graft rejection (5). These actions suggest that FK506 can suppress delayed-type hypersensitivity, but the exact mode of action of FK506 is not known.

The potential of FK506 to suppress other immunopathologic conditions, such as glomerulonephritis, is largely unknown, although there has been a recent report (6) that FK506 can suppress the development of spontaneous lupus-like nephropathy in susceptible mice. In those studies, FK506 was demonstrated to suppress the production of anti-DNA antibody and to prevent the development of lupus nephritis by the suppression of both antibody deposition and hyperplasia of lymph nodes when it was given to susceptible mice prior to the onset of the apparent disease or after the appearance of physical evidence of the disease. On the other hand, although it has been reported that FK506 can suppress the progression of active Heymann nephritis (7, 8) and Masugi nephritis (8), the anti-nephritic mechanism of FK506 is unknown on these models as well. Nagamatsu et al. (9) has demonstrated that cyclosporin A suppressed the development and progression of anti-glomerular basement membrane (GBM) nephritis through the inhibition of glomerular infiltration of leukocytes. It was reported that FK506 had a more potent effect than cyclosporin A in in vitro studies, reflecting their immunosuppressive effect (1, 10–12). We also found that FK506 was more effective than cyclosporin A in terms of the suppressive action on IL-2 produced by concanavalin A in mouse spleen cells (unpublished data of K. Hayashi). Therefore, FK506 is expected to exert a greater antinephritic effect than cyclosporin A, through antinephritic mechanisms similar to cyclosporin A.

In the present report, we have shown the efficacy of FK506 in preventing the immunopathological insult of
crescentic-type anti-GBM nephritis in rats, which mimics rapidly progressive glomerulonephritis in humans. Moreover, we have demonstrated the antinephritic mechanisms of FK506 in both in vivo and in vitro studies.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley strain rats, weighing approximately 160 g (Nihon SLC, Hamamatsu), were used in all experiments. These animals were housed in an air-conditioned room at 23 ± 1°C during the experimental period.

Drugs

FK506 (Fujisawa Pharm. Co., Ltd., Osaka) dissolved in ethanol with 20% Tween 80 was diluted in sterile saline at concentrations of 0.2, 0.8 or 2.0 mg/kg per 0.5 ml. Cyclosporin A (Sandoz, Tokyo) was dissolved in olive oil with 1% ethanol at the concentration of 20 mg/kg in a volume of 1 ml per 100 mg of body weight. For the in vitro studies, FK506 was dissolved in ethanol at 1 x 10^{-2} M. Cyclosporin A was dissolved in ethanol with 20% Tween 80 at 1 x 10^{-2} M. H-7 (Seikagaku Kogyo Co., Ltd., Tokyo) was dissolved in RPMI 1640 at 1 x 10^{-2} M. Anti-cytokine neutralizing antibodies (rabbit anti-human tumor necrosis factor (TNF)-α polyclonal antibody and rabbit anti-human interleukin-1 (IL-1) β polyclonal antibody) and recombinant human TNF-α were purchased from Genzyme Co., Ltd. (Cambridge, MA, USA).

Induction of crescentic-type anti-GBM nephritis

Crescentic-type anti-GBM nephritis was induced by immunizing the rats, which had received a nephritogenic dose of rabbit anti-rat GBM (anti-GBM) serum, with rabbit γ-globulin according to a slight modification of the previously reported method (13). In this experiment, rats were administered 0.9 ml/animal of anti-GBM serum into the tail vein.

The effect of test drugs was estimated by administering them from 1 day after the anti-GBM serum injection (heterologous phase) or from 20 days after the anti-GBM serum injection (autologous phases). In the experiments, 24-hr urine samples were collected, and the rats were then divided into 4 or 5 groups of 7 rats, so that the average protein content in the 24-hr urine in each group was at a similar level.

Evaluation of antinephritic effect of the test drugs

Three groups were administered subcutaneously 0.2, 0.8 or 2.0 mg/kg/day of FK506, respectively, in a volume of 0.5 ml per rat, daily from 1 or 20 days after the anti-GBM serum injection to 40 days. The remaining group was administered subcutaneously or orally given the vehicle (saline or olive oil) instead of test drugs and served as the nephritic control. In addition, a nontreated (normal) group was used for comparison with the nephritic groups.

Determinations of urinary protein and plasma cholesterol

The 24-hr urine sample was obtained by keeping each animal in an individual metabolic cage for 24 hr. The urinary protein excretion was determined by the method of Kingsbury et al. (14) and expressed as mg/24-hr urine.

