Lupus (2002) 11, 411–418
www.lupus-journal.com

PAPER

Effect of mycophenolate mofetil on severity of nephritis and nitric oxide production in lupus-prone MRL/lpr mice

SL Lui[1]*, R Tsang[1], D Wong[2], KW Chan[3], TM Chan[1], PCW Fung[2] and KN Lai[1]
[1]Division of Nephrology, University Department of Medicine, Queen Mary Hospital, Hong Kong, Republic of China; [2]Division of Medical Physics, University Department of Medicine, Queen Mary Hospital, Hong Kong, Republic of China; and [3]University Department of Pathology, Queen Mary Hospital, Hong Kong, Republic of China

Mycophenolate mofetil (MMF), an immunosuppressive drug commonly used in organ transplantation, is increasingly being used to treat autoimmune diseases including systemic lupus erythematosus (SLE). Excessive production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) has been implicated in the pathogenesis of lupus nephritis. We evaluated the effect of MMF on the severity of nephritis and the production of NO in lupus-prone MRL/lpr mice.

Eight-week-old female MRL/lpr mice ($n = 20$) were treated with MMF (100 mg/kg/day) by oral gavage for 12 weeks. Control mice ($n = 20$) received vehicle on the same schedule. The mice were killed after 12 weeks of treatment. Treatment with MMF significantly decreased the amount of proteinuria, prolonged survival and reduced the histological severity of glomerulonephritis. Urinary nitrite/nitrate excretion in the MMF-treated mice was significantly reduced during the first 8 weeks of treatment. However, by the end of the 12 weeks' treatment period, there was no significant difference between vehicle and MMF-treated mice in terms of urinary nitrite/nitrate excretion, intra-renal production of NO, expression of iNOS protein and induction of iNOS mRNA.

We conclude that MMF is effective in attenuating the severity of nephritis in MRL/lpr mice. The beneficial effects of MMF on lupus nephritis during the early phase of the disease might be partly attributed to the inhibition of NO production. The inhibitory effect of MMF on NO production diminishes as the disease progresses. MMF probably has additional, as yet undefined mode of actions to fully account for its beneficial effects on lupus nephritis. *Lupus* (2002) **11,** 411–418.

Key words: mycophenolate mofetil; nitric oxide; renal; systemic lupus erythematosus

Introduction

Mycophenolate mofetil (Cellcept®, MMF), a prodrug of mycophenolic acid, is a lymphocyte selective antiproliferative agent, which acts by inhibiting inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the *de novo* purine synthesis pathway.[1] Mycophenolate mofetil is commonly used for the prevention of acute rejection after organ transplantation.[2–4] In addition, it is increasingly being used to treat various autoimmune diseases including systemic lupus erythematosus (SLE). In murine models of lupus nephritis, MMF has been shown to attenuate the severity of glomerulonephritis and to improve survival.[5–8] The therapeutic effect of MMF on human lupus nephritis has been demonstrated in a recent clinical trial, which showed that combination of MMF and prednisolone was as effective as a regimen of cyclophosphamide and prednislolone for the treatment of diffuse proliferative lupus nephritis.[9] However, the exact mechanism of action of MMF in the treatment of lupus nephritis has not been completely elucidated.

Nitric oxide (NO) is a labile gaseous free radical, which plays an important role in a wide range of physiological and pathological conditions.[10,11] There is increasing evidence to suggest that excessive production of NO by the inducible isoform of nitric oxide synthase (iNOS) plays an important role in the pathogenesis of lupus nephritis.[12] Increased iNOS expression and NO production have been demonstrated in animal models of lupus nephritis.[13] Suppression of NO production by NOS inhibitor has been shown to attenuate the severity of nephritis in lupus-prone

*Correspondence: SL Lui, Division of Nephrology, University Department of Medicine, Queen Mary Hospital, Pokfulam, Hong Kong, Republic of China.
E-mail: sllui@hkucc.hku.hk
Received 9 January 2002; accepted 15 April 2002

10.1191/0961203302lu214oa

mice.[14,15] In patients with SLE, NO production was found to correlate with lupus disease activity, especially nephritis.[16–18] The detrimental effect of NO is mainly mediated through the interaction of NO with superoxide anion to form peroxynitrite, which induces renal injury *via* direct oxidant injury and protein tyrosine nitration.[19]

Mycophenolate mofetil has been shown to suppress cytokine-induced NO biosynthesis *in vitro*.[20] We have recently shown that MMF is capable of inhibiting NO production *in vivo* after renal ischemia-reperfusion injury.[21] This raises the possibility that the beneficial effects of MMF on lupus nephritis may be mediated through the inhibition of NO production.

