Mycophenolic Acid: A New Approach to the Therapy of Experimental Mesangial Proliferative Glomerulonephritis

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Abstract. Mycophenolate mofetil (MMF) represents a powerful immunosuppressant in organ transplantation. The aim of this study was to determine the anti-inflammatory effects of MMF on mesangial cells. Cultured rat mesangial cells were exposed to mycophenolic acid (MPA) in concentrations of 0.1 to 10 μM. MPA inhibited the proliferation of these cells in a dose-dependent manner. A maximum of 98% inhibition was obtained by a 2-d exposure of mesangial cells to ≥5 μM MPA. As expected, the addition of ≥75 μM guanosine prevented the antiproliferative effect of MPA completely. Subsequently, in vivo studies were performed in the anti-Thy1.1 nephritis model. Sixty-six male Wistar rats were investigated: healthy rats (n = 15), treated healthy rats (n = 6), nephritic rats (n = 15), and treated nephritic rats (n = 30). MMF therapy (40 mg/kg body wt per d) of nephritic animals was initiated 2 d before (n = 3) and 6 h (n = 15) or 2 d (n = 12) after induction of nephritis. Renal histology was analyzed at days +6 and +9 after initiation of disease. Therapy of nephritic rats by MMF resulted in a significant amelioration of glomerular histology, assessed by glomerular cellularity, synthesis of α-smooth muscle actin, extracellular matrix deposition, and glomerular hypertrophy. Proteinuria, expressed as areas under the curve of protein/creatinine ratios versus time, showed a clear tendency toward a reduction by MMF therapy. Healthy control rats were not negatively affected by exposure to MMF. In summary, this study shows that mesangial cell proliferation can be significantly inhibited by MPA in vitro and in vivo. MMF represents a new approach to the therapy of experimental mesangial cell-mediated forms of glomerulonephritis.

Mycophenolate mofetil (MMF) represents a powerful new immunosuppressive agent for the prevention and possibly even for the therapy of acute allograft rejection. MMF is rapidly metabolized to its active component mycophenolic acid (MPA) by esterases in the intestine and in the blood (1,2). MPA reversibly inhibits the inosine monophosphate dehydrogenase (IMPDH). IMPDH exists in two isoforms: the constitutively active type I, and the inducible type II, which is significantly more susceptible to MPA (3). IMPDH is responsible for the de novo synthesis of guanosine (2). Because this pathway is essential for B and T lymphocytes, the proliferation of these cells is particularly sensitive to the inhibition by MPA. Furthermore, MPA inhibits the transfer of fucose and mannose to membrane glycoproteins, which are components of adhesion molecules (1,2). Thereby, MPA may suppress the influx of lymphocytes and monocytes into areas of graft rejection or inflammation (2).

Mesangial hypercellularity and accumulation of extracellular matrix (ECM) proteins are important features of many forms of glomerular inflammatory diseases, such as IgA nephritis and membranoproliferative glomerulonephritis. In both of these diseases, mesangial cells (MC) are believed to play an active role (4). The search for agents favorably regulating MC proliferation and deposition of ECM proteins is therefore of substantial clinical importance. In this respect, MMF is a particularly interesting compound. It has been shown recently that clinically achievable concentrations of MPA of 1 to 10 μM can effectively inhibit the proliferation of cultured arterial smooth muscle cells (2). In the rat aortic allograft model, MMF significantly suppressed the replication rate and the α-actin expression of smooth muscle cells in the media of the arterial wall (1).

Analyzing several rat models of MC-mediated glomerulonephritis, Johnson et al. demonstrated that proliferating MC undergo a phenotypic change and adopt smooth muscle cell characteristics, such as the expression of α-smooth muscle actin and the increased synthesis of desmin and vimentin (4). Because MC and arterial smooth muscle cells can display such similarities, MMF may also have potential as an anti-inflammatory agent in the therapy of glomerulonephritis. In the present study, we first investigated the effect of MPA on the proliferation of cultured rat MC. Subsequently, we evaluated MMF as an anti-inflammatory agent in the anti-Thy1.1 nephritis, a well known rat model of mesangial proliferative glomerulonephritis (5–7).
Materials and Methods

**MPA and MMF**

A stock solution of 20 mM MPA (Merck, Darmstadt, Germany) was prepared in DMSO and stored in aliquots of 20 μl at −20°C. For cell culture experiments, the stock solution was serially diluted and added to tissue culture medium to yield final concentrations of 0.1 to 10 μM MPA in 0.05% DMSO. Dr. J. Aeberli (Roche Products, Basel, Switzerland) provided MMF as a gift. MMF was dissolved in a solution of 5% glucose to produce a stock solution of 10 mg/ml.

**Mesangial Cell Cultures**

MC from Wistar rats were isolated as described previously (8–10). Furthermore, the cells were characterized by a homogeneous positive staining for the Thy1.1 antigen (5).

MC from Wistar rats (14th and 15th passage) were propagated in culture medium as reported previously (11,12). RPMI 1640 (Life Technologies, Basel, Switzerland) was supplemented with 1% non-essential amino acids (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin/100 μg/ml streptomycin (Life Technologies), and 1 or 10% heat-inactivated fetal calf serum (FCS; Life Technologies), as required.

In all experiments, MC were distributed into 24-well plates (2 cm²/well; Falcon, Wingen, Switzerland) at a density of 1.8 × 10⁴ cells per well. Subsequently, cells were propagated in culture medium (0.6 ml/well) containing 10% FCS for 3 d to reach subconfluence (75% confluence) or for a shorter period to reach a state of only 60% confluence, as indicated. All experiments were done in triplicate.

**Mesangial Cell Proliferation**

Subconfluent MC (75% confluence) were washed twice with serum-free RPMI 1640 and synchronized in culture medium containing 1% FCS for 2 d. Subsequently, the cells were exposed to medium containing 10% FCS supplemented either with 0.1, 0.25, 0.5, 0.75, 1, 5, and 10 μM MPA in 0.05% DMSO or with 0.05% DMSO only for 2 d.