Blood was drawn from eyebound of conscious animal with a heparinized hematocrit capillary. The cholesterol content was determined with a commercial assay kit (Determina TC-5; Kyowa Medex Co., Ltd., Tokyo) (15).

Measurement of plasma antibody against γ-G

The measurement of rat anti-rabbit IgG antibody was performed by enzyme-linked immunosorbent assay (ELISA) (13).

Assessment of histopathological parameters

For the light microscopic study, kidneys were isolated from rats anesthetized with pentobarbital (50 mg/ml), then fixed in 10% formalin in 0.01 M phosphate buffer saline (PBS), pH 7.4, and dehydrated by immersing the tissues stepwise into various concentrations of ethyl alcohol from low to high. The tissues were then embedded in paraffin and sectioned into 2- to 3-μm-thick slices. In the studies of crescentic-type anti-GBM nephritis, the sections were stained with Masson’s trichrome. Crescent formation, adhesion of Bowman’s capsule to the capillary wall (adhesion) and fibrinoid necrosis in the glomeruli were observed under a light microscope. For assessing these parameters, an equatorial cross section was selected by random sampling methods. Fifty glomeruli/section were observed, and the appearance rate of crescent formation, adhesion and fibrinoid necrosis are expressed as the percentages of the glomeruli (incidence) having these morphological alterations as the previously reported method (13). For assessing hypercellularity, an equatorial cross-section was selected by a random sampling method. The number of nuclei (including the nuclei of glomerular cells and exudative leukocytes) was counted and the results are expressed as the mean number per glomerular cross section (G.C.S.) in 30 glomeruli/section. The evaluation was performed by a different person in blind fashion.

Immunohistochemistry

In tissues for immunoenzymatic staining of rat-IgG, leukocytes, intercellular adhesion molecule-1 (ICAM-1), proliferating cell nuclear antigen (PCNA, a marker for cell proliferation) and mesangial area (Thy1.1-positive area), the paraffin sections were cut as described above,
and the sections were labeled with monoclonal antibodies (mAbs) as previously described (16). mAbs used in this study were as follows: anti-rat IgG mouse mAb, OX-1 (leukocyte common antigen, total leukocyte), W3/25 (CD4, helper/inducer T cells), OX-8 (CD8, cytotoxic/suppressor T cells), ED-1 (most macrophages and some dendritic cells), OX-39 (IL-2 receptor, activated T cells), OX-18 (MHC class II, Ia-positive cells) and anti-Thy1.1 mAb (Serotec, Oxford, England); anti-leukocyte function associated antigen-1 (CD11a, LFA-1) mAb and anti-ICAM-1 mAb (Seikagaku Kogyo Co., Ltd.); anti-PCNA mAb (192A; Coulter Immunology, Hialeah, FL, USA).

Additionally, for rat-C3, the cryostat sections were incubated peroxidase-conjugated goat-IgG fraction to rat complement C3 with DAB (Cappel, West Chester, PA, USA) after washing in PBS.

Quantification of positive area of rat-IgG, rat-C3, ICAM-1 and Thy1.1, PCNA-positive cells, and leukocyte subsets on tissue sections

The total area showing positive staining for immunoreactive rat-IgG, rat-C3, ICAM-1 and Thy1.1 in the glomerulus was measured in 30 glomeruli per section using an image analyzer (Toyobo Image Analyzer V1; Toyobo Co., Ltd., Tokyo) and the results are presented as mm²/G.C.S. PCNA-positive cells and leukocytes within each glomerulus were counted with the image analyzer, and the results are expressed as the number of cells/G.C.S.

Cell ELISA

HUVEC: Human umbilical vein endothelial cells (HUVECs) were obtained from Curabou (Neyagawa). The cells were suspended in the culture medium (MCDB131 with 2% fetal bovine serum (FBS), 10 μg heparin/ml, 10 μg endothelial cell growth supplement/ml, 10 μg epidermal growth factor/ml, 1 μg hydrocortisone/ml, 50 μg gentamicin/ml, 0.25 μg amphotericin B/ml) (Curabou) and grown in 75 cm² tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA). The culture medium was changed twice weekly. HUVECs were trypsinized when subconfluent, resuspended in the culture medium, and either seeded into new culture flasks or collagen-coated, 96-well-plates. HUVECs were used from passage 3-6.