MRL/lpr mice spontaneously develop an autoimmune disease that resembles human SLE and is characterized by immune-complex mediated glomerulonephritis, splenomegaly, lymphadenopathy and autoantibody formation. These mice usually begin to develop glomerulonephritis at age of around 12 weeks and die of renal failure when they are 3–6 months of age.[22]

The aim of this study was to determine the effect of MMF on the severity of glomerulonephritis and the production of NO in the lupus prone MRL/lpr mice.

Materials and methods

Animals

MRL/lpr (MRL/lpr/lpr) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained in animal colony of the Laboratory Animal Unit of the University of Hong Kong under standard conditions. All experiments conformed to approved animal care protocols.

Drug

Mycophenolate mofetil was obtained in powder form as a gift from Hoffmann-La Roche Inc. A suspension of MMF in carboxymethyl-cellulose (CMC) in saline vehicle was prepared fresh daily before use. The MMF suspension was well sonicated before administration.

Treatment protocols

Eight-week-old female MRL/lpr mice were treated with MMF (100 mg/kg/day) in CMC vehicle by oral gavage ($n = 20$). Control mice received equal volume of CMC vehicle alone on the same schedule ($n = 20$). The total duration of treatment was 12 weeks (from the age of 8 weeks to 20 weeks). The mice did not display signs of glomerulonephritis at the start of the treatment. Twenty-four-hour urinary collection was performed at weeks 4, 8 and 12 after the commencement of treatment to determine the amount of proteinuria and urinary nitrite/nitrate excretion. The mice were sacrificed after 12 weeks of treatment (at the age of 20 weeks). After sacrifice, the left and right kidneys were harvested for histological, immunohistochemical, electron paramagnetic resonance (EPR) and semi-quantitative gene expression studies.

Assessment of disease activity

Urinary protein excretion was assessed by collection of 24 h urine samples in metabolic cages at week 4, 8 and 12 after the initiation of treatment. Urinary protein concentration was determined by colorimetric assay and was expressed as milligrams per mouse per 24 h.

For histological assessment of the severity of glomerulonephritis, kidney tissue fragments obtained at the time of death (20 weeks of age) were embedded in paraffin. Four-micrometer sections were stained with haematoxylin and eosin. The kidney sections were coded and then examined by a single pathologist who was blinded to the treatment groups. The histological changes for mesangial proliferation, leukocytes in the glomeruli, hyaline deposition and interstitial infiltration of inflammatory cells were evaluated in a semi-quantitative fashion by a scoring system from 0 to 3+ (0 = no changes; 1+ = mild; 2+ = moderate; and 3+ = severe). Crescent formation was graded from 0 to 5+ (0 = no crescent; 1+ = < 20%, 2+ = 20–39%, 3+ = 40–59%, 4+ = 60–79%, 5+ = 80–100% of the glomeruli in the sample being affected). At least 50 glomeruli were examined for each sample.

Urinary nitrite/nitrate excretion

Measurement of urinary concentration of total nitrites and nitrates was performed by chemiluminescence using a NO analyzer (Sievers 280, USA) according to previously described methodology.[23] The procedure is briefly described as follows. Urinary samples were deproteinized by zinc sulfate and the supernatants after deproteinization were collected for further analysis. Assay of urinary nitrites and nitrates were then carried out according to the NO analyzer manufacturer's instructions. Vanadium (III) chloride was used as the reducing agent in the system. A sodium nitrate (100 mM) solution ($NaNO_3$) was prepared and diluted to various concentrations for the calibration test. Ten microliters of a standard concentration of $NaNO_3$ was injected into the radical purger, which was linked to the NO analyser, to obtain the calibration curve and the peak area for each standard

concentration was measured. Deproteinized urinary samples were then injected and the NO concentrations measured after correction for background noise.

Immunohistochemical staining for iNOS

Paraffin-embedded kidney sections (4 μm) were dewaxed, rehydrated and incubated in TBS (0.05 M Tris–HCl, 0.15 M NaCl, pH 7.4) for 5 min. The sections were then treated with peroxidase block (DAKO EnVision+ System, Denmark) for 30 min. After rinsing in TBS, the sections were incubated with primary mouse monoclonal antibodies in a dilution of 1:400 for iNOS (Transduction Laboratories) at 4°C overnight. After being rinsed with TBS, the sections were incubated with peroxidase labeled polymer conjugated to goat anti-rabbit immunoglobulins (DAKO EnVision+ System, Denmark) for 30 min. Finally, the sections were rinsed and incubated with Liquid DAB+ substrate-chromogen solution (DAKO EnVision+ System, Denmark) for 15 min. The sections were coded and examined by the same pathologist in a single-blinded fashion. The intensity of the glomerular staining for iNOS from 50 glomeruli in each mouse kidney was graded on a scale of 0–4+.