In a separate but otherwise identical experiment, subconfluent MC were exposed to 0.25, 0.5, 0.75, 1, and 5 μM MPA in 0.05% DMSO or to 0.05% DMSO only, in the presence or absence of 10 ng/ml platelet-derived growth factor-AB (PDGF-AB; Pharma Biotechnologie, Hannover, Germany) for 2 d in culture medium containing 1% FCS only.

To quantify cell proliferation, (methyl-³H)thymidine (1 μCi/ml, specific activity 47.0 Ci/mmol; Amersham, Zürich, Switzerland) was present in the cell culture medium together with MPA for the entire observation period of 2 d. Thereafter, MC cultures were terminated by removal of the supernatant and the addition of 1 ml/well of ice-cold 10% (w/v) TCA. Quantification of (methyl-³H)thymidine incorporation was assayed as described by us and others previously (13,14). Furthermore, MC were counted with the Coulter Counter® apparatus (Instrumenten-Gesellschaft, Zürich, Switzerland) to confirm the results obtained by the analyses of (methyl-³H)thymidine incorporation.

**Prevention of the Antiproliferative Effect of MPA by Guanosine**

Subconfluent MC (75% confluence) were kept for 2 d in culture medium containing 1% FCS, as described above. Thereafter, the cells were propagated for 2 d in culture medium containing 10% FCS supplemented with 0, 0.1, 10, 50, 75, 100, 200, or 300 μM guanosine (3 mM stock solution in RPMI 1640; Sigma), in the presence or absence of 1 μM MPA. Fresh guanosine was added after 24 h (15,16). MC proliferation was assessed by the incorporation of (methyl-³H)thymidine that was present in the medium during the entire observation period of 2 d.

Reversibility of the Antiproliferative Effect of MPA

Subconfluent (60% confluence) MC were synchronized for 2 d in culture medium containing 1% FCS, as described above. Subsequently, the cells were divided into four equal groups. The MC of group a served as controls, whereas MC of groups b, c, and d were exposed to 1 μM MPA in cell culture medium containing 10% FCS for 2 d. Thereafter, cells were washed two times with pure RPMI 1640, and fresh culture medium containing 10% FCS was added for a second period of 2 d. This time, MC of groups a and b were kept in culture medium alone. The culture medium of MC belonging to group c was again supplemented with 1 μM MPA, and the medium of group d was supplemented with 1 μM MPA and 100 μM guanosine. MC proliferation was assessed by the incorporation of (methyl-³H)thymidine for each period separately, as described above.

**Mesangial Cell Viability**

The effect of MPA and guanosine on MC viability was assessed by light microscopy, trypan blue exclusion, and release of lactate dehydrogenase (LDH) in cell culture supernatant after centrifugation (17). Values for LDH present in the supernatant were expressed as percentage of total LDH release, obtained by twice freezing and thawing of the respective MC.

**Reverse Transcription-PCR of Fibronectin and Collagen Type I and IV**

Cultured MC were analyzed in four groups: untreated controls and cells exposed for 2 d to either 0.05% DMSO, 5 μM MPA, or 5 μM MPA and 100 μM guanosine, as described above. Total RNA was extracted using TRIzol® reagent (Life Technologies). Thereafter, the expression of fibronectin and of collagen type I and IV was analyzed by semiquantitative reverse transcription-PCR, using [α³²P]-dCTP, as reported previously by members of our institution (18). To perform PCR, the following primers were used: mouse α2 (I) collagen: sense primer 5'-TGT TCG TGG TTC TGA GAG TGG TAG-3' and antisense primer 5'-TTG TCG TAG CAG GGT TCT TTC-3' encoding for a 254-bp fragment (19); mouse α1 (IV) collagen: sense primer 5'-TCG GCT ATT CCT TCG TGA TG-3' and antisense primer 5'-TCT CGC TTC TCT CTA TGG TG-3' encoding for a 185-bp fragment (19); rat fibronectin: sense primer 5'-CGT GAA TTC CAG CCA CTG ACT ACA AGA-3' and antisense primer 5'-CGG TCA CTC GAG CGG GTA CAT AGA TGG-3' encoding for a 420-bp fragment (20); and GAPDH primers as described previously (18). PCR was run in a DNA thermal cycler (Perkin-Elmer Cetus, Rotkreuz, Switzerland) for 26 cycles; exponential kinetics of PCR products were present up to this number of cycles, as analyzed separately. The following temperature profile was used: denaturation at 94°C for 1 min 15 s, annealing for 2 min at 56°C (collagen type I) or for 2 min at 60°C (collagen type IV, fibronectin, and GAPDH), and extension for 3 min at 72°C.

**Animals and Antibodies**

Male Wistar rats were purchased from Charles River, Inc. (Sulzberg, Germany). Approval for animal studies was obtained from the commission for animal studies, a local government agency. Anti-Thy1.1 IgG (OX-7) synthesizing hybridoma cells (European Collection of Animal Cell Cultures, cell line ECACC no: 84112008) were a gift from J. Floege (Division of Nephrology, Medizinische Hoch-
Glomerulonephritis and Mycophenolic Acid

Anti-Thyl.1 Nephritis: Experimental Design

Sixty-six male Wistar rats (180 g body wt at day 0) divided into six groups were studied according to the diagram outlined in Figure 5. Group A: healthy rats (n = 15); group B: healthy rats exposed to MMF from day 0 (n = 6); group C: nephritic rats (n = 15); group D: nephritic rats with MMF treatment initiated early (6 h) after induction of disease (n = 15); group E: nephritic rats with MMF therapy started 2 d after disease induction (n = 12); and group F: nephritic rats with MMF application commenced 2 d before disease induction (n = 3). Each animal was kept separately in a metabolic cage with free access to food and water. An initial period of 2 d was used to get the animals adjusted to the cages (days -5 to -3).

Anti-Thyl.1 nephritis was induced at day 0 (groups C through F) by injection of anti-Thyl.1 IgG (1 mg/kg body wt in 500 µl of PBS) into a tail vein. Healthy control rats (groups A and B) received the respective amount of PBS only.