Cell ELISA: HUVECs (1x10^4 cells/well) were seeded into 96-well, flat-bottomed type-I-collagen-coated plates (Sumitomo Bakelite Co., Ltd., Tokyo) in 100 μl of M199 (Nissui Pharmaceutical Co., Ltd., Tokyo) and allowed to reach subconfluence (approximately 10^5 cells/well). When HUVECs were subconfluent, 100 μl of the medium from each well was removed, and then 50 μl of the RPMI 1640 or various concentrations of FK506, cyclosporin A, H-7 or anti-cytokine antibodies was added to the appropriate wells. Then 50 μl of recombinant human TNF-α (final concentration: 100 U/ml) or medium were added to each appropriate well to yield a final volume of 100 μl/well. The cultured cells were incubated for 20 hr at 37°C in 5% CO₂−95% air. The cell monolayers were washed twice with Hank's balanced salt solution (HBSS) (Sanko Junyaku Co., Ltd., Tokyo) and then fixed with 1% paraformaldehyde for 15 min at room temperature. After washing the fixed HUVECs three times with HBSS, the unbound sites were blocked by casein (Block A®; Yukizirushi Co., Ltd., Sapporo) diluted in HBSS and incubated at 37°C for 1 hr in 5% CO₂−95% air. The blocking solution was removed from the plates by aspiration. A total of 100 μl of anti-human ICAM-1 mAb, which was kindly supplied by Dr. Yagita (Department of Immunology, Juntendo University School of Medicine, Tokyo) was added into each well and the plates were incubated at 37°C for 1 hr in 5% CO₂−95% air. Anti-human ICAM-1 mAb was removed by aspiration from the plates and the culture wells were washed three times with HBSS. After the last washing, 100 μl of a 1/2000 dilution of the secondary antibody (goat anti-mouse IgG (H+L) horseradish peroxidase conjugate; Bio-Rad Lab. Co., Ltd., Richmond, CA, USA) in RPMI 1640 was added into each well. The plates were then incubated for 1 hr at 37°C in 5% CO₂−95% air. After washing with PBS, o-phenylene-deamine development was determined by measuring the optical density at 490 nm with a Microplate reader (model 3550 Microplate reader; Bio-Rad Lab. Co., Ltd.).

Measurement of IL-2 activity

Cell line: CTLL-2 (cytotoxic T cell, No. TIB 214; American Type Culture Collection, Rockville, MD, USA) was cultured in MEDIA II (Immuno-Biological Lab., Fuzioka) with 10% FBS (Gibco, Grand Island, NY, USA), penicillin (100 μg/ml) (Sigma, St. Louis, MO, USA), streptomycin (100 μg/ml) (Sigma), glutamine (300 μg/ml) (Gibco) and 20% rat IL-2 (Immuno-Biological Lab.). Cultured cells were maintained in a humidified incubator at 37°C in 5% CO₂−95% air. They were passaged every 4 days.

Preparation of spleen cell suspension: The spleens were aseptically removed from normal, nephritic control or FK506- or cyclosporin A-treated rats, and the spleen cells were gently dissociated in ice-cold RPMI 1640. The cell suspensions were centrifuged, and the pellets were depleted of contaminating red cells by treatment with ACT buffer (tris-HCl buffer with 0.83% NH₄Cl). After washing, the spleen cells were suspended in complete culture medium (RPMI 1640 with 10% FBS, penicillin...
(100 µg/ml), streptomycin (100 µg/ml), glutamine (300 µg/ml), 2-ME (5 x 10^{-5} M) at 1 x 10^7 cells/ml.

Spleen cell culture and IL-2 induction: Cultures were set up in flat-bottom, 48-well plates (Costar, Cambridge, MA, USA). Each well received 100 µl of cell suspension at 1 x 10^7 cells/ml. The plates were incubated at 37°C in 5% CO₂-95% air for 24–36 hr.

Measurement of IL-2 activity: Supernatants from non-stimulated spleen cells were collected and centrifuged at 220 x g for 5 min to remove the spleen cells from the supernatants. The assessment of IL-2 activity was performed as described by Gills et al. (17) with the use of the IL-2-dependent cell line (CTLL-2).

Statistical analyses
The data represent means±S.D. or S.E., and the results were statistically evaluated by ANOVA. When these results were parametric, they were statistically evaluated by the Duncan multiple-range test. When the results were non-parametric, they were statistically evaluated by the Kruskal-Wallis test.