Spin-trapping EPR spectroscopy

Spin-trapping electron paramagnetic resonance (EPR) spectroscopy is a well-established technique to measure *in vivo* production of NO, which has a very short half-life, in various biological models.[21,24,25] We utilized this technique to detect the intra-renal production of NO *in vivo* in the MRL/lpr mice as previously described.[21,25] Briefly, two spin trapping agents, diethyldithiocarbamate (DETC; 500 mg/kg), intraperitoneally and iron sulfate (50 mg/kg)/sodium citrate (250 mg/kg) subcutaneously were administered to each mouse 20 min prior to harvest of the kidneys. These spin trapping agents served to trap the NO produced in the kidney and form with it stable paramagnetic mononitroysl complex, which can then be detected by an EPR spectrometer. After harvest, the whole left kidney from six mice in each group was then sliced into small pieces, fitted into plastic EPR tubes and snapped frozen in liquid nitrogen for EPR analysis.

EPR spectra were measured using a Bruker ER300E spectrometer. The EPR measuring conditions were: 10 mW microwave power, 9.34 GHz microwave frequency, 100 kHz modulation frequency, 0.52 mT modulation amplitude, 10.24 s time constant, 0.48 mT/s sweep rate and 2.0×10^4 receiver gain. The NO-Fe-DETC complex is characterized by a triplet EPR signal with $\mathbf{g}_{\perp}=2.035$ and $\mathbf{g}_{||}=2.02$. The size of the NO signal was obtained by measuring the height of the first peak of the standard triplet waveform from the baseline.[21,25] It has been shown that the concentration of NOFe (DETC) corresponds directly to the height of the first peak of the standard triplet waveform.[24] We have verified this method by showing that the height of the EPR signal has a linear relationship with NO concentration up to at least 20 μmol/l using saturated NO solution.

RNA isolation

Total RNA from kidney specimen was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction.[26] The total RNA was air-dried and re-suspended in diethyl pyrocarbonate-treated water. The concentration of RNA was quantified by measuring the absorbance at 260 nm. The quality of the RNA was monitored by the optical density (OD) 260/280 ratio. The RNA was electrophoresed through formaldehyde agarose gel and the RNA integrity was verified by examination of the 28S and 18S ribosomal RNA bands under ultraviolet irradiation after staining with ethidium bromide.

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described.[27] Briefly, 4 μg of total RNA was reverse-transcribed in a final volume of 20 μl containing 10 mM dithiothreitol, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 25 mM dNTPs, 75 ng random hexamer, 40 U RNase inhibitor (Promega, Madison, WI, USA) and 200 U of Superscript II RNase H^- reverse transcriptase (Gibco BRL, Grand Island, NY, USA). The RNA sample was heat-denatured at 95°C before the addition of reverse transcriptase and RNase inhibitor. The reaction was incubated for 60 min at 42°C. PCR was performed in a 20 μl reaction volume containing 1 μl of cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ 200 μM dNTPs, 1 U of Ampli*Taq* gold DNA polymerase (Perkin Elmer-Cetus, Foster City, CA, USA) and 0.8 μM, or 1.2 μM of sense and antisense primers for β-actin and iNOS, respectively. Samples were overlaid with mineral oil, heated to 94°C for 10 min, and then amplified for 25 cycles (β-actin) or 37 cycles (iNOS) consisting of 45 s denaturation at 94°C, 45 s annealing at 63°C (β-actin) or at 58°C (iNOS) and 5 min extension at 72°C. The experimental conditions and number of cycles of PCR were predetermined to ensure that the amount of iNOS and β-actin amplicons were within the linear range of amplification. The sequences of the PCR sense and antisense primers and the size of amplicons were as follows:

mRNA	*Sequences*	*Size of amplicon*
β-actin	5′-GGACTCCTATGTGGGTGACGAGG-3′ 5′-GGGAGAGCATAGCCCTCGTAGAT-3′	367 bp
iNOS	5′-AAGCTGCATGTGACATCGACCCGT-3′ 5′-GCATCTGGTAGCCAGCGTACCGG-3′.	598 bp

Semi-quantitative measurement of iNOS mRNA

The iNOS mRNA was semi-quantified by normalizing the differences occurring during reverse transcription and PCR using a housekeeping gene, β-actin. After amplification, 20 µl of each PCR reaction mixture were electrophoresed through a 1.8% agarose gel with ethidium bromide (0.5 µg/ml). The PCR products of β-actin and iNOS were electrophoresed in the same gel to eliminate gel to gel variation. The gel image was captured by video gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA) under the same exposure and integration. The computer image was analysed by IPLab gel software from Signal Analytics Corporation (VA, USA) for quantitation. The results were expressed as a ratio calculated from the integrated signal of iNOS amplicon over that of the β-actin amplicon.