MMF in a dose of 40 mg/kg body wt dissolved in a solution of 5% glucose (groups B and D through F) or the respective amount of 5% glucose only (groups A and C) was given gavage-fed in a volume of 1 ml/250 g body wt once daily until nephrectomy. Nephrectomy was performed at three different time points: at day +2 in three animals per group (A, C, and D); at day +6 in six animals per group (A and C through E); and at day +9 in six animals per group (A through E) in the three animals of group F. Anesthesia in all rats was achieved by intraperitoneal injection of pentobarbitone (50 mg/kg body wt; Abbott, Cham, Switzerland). Euthanasia was performed by exsanguination at the time of nephrectomy. In addition, rats of groups A through E received 5-bromo-2'-deoxyuridine (BrdU; 100 mg/kg body wt intraperitoneally; Sigma) 4 h before nephrectomy at day +9 (20).

Laboratory Analyses

Protein/creatinine ratios (g of protein/mmol creatinine) of 24-h urine specimens were measured daily from days -3 to +6 in animals of groups A through E. Estimation of the areas under the curve (AUC) of protein/creatinine ratios versus time was performed using the trapézoidal rule (24,25). The body weight of all rats was assessed daily from day -5 until sacrifice. Blood samples were taken at the time of nephrectomy on days +6 and +9 for the analysis of creatinine, potassium, and sodium in groups A through E. Leukocyte counts were measured at day +9 in the blood of animals belonging to groups A, B, and D (Cell-Dyn® 3500, Abbott). Plasma levels of MPA were determined at days +2, +6, and +9 by Dr. M. Fatth at the central laboratory of the University Hospital of Geneva, Switzerland. These analyses were performed with a Cobas Mira Plus (Roche Diagnosticas, Basel, Switzerland), using the Emit MPA assay (Syva, Dade Behring, Miami, FL).

Renal Histology

Renal tissues were fixed for 48 h in 5% neutral-buffered formalin and then embedded in paraffin. Subsequently, 3-µm sections were stained with periodic acid-Schiff reagent. All histologic analyses of equally distributed glomerular cross sections were performed by one blinded observer (R. Ziswiler). Glomerular cross sections containing only a minor portion of the glomerular tuft (<20 capillary segments/cross section) were excluded from the investigations (4). The analyses were performed using a video camera mounted on a Leitz microscope (Dialux 20; Wetzlar, Germany) and a color monitor.

Cell nuclei were counted manually to determine the total cell number per glomerular cross section (180 sections per animal group) at day +2 (groups A, C, and D), at day +6 (groups A and C through E), and at day +9 (groups A through F). Animals of group F were used exclusively for these analyses.

Glomerular hypertrophy was assessed by measurements of the maximal diameter of 180 glomerular cross sections per animal group at day +6 (A and C through E) and at day +9 (A through E), as described by us previously (25). In brief, the image of the glomerulus on the monitor screen was overlaid with a grid of 16 vertical and 12 horizontal lines (6.1 cm on the screen were equivalent to 0.05 mm on the kidney specimen).

ECM deposition in the mesangium was investigated in 180 glomerular cross sections per animal group (periodic acid-Schiff staining) at day +6 (groups A and C through E) and day +9 (A through E). Semi-quantitative grading (0 to 4) was performed according to a method published by Floege et al. (6): 0, very weak or absent mesangial staining; 1, to 25% of the glomerular tuft demonstrating focally increased staining; 2, 25 to 50% of the glomerular tuft displaying a focal strong staining; 3, 50 to 75% of the glomerular tuft with a focal strong staining; and 4, 75 to 100% of glomerular tuft displaying a strong staining. The mean mesangial matrix score was calculated for each animal group.

Immunohistochemistry

For immunohistochemical analyses, renal tissues were fixed for 24 h in methanol with 4% glacial acetic acid and then embedded in paraffin. Kidney sections of 2 µm were stained with mouse monoclonal antibodies against α-smooth muscle actin (1A4, dilution 1:1200; Sigma), monocytes/macrophages (ED-1, dilution 1:100; Chemicon, Temecula, CA), and BrdU (BU51, dilution 1:100; Progen, Heidelberg, Germany), as well as with rabbit polyclonal antibodies against collagen type IV (dilution 1:100; Collaborative Biomedical Products, Bedford, MA) and fibronectin (dilution 1:100; Life Technologies). In negative controls, the primary antibody was replaced by buffer only. Briefly, sections were dewaxed, rehydrated, and pretreated by boiling in 10 mM citrate buffer, pH 6.0, either in a pressure cooker for 5 min (ED-1) or in a microwave oven for 3 × 5 min (BrdU and negative control). Samples for actin, collagen type IV, and fibronectin staining did not undergo such a pretreatment. Thereafter and following all subsequent steps, kidney sections were washed in Tris-buffered saline (TBS), pH 7.4. Incubations with primary antibody diluted in TBS supplemented with 0.5% casein and 5% normal rabbit serum (for monoclonal antibodies) or 5% normal swine serum (for polyclonal antibodies) were carried out for 60 min at room temperature. Afterward, sections were incubated in a 1:200 dilution (same buffer as primary antibody) of biotinylated rabbit anti-mouse immunoglobulin absorbed with rat immunoglobulin (Dako, Glostrup, Denmark) or with biotinylated swine anti-rabbit immunoglobulin (Dako) and thereafter with avidin-biotin-complex/alkaline phosphatase (Dako) diluted 1:200 in TBS. Finally, sections were developed in new fuchsin-naphthol AS-BI (Sigma) and counterstained with hematoxylin.

Cell proliferation was assessed by counting BrdU-positive nuclei (20) in 900 glomerular cross sections in each of the five groups A through E at day +9. BrdU-positive cells were further characterized by double staining using antibodies against Thy1.1, an MC marker (5), and against Factor VIII-related antigen, a marker for endothelial cells (26). For these analyses, 2-µm sections from renal tissues fixed
for 24 h in 4% neutral-buffered formaldehyde were dewaxed and digested for 6 min at 37°C with a solution of 0.1% Pronase (Boehringer Mannheim, Mannheim, Germany) in TBS. Thereafter, either a monoclonal antibody against Thy1.1 (OX-7) or a polyclonal antibody against Factor VIII-related antigen (dilution 1:100; Dako) was applied, and slides were stained as outlined above, except that an avidin-biotin-complex/horseradish peroxidase (Dako) was used and slides were developed in 3,3′-diaminobenzidine (Sigma). Antibodies from the first staining were then stripped by immersing slides for 30 min into 50 mM glycine buffer, pH 2.2. After extensive washing, slides were pretreated by boiling in 10 mM citrate buffer, pH 6.0, in a microwave oven for 3 × 5 min. Subsequent steps followed exactly the protocol for the avidin-biotin-complex/alkaline phosphatase method described above, with anti-BrdU as the primary antibody.