RESULTS

Effect of FK506 on crescentic-type anti-GBM nephritis

Urinary protein excretion (Fig. 1): When the treatment of FK506 was started from the day after the anti-GBM serum injection (heterologous phase), the first significant suppression of urinary protein was observed in 10 days at 2.0 mg/kg, s.c.; in 20 days at 0.8 mg/kg, s.c.; and in 40 days at 0.2 mg/kg, s.c.

When FK506 was given from 20 days after the anti-

![Fig. 1. Effect of FK506 on urinary protein in crescentic-type anti-GBM nephritis in rats. Test drugs were given p.o. daily during the period from 1 day (A) or 20 days (B) after i.v. injection of anti-GBM serum to 40 days. Each plot denotes the mean±S.D. of mg/day. O: normal (n=5), •: nephritic control (n=7), □: nephritis + FK506 (0.2 mg/kg, s.c.) (n=7), △: nephritis + FK506 (0.8 mg/kg, s.c.) (n=7), □: nephritis + FK506 (2.0 mg/kg, s.c.) (n=7), Δ: nephritis + cyclosporin A (20 mg/kg, p.o.) (n=7). *P<0.05, **P<0.01, compared to the nephritic control rats.]

Table 1. Effects of FK506 and cyclosporin A on total glomerular cellularity, proliferating (PCNA-positive) cells and mesangial (Thy1.1-positive) area in crescentic-type anti-GBM nephritis in rats

<table>
<thead>
<tr>
<th></th>
<th>Total cells (cell/G.C.S.)</th>
<th>Proliferating cell (cells/G.C.S.)</th>
<th>Thy1.1 positive area (x 10^{-5} mm²/G.C.S.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>25.8±2.0</td>
<td>0.9±0.1</td>
<td>4.8±1.4</td>
</tr>
<tr>
<td>Nephritic control</td>
<td>40.7±2.0</td>
<td>2.3±0.3</td>
<td>6.8±1.2</td>
</tr>
<tr>
<td>FK506 (0.8 mg/kg)</td>
<td>30.7±2.5**</td>
<td>1.3±0.2**</td>
<td>4.3±1.4*</td>
</tr>
<tr>
<td>Cyclosporin A (20 mg/kg)</td>
<td>35.7±2.6**</td>
<td>1.6±0.2**</td>
<td>5.0±1.0</td>
</tr>
</tbody>
</table>

For assessing these parameters, an equatorial cross section was selected by a random sampling method. The number of nuclei (including nuclei of glomerular cells and exudate leukocytes) was counted and the results are expressed as the mean number per glomerular cross section (G.C.S.) in 30 glomeruli/sections. PCNA-positive cells and Thy1.1-positive area were measured by an image analyzer, and the results are expressed as the number of cells/G.C.S. and 10^{-5} mm²/G.C.S. in 30 glomeruli/sections, respectively. FK506 at 0.8 mg/kg, s.c. and cyclosporin A at 20 mg/kg, p.o. was given from 1 day after i.v. injection of anti-GBM serum. The kidney was taken at 15 days. The values are expressed as means±S.D. of 5 rats. *P<0.05, **P<0.01, compared to the nephritic control rats.
GBM serum injection (autologous phase), the elevation of the protein excretion into urine was inhibited with FK506 at 2.0 mg/kg by 25 days and at 0.8 mg/kg by 36 days. On the other hand, the suppression of urinary protein by cyclosporin A at 20 mg/kg, p.o. was as potent as that by FK506 at 0.8 mg/kg, s.c.

**Histological observation (Table 1; Figs. 2, 3):** Light microscopic examination of the nephritic glomeruli revealed lesions characterized by crescent formation, adhesion and fibrinoid necrosis. Histological observation demonstrated that FK506 (from both the heterologous (Fig. 2, a–c; Fig. 3) and autologous phases (data not shown)) inhibited the incidence of crescentic formation, adhesion and fibrinoid necrosis in the glomeruli. The lesion of cyclosporin A-treated nephritic rats (from autologous phase) was also less than those of the nephritic control rats (data not shown).

On the 15th day, the number of nuclei (total cells), PCNA-positive cells (proliferating cells) and the amount of Thy1.1-positive area (mesangial area) in the glomeruli

![Fig. 2. Light micrographs of glomeruli from rats of the normal group (a, d, g); nephritic control group (b, e, h); group given FK506, 0.8 mg/kg, s.c. (c, f, i). The rats were examined at 40 days after i.v. injection of anti-GBM serum (Masson's trichrome stain) (a, b, c). Note that crescent formation is markedly less in the group treated with FK506 than in the nephritic control group. Linear staining of rat-IgG (d, e, f) is observed on the GBM. Rat-IgG is negative in the normal glomeruli (d) and is less in the FK506-treated rats (f) than in the nephritic control rats (e). The photographs for the Thy1.1-positive area (g, h, i) show the glomerulus of the kidney isolated at 15 days after i.v. injection of anti-GBM serum. The amount of Thy1.1-positive area was significantly greater in the nephritic control rats than in the normal rats. FK506 suppressed the increase in Thy1.1-positive area. ➞ indicates the Thy1.1-positive area. Original magnification is × 400.](image-url)
were significantly greater in the nephritic control rats than in the normal rats (Table 1; Fig. 2, g–i). FK506 markedly suppressed the increases in total numbers of cells (hypercellularity), numbers of proliferated cells and mesangial area by 67%, 72% and 100%, respectively (Table 1; Fig. 2, g–i). Cyclosporin A also suppressed the increase in total cell numbers and mesangial area by 34% and 100%, respectively.

Table 2. Effects of FK506 on the amount of antibody, rat-IgG and C₃ deposits on the GBM in crescentic-type anti-GBM nephritis in rats

<table>
<thead>
<tr>
<th>Heterologous phase</th>
<th>Antibody level</th>
<th>Rat-IgG deposition</th>
<th>Rat-C₃ deposition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 days</td>
<td>40 days</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>(n = 5)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.01</td>
</tr>
<tr>
<td>Nephritic control</td>
<td>(n = 7)</td>
<td>21.8 ± 28.9</td>
<td>83.2 ± 45.8</td>
</tr>
<tr>
<td>FK506 (0.2 mg/kg)</td>
<td>(n = 7)</td>
<td>0.0 ± 0.0**</td>
<td>1.9 ± 3.3**</td>
</tr>
<tr>
<td>FK506 (0.8 mg/kg)</td>
<td>(n = 7)</td>
<td>0.0 ± 0.0**</td>
<td>0.0 ± 0.1**</td>
</tr>
<tr>
<td>FK506 (2.0 mg/kg)</td>
<td>(n = 7)</td>
<td>0.0 ± 0.0**</td>
<td>0.0 ± 0.0**</td>
</tr>
</tbody>
</table>

FK506 was given from 1 day (heterologous phase) after i.v. injection of anti-GBM serum. Blood was taken at 15 or 40 days. The kidney was taken at 40 days. The amount of antibody against rabbit IgG was determined by ELISA. For assessing rat-IgG and C₃ deposits on the GBM, an equatorial cross section was selected by a random sampling method; rat-IgG and C₃ deposits on the GBM were measured by an image analyzer; and the results are expressed as $10^{-3}$ and $10^{-4}$ mm²/glomerular cross section (G.C.S.) in 30 glomeruli/sections, respectively. The values are expressed as means ± S.D. **P < 0.01, compared to the nephritic control rats.
Mechanisms of FK506 on crescentic-type anti-GBM nephritis

Antibody level against rabbit IgG and the amount of rat-IgG and C3 deposits (Table 2, Fig. 2): Nephritic rats had markedly accelerated antibody production. The accelerated antibody production was suppressed with FK506 (0.2, 0.8 and 2.0 mg/kg) from the heterologous phase on days 15 and 40 (Table 2). However, it was not affected by FK506 from the autologous phase (data not shown).

The deposits of rat-IgG and C3 on the GBM were observed in nephritic control rats, but not in normal rats. FK506 (0.8 and 2.0 mg/kg) from the heterologous phase abrogated rat-IgG and C3 deposition on the GBM (Table 2; Fig. 2, d-f).