Glomerular monocyte/macrophage influx was determined by counting ED-1-positive cells (27,28) in 900 cross sections per animal group at day +2 in groups A, C, and D, at day +6 in groups A and C through E, and at day +9 in groups A through E. Staining for α-smooth muscle actin, collagen type IV, and fibronectin were evaluated in 900 glomerular cross sections per animal group at day +9 (groups A through E), according to a semiquantitative grading system (0 to 4), as described previously (4,6). The mean scores were determined for the different experimental groups.

**Immunofluorescence Microscopy**

Immunofluorescence staining for deposition of rat IgG in glomeruli was performed by adapting a previously published method (25,29). In brief, kidneys from groups A, C, and D obtained at day +9 were fixed in methanol supplemented with 4% acetic acid and cut into 2-μm sections. Thereafter, kidney sections were incubated with FITC-conjugated anti-rat IgG (Dako) overnight. The antibody was diluted 1:25 in a solution containing a 1:1 mixture of 0.5% bovine albumin (Sigma) in PBS, pH 7.2, and 0.05 M Tris buffer (1 part Tris buffer [0.5 M; pH 7.6] and 9 parts 0.88% NaCl). Subsequently, tissue specimens were washed in PBS and counterstained by a 4-min exposure at room temperature to 10× Dapi (4′,6-diamidino-2-phenyl-indol-dihydrochlorid; Fluka, Buchs, Switzerland). All kidney sections were stained simultaneously, and immunofluorescence intensity of 90 individual glomeruli per group were investigated using the fluorescence microscope Orthoplan (Leitz).

**Statistical Analyses**

Results are expressed as mean ± SD. Statistical analyses were performed by ANOVA; comparison of means was done by the Tukey test. For all experiments, P values <0.05 were considered significant. Data of proteinuria are given as median (percentile P25 to percentile P75) with statistical analyses performed by the Wilcoxon rank sum test.

**Results**

**Antiproliferative Effect of MPA on Cultured Mesangial Cells**

Subconfluent rat MC were exposed to 0.1 to 10 μM MPA for 2 d. MPA caused a dose-dependent decrease in MC proliferation. A 50% inhibition of thymidine incorporation occurred at approximately 0.5 μM MPA, and maximal inhibition of 98% occurred at concentrations of ≥5 μM MPA (Figure 1). The addition of 10 ng/ml PDGF-AB did not attenuate the antiproliferative effect of MPA (data not shown). To confirm the inhibitory effect of MPA on cell proliferation, semiautomated cell counting was performed using the Coulter Counter apparatus, in an otherwise identical experiment. After the incubation period of 2 d, 10 μM MPA led to a decrease in cell number of 84 ± 3.8%.

Viability of cells exposed to 10 μM MPA compared with that of control cells (0.05% DMSO) was not affected, as analyzed by light microscopy and trypan blue exclusion (viability in all cases >95%). Furthermore, LDH levels in MC culture supernatant expressed as percentage of total LDH release remained low in both groups: 1.4 ± 0.1% in control cells and 5.4 ± 0.5% in cells exposed to 10 μM MPA. In addition, DMSO at a concentration of 0.05% did not interfere with cell proliferation or viability (data not shown).

**Prevention and Reversibility of the Inhibitory Effect of MPA on Cultured Mesangial Cells**

Mesangial cells were propagated for 2 d in growth medium containing 10% FCS supplemented by 0 to 300 μM guanosine, in the presence or absence of 1 μM MPA. Results are depicted in Figure 2. The addition of guanosine prevented the antiproliferative effect of MPA in a dose-dependent manner. Complete prevention was achieved by ≥75 μM guanosine. Viability of MC was not negatively affected by guanosine, investigated as described above (data not shown).

To possibly exclude cell toxicity of MPA, the recovery of cells from the antiproliferative effect of 1 μM MPA was analyzed, as shown in Figure 3. Recovery experiments were performed by the analyses of four groups of MC. For 2 d, control MC of group a were kept in pure culture medium,
Thereafter, during a second period of 2 d, we analyzed recovery of MC from the inhibitory effect of MPA. After removal of the inhibitor, the MC of group b markedly increased their proliferation rate, to the same extent as MC of group d exposed to 1 \( \mu \)M MPA and 100 \( \mu \)M guanosine. At the end of the observation period, thymidine incorporation in both of these MC groups was only approximately 5% less than in the control group a, never exposed to MPA. Therefore, the inhibitory effect of MPA on the proliferation of cultured MC was clearly reversible. In contrast, MC of group c, again exposed to 1 \( \mu \)M MPA, remained inhibited. Viability of MC at the end of the experiment was not negatively affected, as assessed by the methods mentioned above (data not shown).

**Effects of MPA on Expression of ECM Proteins in Vitro**

Semiquantitative reverse transcription-PCR was used to quantify mRNA of fibronectin, as well as collagen type I and IV. MC were cultured for 2 d in the presence of either plain medium, 0.05% DMSO, 5 \( \mu \)M MPA, or 5 \( \mu \)M MPA with 100 \( \mu \)M guanosine. We applied 5 \( \mu \)M MPA, since this dose already achieves a maximal antiproliferative effect and reflects our in vivo plasma levels more closely than the higher concentration of 10 \( \mu \)M used in other experiments.

MPA failed to decrease mRNA levels of fibronectin and collagen type I and IV, as depicted in Figure 4. These results were confirmed by measurements of the incorporation of \([\alpha^{32}P]\)-dCTP into the cDNA that was standardized to that of the GAPDH mRNA (data not shown).