Glomerular accumulation of leukocytes (Fig. 4): Nephritic rats in two groups were given, once daily, FK506 at 0.8 mg/kg, s.c., a dose that has shown a marked antinephritic action on the early stage of crescentic-type anti-GBM nephritis in rats, and cyclosporin A at 20 mg/kg p.o., respectively, from 1 day after the anti-GBM serum injection to day 5 or 15. On days 5 and 15, the total number of leukocytes and the number of cells in each leukocyte subpopulation (CD4-positive cells, CD8-positive cells, IL-2-receptor-positive cells, ED-1-positive cells and la-positive cells) in the glomeruli were markedly greater in the nephritic control rats than in the normal rats. FK506 markedly suppressed the increase in total leukocytes and ED-1-positive cells on days 5 and 15 by approximately 60–70%. FK506 markedly suppressed the increase in numbers of CD4-positive cells, CD8-positive cells, IL-2-receptor-positive cells and la-positive cells on day 5 by 84%, 61%, 50% and 74%, respectively, and on day 15 by 79%, 70%, 80% and 67%, respectively. Cyclosporin A suppressed the accumulation of total leukocytes, ED-1-positive cells, CD4-positive cells and la-positive cells in nephritic glomeruli on days 5 and 15, and the accumulation of CD8-positive cells and IL-2-receptor-positive cells on day 15.

Glomerular accumulation of LFA-1 positive cells and up-regulation of ICAM-1 in glomeruli (Figs. 5 and 6): On days 5 and 15, the number of LFA-1-positive cells and ICAM-1 expression in the glomeruli were markedly greater in the nephritic control rats than in the normal rats. FK506 suppressed the increase in LFA-1-positive cells and the up-regulation of ICAM-1 expression on both days 5 and 15 by 51% to 74% and 54% to 63%, respectively. Cyclosporin A also suppressed the accumulation of LFA-1-positive cells on day 15 by 59% and the up-regulation of ICAM-1 expression on both days 5 and 15 by 65% to 63%, respectively.

Effect of FK506 on ICAM-1 expression of endothelial
cells (Table 3): In the continuous presence of activator, the surface expression of endothelial ICAM-1 induced with recombinant human TNF-α (100 U/ml) increased by 4 hr and reached an approximately 5 times higher level compared to the control (non-stimulation) level by 12–20 hr (data not shown). Anti-TNF polyclonal antibody excluded ICAM-1 expression on HUVECs in response to TNF-α, not but anti-IL-1 polyclonal antibody (data not shown).

Fig. 5. Photographs of glomeruli immunohistochemically stained with anti-LFA-1 (CD11a) (a, b, c) and anti-ICAM-1 (d, e, f) mAbs. Glomeruli were obtained on day 15 after i.v. injection of anti-GBM serum. a and d: normal, b and e: nephritic control, c and f: nephritis + FK506 (0.8 mg/kg, s.c.). → and ← indicate LFA-1-positive cells and ICAM-1, respectively. Original magnification is × 400.

Fig. 6. Effects of FK506 and cyclosporin A on the accumulation of LFA-1-positive cells and the up-regulation of ICAM-1 in the glomeruli of rats with crescentic-type anti-GBM nephritis. LFA-1-positive cells and ICAM-1 expression were analyzed on days 5 and 15 after i.v. injection of anti-GBM serum. a: normal, b: nephritic control, c: nephritis + FK506 (0.8 mg/kg, s.c.), d: nephritis + cyclosporin A (20 mg/kg, p.o.). G.C.S.: glomerular cross section. Each column denotes the mean ± S.D. of 5 rats. *P < 0.05, **P < 0.01, compared to the nephritic control rats.
Table 3. Effects of FK506, cyclosporin A and H-7 on endothelial ICAM-1 expression induced by TNF-α

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent of control</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (100 U/ml)</td>
<td>100.0± 0.0</td>
<td></td>
</tr>
<tr>
<td>TNF-α + FK506 (1 x 10⁻⁷ M)</td>
<td>103.8± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α + FK506 (1 x 10⁻⁸ M)</td>
<td>100.0± 3.6</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α + FK506 (1 x 10⁻⁷ M)</td>
<td>102.4± 12.6</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α + Cyclosporin A (1 x 10⁻⁷ M)</td>
<td>109.3± 11.0</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α + Cyclosporin A (1 x 10⁻⁶ M)</td>
<td>110.5± 5.5</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α + Cyclosporin A (1 x 10⁻⁵ M)</td>
<td>93.3± 6.0</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α + H-7 (1 x 10⁻⁷ M)</td>
<td>74.6± 3.8</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>TNF-α + H-7 (1 x 10⁻⁶ M)</td>
<td>77.2± 5.1</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>TNF-α + H-7 (1 x 10⁻⁵ M)</td>
<td>46.8± 6.7</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

HUVECs were stimulated with TNF-α (100 units/ml, 20 hr) in the absence or presence of these agents. ICAM-1 expression was determined by cell ELISA. Results are reported relative to the expression induced by TNF-α in the absence of these agents and represent means±S.E. of 5−10 experiments. NS: not significant.

Fig. 7. Effects of FK506 and cyclosporin A on spontaneous IL-2 production from the spleen cells in crescentic-type anti-GBM nephritis in rats. Spleen cells were isolated on day 5 after i.v. injection of anti-GBM serum, and the assessment of IL-2 activity in the culture medium was performed with the use of the IL-2-dependent cell line (CTLL-2). a: normal, b: nephritic control, c: nephritis + FK506 (0.8 mg/kg, s.c.), d: nephritis + cyclosporin A (20 mg/kg, p.o.). Each column denotes the mean±S.D. of 5 rats. **P<0.01, compared to the nephritic control rats.

As shown on Table 3, FK506 and cyclosporin A failed to abrogate TNF-α induced ICAM-1 expression on the surface of HUVECs at a nontoxic concentration. On the other hand, H-7 (1 x 10⁻⁵ - 1 x 10⁻⁷ M) prevented ICAM-1 expression of HUVECs in response to TNF-α for 20 hr.

Effect of FK506 on IL-2 production from spleen cells of nephritic rats (Fig. 7): Spontaneous IL-2 production in cultures of normal rat spleen cells was 213±52.8 units/ml. In contrast, on day 5 after the anti-GBM serum injection, the isolated spleen cells of the nephritic control rats had augmented IL-2 production (604.7±120.0 units/ml). When nephritic rats were treated with FK506 (0.8 mg/kg, s.c.), the IL-2 production was abrogated in the isolated spleen cells. The suppression of IL-2 production by cyclosporin A was as potent as that by FK506.

DISCUSSION

Crescentic-type anti-GBM nephritis represents a very severe form glomerulonephritis and mimics many features of rapidly progressive glomerulonephritis and kidney disease in Goodpasture's syndrome in humans (18). The role of leukocytes in the progression of anti-GBM antibody-induced nephritis in rats is well-established (19). Hattori et al. (20) have previously shown that intraglomerular leukocytic infiltration and activation play an important role in disease progression in this model. The results obtained from this investigation indicate that FK506 has an anti-nephritic effect in this model through the immunosuppressive action of this agent. This notion is supported by the following findings: 1) suppression of urinary protein excretion (Fig. 1); 2) prevention of progressive histological damage including crescent formation (Figs. 2 and 3); 3) inhibition of autologous antibody (rat anti-rabbit IgG antibody) production and prevention of deposition of autologous antibody and rat C3 on the GBM (Table 2 and Fig. 2); 4) inhibition of the accumulation of CD4-positive cells, CD8-positive cells, IL-2-receptor-positive cells, activated T cells and ED-1-positive cells in nephritic glomeruli (Fig. 4); 5) inhibition of the accumulation of LFA-1-positive cells and the up-regulation of ICAM-1 expression in the glomeruli (Figs. 5 and 6).
Several studies showed that FK506 suppressed the antibody production against autologous and heterologous antigen (21–23). In these studies, FK506 inhibited the anti-rabbit IgG antibody production when FK506 treatment was carried out from the heterologous phase (Table 2 and Fig. 2). However, there has been no report showing the direct inhibitory effect of FK506 on B cells, and antibody production has not been affected by treatment with FK506 from the autologous phase in which the antibody level against rabbit IgG had been elevated in this experiment (data not shown). From the experiments using polyclonal B cell activators and pokeweed mitogen, Suzuki et al. (24) reported that the suppressive effects of FK506 on the antibody production could be due to the interference of interactions between T cells and B cells. Antigen-specific immunosuppression by FK506 was also suggested in Heymann nephritis (25). We also found that FK506 suppressed the induction and progression of accelerated passive Heymann nephritis (unpublished data of K. Hayashi et al.). On the other hand, we previously reported that the anti-platelet agent dipyridamole suppressed urinary protein excretion and histological changes, but not antibody production against rabbit IgG, in this model (13). Therefore, it is suggested that the inhibition of antibody production is one of the antinephritic actions of FK506 and is not the result of the anti-nephritic action of FK506.