**Effects of MMF on Glomerular Histology in Anti-Thyl.1 Nephritis**

Anti-Thyl.1 nephritis is characterized by an initial phase of complement-dependent mesangiolysis that is followed by a marked proliferative response of residual MC, associated with

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**Figure 2.** Prevention of the antiproliferative effect of MPA by guanosine. MC were propagated for 2 d in growth medium containing 10% FCS supplemented with 0 to 300 \( \mu \)M guanosine, in the presence of 1 \( \mu \)M MPA (\( \bullet \)) or in the absence of this agent (\( \bigcirc \)). Each point represents the mean \( \pm \) SD of three assays.

**Figure 3.** Reversibility of the antiproliferative effect of MPA. MC were propagated in culture medium containing 10% FCS in the absence (group a: \( \bigcirc \)) or in the presence of 1 \( \mu \)M MPA (group b: \( \times \); group c: \( \square \); group d: \( \bigcirc \)) for the first period of 2 d. For the second period of 2 d, fresh growth medium containing 10% FCS was added, either medium alone (groups a and b) or medium supplemented with 1 \( \mu \)M MPA (group c) or with 1 \( \mu \)M MPA and 100 \( \mu \)M guanosine (group d). Each point represents the mean \( \pm \) SD of three assays.

whereas MC of groups b, c, and d were exposed to 1 \( \mu \)M MPA. During this time period, the thymidine incorporation in groups b, c, and d decreased to 16.2 \( \pm \) 0.3% of the control group a.
accumulation of ECM proteins (30). A preliminary experiment demonstrated a maximum of these features at around day +9 (data not shown). Therefore, day +9 and an earlier time point (day +6) were chosen for nephrectomy and analyses of renal histology (Figure 5). The treatment of nephritic rats with MPA resulted in an obvious amelioration of the glomerular histology, as shown in Figure 6, A through D. Subsequently, the beneficial effects of MPA treatment were quantified by morphometric analyses and semiquantitative grading.

Mesangial cell proliferation was reflected by changes in total glomerular cell numbers that were calculated by counting all nuclei present in the glomerular cross sections at days +6 and +9. Results obtained at the latter time point are depicted in Figure 7A. Healthy rats (group A) demonstrated 57 ± 9 (day +6) and 54 ± 5 (day +9) cells; the exposure of these rats to MPA (group B) did not significantly alter glomerular cellularity, as reflected by a cell number of 58 ± 7 at day +9. As expected, total cell number in glomerular cross sections of nephritic rats (group C) significantly increased to 75 ± 4 (day +6) and 86 ± 8 (day +9) (P < 0.001 versus healthy rats). Early treatment of nephritic animals with MPA (group D) at both analyzed time points resulted in significantly decreased cell numbers of 65 ± 9 (day +6) and 68 ± 6 (day +9) (P < 0.001 versus nephritic rats). Almost identical results were seen in the delayed treated nephritic rats (group E) that displayed cell numbers of 70 ± 10 (day +6) and 66 ± 7 (day +9). However, only the result obtained at the latter time point reached statistical significance (P < 0.001 versus nephritic rats). Interestingly, similar results were obtained from the pretreated rats of group F, with a cell number of 68 ± 9, analyzed at day +9 only (P < 0.001 versus nephritic rats).

To validate these findings and to more specifically assess cell proliferation, BrdU-positive cells were counted in glomerular cross sections obtained at day +9. Results were expressed as number of positive cells per 100 glomerular cross sections, as depicted in Figure 7B. Nephritic rats (group C) displayed 152 ± 46 positive cells. MMF therapy instituted early or delayed resulted in a significant decrease of BrdU-positive cells, as reflected by the lower numbers of 67 ± 24 (group D) and 72 ± 42 (group E), respectively (P = 0.001 versus nephritic rats). The two groups of healthy rats were identical and demonstrated minimal cell proliferation of only 24 ± 11 (group A) and 20 ± 11 (group B). The BrdU-positive cells were further characterized by double staining with antibodies against either Thy1.1 antigen or Factor VIII-related antigen. As expected, none of the cells was double positive for BrdU and Factor VIII. Interestingly, in all animal groups at least 93% BrdU-positive cells also stained positive for the Thy1.1 antigen; most of the remaining cells could not be classified precisely. Representative examples of double-positive MC are depicted in Figure 6E.

To analyze for an effect of MMF on invasion of monocytes/macrophages, we counted the ED-1-positive cells in glomerular cross sections (Table 1). The number of positive cells was not statistically different at day +6 between animals of groups C (5 ± 1), D (6 ± 1), and E (6 ± 1), as well as at day +9 between the same groups (C: 4 ± 1; D: 3 ± 1; and E: 4 ± 1). Healthy animals of group A demonstrated only 1 ± 0 ED-1-positive cells per glomerular cross section at days +6 and +9. MMF exposure of healthy rats (group B) had no effect, as reflected by a number of 1 ± 0 ED-1-positive cells at day +9. Therefore, MMF did not attenuate the glomerular influx of monocytes/macrophages.

Furthermore, to exclude an interference of early MMF therapy with the initial development of the disease, animals of groups A, C, and D were analyzed at day +2 also. As expected, no effect of MMF on increase of glomerular influx of ED-1-positive cells and on mesangiolysis, reflected by decreased glomerular cellularity, was observed (data not shown).

Glomerular size was assessed at days +6 and +9 by measurements of the maximal diameter in glomerular cross sections. As analyzed at day +9 (Table 2), the two healthy control groups A and B demonstrated almost identical maximal diameters of 115 ± 4 and 110 ± 4 μm, respectively. As expected,

Figure 5. Structural diagram of the experimental design. A detailed description is given in Materials and Methods. The → indicates duration of mycophenolate mofetil (MMF) therapy; asterisks denote time points of nephrectomy while respective animals were sacrificed.
Figure 6. Glomerular histology 9 d after induction of anti-Thyl.1 nephritis. (A) Glomerulus of a healthy rat. (B) Glomerulus of a nephritic rat with marked mesangial hypercellularity and excess of matrix deposition. Treatment with MMF, either given 6 h (C) or 2 d (D) after induction of nephritis, resulted in a similar attenuation of MC proliferation and matrix accumulation. Periodic acid-Schiff stain. Magnification, ×400. (E) Proliferating MC staining positive for 5-bromo-2’-deoxyuridine (BrdU) and Thyl.1 antigen (arrows) from a nephritic rat. Magnification, ×400.

A significantly higher diameter of 136 ± 13 μm was obtained in nephritic animals (group C; \( P < 0.001 \) versus healthy rats). MMF therapy of nephritic rats resulted in a significant decrease of glomerular size, as reflected by diameters of 125 ± 8 μm (group D; \( P < 0.001 \) versus nephritic rats) and 127 ± 5 μm (group E; \( P < 0.05 \) versus nephritic rats). Assessments performed at day +6 demonstrated no significant effect of MMF therapy on glomerular size in any of the treated experimental groups (data not shown).

ECM accumulation was investigated by semiquantitative scoring (0 to 4) of glomerular cross sections at days +6 and +9. Results of day +9 are shown in Figure 8A. Healthy rats (group A) demonstrated a mean mesangial matrix score of 0.9 ± 0.6 at day +6 and 1.0 ± 0.6 at day +9. The exposure of these rats to MMF (group B) did not significantly alter matrix content, as reflected by a score of 0.9 ± 0.2 at day +9. As expected, matrix scores in glomerular cross sections of nephritic rats (group C) significantly increased to 2.1 ± 0.4 at day +6 and 3.0 ± 0.4 at day +9 (\( P < 0.001 \) versus healthy rats). MMF therapy led to a significant attenuation of ECM accumulation in nephritic groups at day +9, as reflected by the lower matrix scores of 2.4 ± 0.5 (group D) and 2.2 ± 0.5 (group E) (\( P < 0.001 \) versus nephritic rats). A similar effect of MMF treatment was observed at day +6. The matrix score of early treated animals was 1.7 ± 0.9 (group D; \( P < 0.05 \) versus nephritic rats), and of delayed treated rats 1.2 ± 0.8 (group E; \( P < 0.001 \) versus nephritic rats).

Semiquantitative scoring of the staining for collagen type IV and fibronectin was performed at day +9 only. MMF-treated nephritic rats showed only a tendency toward a reduction of the scores for these two matrix proteins (data not shown).

In rat MC, the level of expression of α-smooth muscle actin
may be associated with cell proliferation and represents a marker of cell activation (4,31). Therefore, expression of α-smooth muscle cell actin was analyzed at day +9, the time of maximal cell proliferation. A semiquantitative scoring system (0 to 4) was used for these analyses, as depicted in Figure 8B. Healthy rats (group A) displayed a mean actin score of only 0.6 ± 0.2, almost identical to animals of group B with 0.7 ± 0.1. Predictably, the actin score in glomerular cross sections of nephritic rats (group C) significantly increased to 2.7 ± 0.2 (P < 0.001 versus healthy rats). MMF treatment of nephritic animals resulted in a decreased expression of α-actin, reflected by significantly lower scores of 2.1 ± 0.2 in group D, and of 2.3 ± 0.3 in group E (P < 0.001 versus nephritic rats).

**Immunofluorescence Analyses**

The presence of an autologous phase that may have been influenced by MMF was investigated at day +9. Immunofluorescence staining failed to detect rat IgG in glomeruli of the three investigated animal groups (A, C, and D). There was only occasional faint positive staining in small parts of glomeruli and tubules, which occurred to an identical degree in all groups (data not shown).

**Effect of MMF on Proteinuria in Anti-Thy1.1 Nephritis**

Proteinuria was analyzed in all rats of groups A through E that were subsequently nephrectomized at days +6 and +9. Protein excretion in 24-h urine specimens was expressed as protein/creatinine ratios (g/mmol) from days −3 to +6. Before any intervention, baseline proteinuria from days −3 to 0 was found to be minimal and was almost identical in all animals.

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### Table 1. Effect of MMF on invasion of monocytes/macrophages (ED-1⁺ cells) per glomerular cross section

<table>
<thead>
<tr>
<th>Group</th>
<th>ED-1⁺ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day +6</td>
</tr>
<tr>
<td>(A) Healthy rats</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>(B) Treated healthy rats</td>
<td>Not done</td>
</tr>
<tr>
<td>(C) Nephritic rats</td>
<td>5 ± 1ᵇ</td>
</tr>
<tr>
<td>(D) Early treated nephritic rats</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>(E) Delayed treated nephritic rats</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

* Analyses of 900 glomerular cross sections per animal group. Values represent mean ± SD of six animals per group. MMF, mycophenolate mofetil.  
ᵇ P = 0.001 versus healthy rats.

### Table 2. Effect of MMF on glomerular hypertrophy

<table>
<thead>
<tr>
<th>Group</th>
<th>Maximal Glomerular Diameter at Day +9 (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Healthy rats</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>(B) Treated healthy rats</td>
<td>110 ± 4</td>
</tr>
<tr>
<td>(C) Nephritic rats</td>
<td>136 ± 13ᵇ</td>
</tr>
<tr>
<td>(D) Early treated nephritic rats</td>
<td>125 ± 8ᶜ</td>
</tr>
<tr>
<td>(E) Delayed treated nephritic rats</td>
<td>127 ± 5ᵈ</td>
</tr>
</tbody>
</table>

* Glomerular hypertrophy is reflected by the analyses of the maximal diameter of 180 glomerular cross sections per animal group. Values represent mean ± SD of six animals per group.  
b P < 0.001 versus healthy rats.  
c P < 0.001 versus nephritic rats.  
d P < 0.05 versus nephritic rats.
Proteinuria

Early glomerular damage was characterized by proteinuria (Table 3) as reflected by the median AUC(0-τ) of 0.21 (0.17 to 0.27) g/mmol × d in group A, and 0.04 (0.03 to 0.04) g/mmol × d in group B. As expected, the median AUC(0-τ) of nephritic rats (group C) significantly increased to 2.81 (1.49 to 4.13) g/mmol × d (P < 0.05 versus healthy rats). Interestingly, MMF treatment of nephritic rats demonstrated a distinct tendency toward a reduction, with a median AUC(0,τ) of 1.46 (0.66 to 2.51) g/mmol × d in group D and 2.00 (1.05 to 3.59) g/mmol × d in group E.