In vitro studies showed that FK506 inhibited the production of IL-2 (25) and affected an early stage of T cell activation (26) through the inhibition of the serine/threonine phosphatase, so-called calcineurin, the target for FK506-FK binding protein complexes (27). We also observed that FK506 at 10⁻⁶–10⁻⁹ M inhibited the IL-2 production from isolated mouse spleen cells stimulated by concanavalin A by 87–99% (data not shown). It has been reported that IL-2 production in peripheral blood mononuclear cells increase in nephritic patients (28) and a patient receiving immunotherapy with recombinant IL-2 develops acute interstitial nephritis with an interstitial leukocytic infiltration (29). On the other hand, IL-2 induces T cell (CD4- or CD8-positive cells) proliferation (30) and activation, including up-regulation of IL-2 receptor expression (25, 26). Furthermore, Li et al. (31) have reported that IL-2-receptor-positive cells in the glomeruli are closely associated with decreased renal function in IgA nephritis with crescent formation. It has been demonstrated that depletion of CD8-positive cells, including IL-2-positive cells, suppresses the development of experimental nephritis (20, 32). In the present study, FK506 inhibited the basal production of IL-2 from the isolated spleen cells of nephritic rats (Fig. 7) and suppressed the accumulation of IL-2-receptor-positive cells, activated T cells, in nephritic glomeruli in this model (Fig. 4). Taking these results together, we consider that the anti-nephritic action of FK506 is partly due to the suppressed accumulation of leukocytes, including IL-2-receptor-positive cells, in the nephritic glomeruli through the inhibition of T cell proliferation mediated by suppression of IL-2 production.

We further investigated the mechanism by which FK506 inhibits the accumulation of leukocytes in nephritic glomeruli. Recently, with respect to the development of experimental and human nephritis, it has been reported that the up-regulation of ICAM-1 expression was observed in glomeruli (33), and treatment of anti-ICAM-1 or LFA-1 antibody suppressed the renal injury (34, 35). FK506 inhibited the accumulation of LFA-1-positive cells and the up-regulation of ICAM-1 expression in the nephritic glomeruli (Figs. 5 and 6). However, in the in vitro study, FK506 and cyclosporin A failed to abrogate the TNF-α-induced ICAM-1 expression on the surface of cultured endothelial cells (Table 3). Moreover, FK506 and cyclosporin A did not inhibit the inflammatory cytokine-mediated ELAM-1 and VCAM-1 expressions on HUVECs (data not shown). Yard et al. (36) reported that FK506 and cyclosporin A inhibited the TNF-α production by cultured human proximal tubular cells in response to IL-1α. In other studies by Nguyen et al. (37), it has been shown that cyclosporin A inhibits TNF-α production in murine monocytes. Several reports have demonstrated that TNF-α is produced by intrinsic glomerular cells (38, 39) and participate in the recruitment of inflammatory cells in the glomeruli and in the progression of nephritis. Therefore, it has been considered that the decline in glomerular ICAM-1 expression was associated with an inhibition of TNF-α production in rats treated with FK506 or cyclosporin A. These data suggest that the antinephritic action of FK506 is, at least in part, due to the reduced amount of glomerular influx of leukocytes through the secondary inhibition of ICAM-1 expression by the inhibited production of TNF-α in the nephritic glomeruli.

In the present studies, FK506 markedly suppressed the increase in mesangial area (Table 1 and Fig. 2). Moreover, the increase of total cells (hypercellularity) and proliferating cell numbers, including mesangial cells, were suppressed by FK506 in nephritic glomeruli (Table 1). Glomerular cell proliferation, namely, the increase in the Thy1.1-positive area and PCNA-positive cells, is characteristic of crescentic-type anti-GBM nephritis (13). Musso et al. (40) has demonstrated that IL-2 induces IL-6 production in human monocytes. It has been reported that IL-6 transgenic mouse excretes urinary protein, causing the progression of mesangial proliferative
glomerulonephritis (41). Moreover, in both in vivo and in vitro studies, recombinant IL-6 induced the growth of rat mesangial cells (42–44). These findings suggest that the suppressed glomerular cell proliferation by FK506 is due to the inhibition of IL-6 through the inhibition of IL-2 production.

In conclusion, we have shown that FK506 is a potent and effective immunosuppressant, preventing both development and progression of crescentic-type anti-GBM nephritis in rats. In the antinephritic mechanisms of FK506, two aspects can be considered: firstly, the effect of FK506 on the humoral immune response may result in the reduction of both antibody production against rabbit IgG and the deposition of autologous antibody on the GBM; secondly, the immunosuppressive effects of FK506 on cellular immunity may suppress intraglomerular leukocyte accumulation through the inhibition of both T cell proliferation by IL-2 and up-regulation of ICAM-1 expression by inflammatory cytokines.

REFERENCES