Hematologic and Biochemical Blood Analyses

Leukocytes were measured at day +9 in the blood of healthy rats (group A) and in the two groups B and D that received MMF for 9 d. MMF did not cause leukopenia, as reflected by leukocyte counts of 7.5 ± 1.5 × 10⁹/L (group A), 8.8 ± 1.7 × 10⁹/L (group B), and 5.2 ± 1.5 × 10⁹/L (group D). There were no significant differences between any of these groups, and all values remained essentially within the normal range of 4 to 8 × 10⁹/L, reported for healthy adult male Wistar rats (32). Furthermore, glomerular monocyte/macrophage influx occurs mainly during the early days of anti-Thy1.1 nephritis (33), a time point unlikely to be affected by MMF with respect to leukopenia.

At day +2, MPA plasma levels of animals belonging to group D remained minimal: 0.18 ± 0.08 µg/ml. This value was almost identical to the background measurements of 0.12 ± 0.03 µg/ml (group A) and 0.13 ± 0.06 µg/ml (group C). Significant MPA concentrations were achieved by day +6: 1.86 ± 0.99 µg/ml (group D) and 1.46 ± 0.93 µg/ml (group E). Levels in a similar range without further increase were obtained at day +9 (data not shown).

Analyzed at days +6 and +9, mean plasma levels of creatinine in the different animal groups remained within normal limits (data not shown) (34,35). Sodium and potassium levels were essentially identical in all animals (data not shown).

Kidney and Body Weight

The weight of each kidney at day +9 was expressed as percentage of total body weight (25). Healthy rats (groups A and B) showed 0.38 ± 0.07%. As expected, nephritic animals

**Table 3. Effect of MMF on proteinuria**

<table>
<thead>
<tr>
<th>Group</th>
<th>Proteinuria AUC Day(0,τ) (g protein/mmol creatinine × d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Healthy rats</td>
<td>0.21 (0.17 to 0.27)</td>
</tr>
<tr>
<td>(B) Treated healthy rats</td>
<td>0.04 (0.03 to 0.04)</td>
</tr>
<tr>
<td>(C) Nephritic</td>
<td>2.81 (1.49 to 4.13)</td>
</tr>
<tr>
<td>(D) Early treated nephritic rats</td>
<td>1.46 (0.66 to 2.51)</td>
</tr>
<tr>
<td>(E) Delayed treated nephritic rats</td>
<td>2.00 (1.05 to 3.59)</td>
</tr>
</tbody>
</table>

*Proteinuria reflected by the areas under the curve (AUC) of the protein/creatinine ratios versus time in 24-h urine specimens from day 0 to +6 (AUC(0,τ)). Values represent median (percentile P₂₅ to P₇₅) of 12 animals per group (group B: 6 animals only).

bP < 0.05: versus healthy rats.

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**Figure 8.** Effect of MMF treatment on extracellular matrix (ECM) accumulation (A) and on α-smooth muscle actin expression (B) in glomerular cross sections at day +9. Analyses of 180 glomeruli (ECM) or of 900 glomeruli (actin) were performed in each of the five groups A through E. ECM deposition and actin staining were graded by semiquantitative scores (0 to 4), as described in Materials and Methods. Column A: healthy rats (group A); column B: treated healthy rats (group B); column C: nephritic rats (group C); column D: early treated nephritic rats (group D); and column E: delayed treated nephritic rats (group E). The values are expressed as mean ± SD of six animals per group. *P < 0.001 versus nephritic animals (group C); **P < 0.001 versus healthy rats (groups A and B).

The obtained values were subtracted from all subsequent measurements. The AUC of the protein/creatinine ratios versus time were calculated from day 0 to +6 for each rat separately. As shown in Table 3, from the AUC(0,τ) of the individual rats, a median AUC(0,τ) for each animal group was obtained (percentile P₂₅ to P₇₅) (25).

Proteinuria was minimal in both groups of healthy animals, as reflected by the median AUC(0,τ) of 0.21 (0.17 to 0.27) g/mmol × d in group A, and 0.04 (0.03 to 0.04) g/mmol × d in group B. As expected, the median AUC(0,τ) of nephritic rats (group C) significantly increased to 2.81 (1.49 to 4.13) g/mmol × d (P < 0.05 versus healthy rats). Interestingly, MMF treatment of nephritic rats demonstrated a distinct tendency toward a reduction, with a median AUC(0,τ) of 1.46 (0.66 to 2.51) g/mmol × d in group D and 2.00 (1.05 to 3.59) g/mmol × d in group E.
(group C) demonstrated a slightly increased number: 0.45 ± 0.06%. Compared with these animals, MMF therapy in nephritic rats led to values of 0.42 ± 0.05% in group D and 0.5 ± 0.09% in group E. However, none of the differences between any of the five groups reached statistical significance. In addition, the body weight of the rats belonging to these groups did not significantly differ from each other, as analyzed at day +9 (mean body weight of all rats: 222 ± 23 g).

Discussion

The present study describes a novel role for MMF in the biology of MC, including inhibition of proliferation rates in cell culture and anti-inflammatory effects in the acute anti-Thy1.1 nephritis in the rat. This nephritis provides a well characterized animal model of immune complex-mediated, mesangial proliferative glomerulonephritis (5–7). In humans, mesangial proliferative glomerulonephritis, such as IgA nephritis, is an important cause of kidney failure. IgA nephropathy often manifests itself in children and young adults. According to the natural history of untreated disease, it can be expected that approximately 30 to 50% of patients suffering from IgA nephropathy finally develop end-stage renal disease during an observation period of 25 yr (36). Currently, no clear consensus exists about the treatment of this disease, since none of the current treatment regimens has been proven to demonstrate a consistent and convincing therapeutic effect (36–38). Therefore, new investigations aimed at modifying abnormal MC behavior in mesangial proliferative glomerulonephritis are of pivotal clinical importance.

MPA, the active metabolite of the prodrug MMF, was first developed in the 1960s (39). The potential antibiotic antineoplastic, and antipsoriatic effects of MPA were investigated before its use as an immunosuppressive drug in transplantation. Up to this date, it is largely unknown how MPA affects glomerular MC. The investigation of the behavior of MC exposed to MPA has implications in the fields of inflammation and possibly also of transplantation. First, we investigated the effect of MPA in vitro. MPA dose dependently inhibited the growth of cultured MC, leading to an almost total arrest of cell proliferation. The antiproliferative effect of MPA was reversible, prevented by guanosine, and not attenuated by PDGF-AB. Thereafter, we administered MMF to the nephritic animals. The main aim of this part of the study was to determine whether the application of MMF demonstrates anti-inflammatory effects in acute anti-Thy1.1 nephritis. As therapeutic endpoints, we chose glomerular cellularity, ECM accumulation, expression of α-smooth muscle actin, glomerular hypertrophy, and proteinuria.

MMF attenuated all investigated inflammatory features. Clearly, the most significant finding was the reduction in total glomerular cellularity, paralleled by a decrease in proliferating BrdU-positive cells. The overwhelming majority of the latter cells were also positive for the Thy1.1 antigen. Therefore, MC proliferation was significantly inhibited by MMF. The anti-inflammatory effect of MMF on these cells was confirmed by a decrease in the expression of α-smooth muscle actin. In anti-Thy1.1 nephritis, expression of this actin isoform was found to be associated with active MC proliferation (4). Because we were unable to detect an autologous phase at the time of nephrectomy, MMF appears to have a direct effect on MC rather than to alter the immune response.

In addition, MMF therapy of nephritic animals moderately attenuated the accumulation of ECM. However, in cultured MC, MPA did not affect the expression of three major matrix proteins: fibronectin and collagen type I and IV. Furthermore, glomerular staining for collagen type IV and fibronectin demonstrated only a slight reduction that did not reach statistical significance. Therefore, the attenuation of matrix deposition in vivo appeared to be the consequence of the reduced cell number rather than of a specific effect of MMF on ECM protein synthesis.

As a consequence of the beneficial effects, MMF led to an amelioration of glomerular hypertrophy, reflected by a decrease in the maximal glomerular diameter. Furthermore, the protective effects of MMF on the glomerular architecture were associated with a clear tendency toward a reduction in proteinuria. Importantly, early and delayed MMF treatment accomplished beneficial effects to a very similar degree. In addition, because even pretreatment of nephritic rats did not enhance the antiproliferative efficacy of MMF, a nonspecific adverse effect of this agent on the development of the early stage of anti-Thy1.1 nephritis is virtually excluded. Therefore, MMF appeared to act during the later phase of mesangial cell proliferation and not during the early stage of mesangiolysis. This hypothesis was confirmed by the analyses performed 2 d after induction of nephritis.

Because MMF interferes with the synthesis of membrane glycoproteins that are recognized by selectins (1,2), this drug may inadvertently decrease tissue invasion of leukocytes. It has been shown previously that MMF inhibits infiltration of lymphocytes and monocyte/macrophages into renal transplants, using a rat model of chronic kidney allograft rejection (40). However, other authors failed to demonstrate an effect of MMF on macrophage markers ED1 and ED3 in rat aortic allografts (41). We performed analyses of ED-1-positive monocytes/macrophages in glomerular cross sections at three different time points. Glomerular infiltration of these cells usually occurs early, with a maximum during the first 2 d after induction of nephritis (33,42). MMF did not affect influx of macrophages, mainly because we did not detect measurable drug levels during this critical time period. Nevertheless, a limited auxiliary anti-inflammatory effect of MMF by inhibition of leukocyte functions cannot be fully excluded. However, MMF did not cause leukopenia, and the in vitro data clearly support a direct effect of MMF on MC behavior.

Interestingly, our results obtained in an experimental situation are in accordance with a recent observation by Nowack et al., demonstrating substantial improvement of proteinuria and in one case also of kidney function in two patients treated with MMF for IgA nephropathy (43). However, the inhibitory effect of MMF on antibody production of B cells may play an additional role in the treatment of this disease (2,43).

MMF is only slowly emerging as an anti-inflammatory agent, as shown by the results of experimental studies. Re-
cently, Corna et al. reported that MMF (60 mg/kg per d) delayed the decrease in kidney function, retarded the development of proteinuria, and prolonged survival in NZBxW lupus mice (44). As in our investigation, the drug was applied as the sole immunosuppressant agent and it was well tolerated. Furthermore, Chanaud et al. demonstrated in Lewis rats that MMF (30 and 60 mg/kg per d) inhibited the development of experimental autoimmune uveoretinitis induced by S-antigen (45). The daily MMF dose used in our study is comparable to these reports. In addition, exactly the same dosing of 40 mg/kg per d in rats was applied by several other investigators without notable side effects, even when administered for somewhat longer time periods (1,46,47). In the therapy of rheumatoid arthritis, the definite role of MMF has not yet been defined. However, reports published some time ago claimed that this agent was effective in the therapy of adjuvant-induced arthritis in rats (48) and even in rheumatoid arthritis in humans (48,49). Furthermore, MMF may be a useful form of systemic therapy in some patients with moderate to severe psoriasis (50). With regard to glomerulonephritis, MMF therapy was shown to significantly decrease proteinuria during the induction of active Heymann nephritis, but not in established disease (51). MMF apparently was well tolerated in all cited studies, as it was in our investigation. We could not find clinically observable side effects (such as decreased food or water intake), and weight gain in MMF-treated rats was not impaired.

In summary, this is the first study demonstrating anti-inflammatory effects of MMF in MC-mediated glomerulonephritis. MMF successfully inhibited proliferation and activation of MC, as well as ECM accumulation and glomerular hypertrophy. The preservation of the glomerular structure displayed limited functional consequences, such as the slight reduction in proteinuria. In conclusion, MMF represents a new and highly promising approach to the treatment of certain forms of glomerular inflammatory diseases. The present investigation provides the rationale for a formal study analyzing the effect of MMF on mesangial proliferative glomerulonephritis in humans.

Acknowledgments
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