Statistical analysis

Data were expressed as mean ± s.d. Survival curves were analysed by log-rank test. Statistical differences between vehicle and MMF-treated groups were analysed using Mann–Whitney test. Statistical significance was defined as a P-value < 0.05.

Results

Disease activity

Figure 1 shows the survival curves of vehicle and MMF-treated mice. By the end of the treatment period (ie at the age of 20 weeks), 20% of the mice in the vehicle-treated group had died. In contrast, all the mice in the MMF-treated group were still alive and the difference in survival between vehicle and MMF-treated mice was statistically significant ($P = 0.037$).

The amount of 24 h urinary protein excretion in vehicle and MMF-treated mice during the treatment period is shown in Figure 2. Treatment with MMF significantly reduced the amount of proteinuria as compared to treatment with vehicle throughout the 12 weeks' treatment period. At 4 weeks, after the commencement of treatment, the amount of proteinuria in the vehicle and MMF-treated mice was 8.4 ± 1.6 mg/day and 3.7 ± 1.2 mg/day respectively ($P < 0.05$). After 8 weeks of treatment, vehicle-treated mice had developed massive proteinuria (31.1 ± 13.6 *vs* 4.7 ± 2.8 mg/day in the MMF-treated mice, $P = 0.005$). By the end of the 12 weeks' treatment period, the amount of proteinuria in the vehicle-treated mice remained at a high level (37.3 ± 14.1 mg/day). The amount of proteinuria in the MMF-treated mice had increased substantially but was still significantly less than that of the vehicle-treated mice (10.5 ± 17.3 mg/day, $P = 0.007$).

Table 1 Effect of MMF treatment on the severity of glomerulonephritis in MRL/lpr mice[a]

	Vehicle-treated	*MMF-treated*	P-*value*
Mesangial cell proliferation	2.4 ± 0.51	1.68 ± 0.58	0.001
Hyaline deposition	1.53 ± 1.06	0.68 ± 0.85	0.016
Leukocyte in glomeruli	2.00 ± 0.85	2.05 ± 0.97	0.66
Interstitial infiltration	0.93 ± 0.96	0.74 ± 0.93	0.55
Crescent	0.93 ± 1.33	0.74 ± 1.24	0.87

[a]The histological changes for mesangial proliferation, hyaline deposition, leukocytes in the glomeruli and interstitial infiltration of inflammatory cells were scored semi-quantitatively on a 0–3+ scale. Crescent formation was graded on a scale of 0–5+. Eight-week-old female MRL/lpr mice ($n = 40$) were treated for 12 weeks with vehicle or MMF (100 mg/kg/day). Values are means ± s.d.

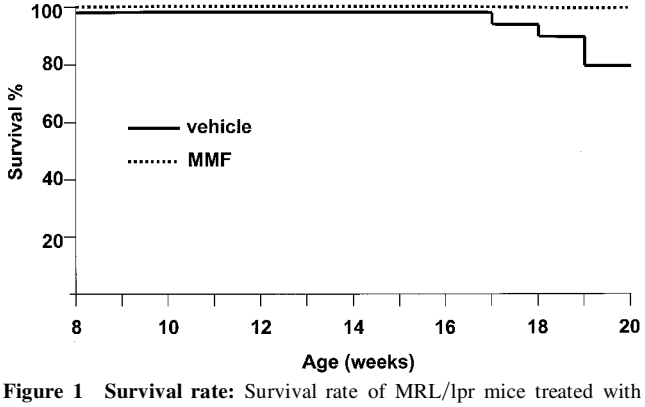


Figure 1 Survival rate: Survival rate of MRL/lpr mice treated with vehicle or MMF for 12 weeks, starting from 8 weeks of age. At 20 weeks of age, all the mice in the MMF-treated group were alive whereas 20% of the mice in the vehicle-treated group had died ($P = 0.037$).

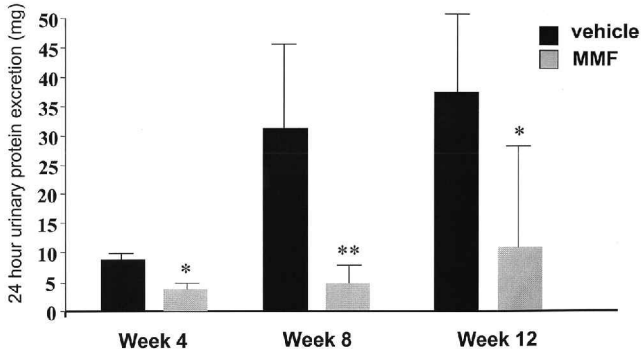
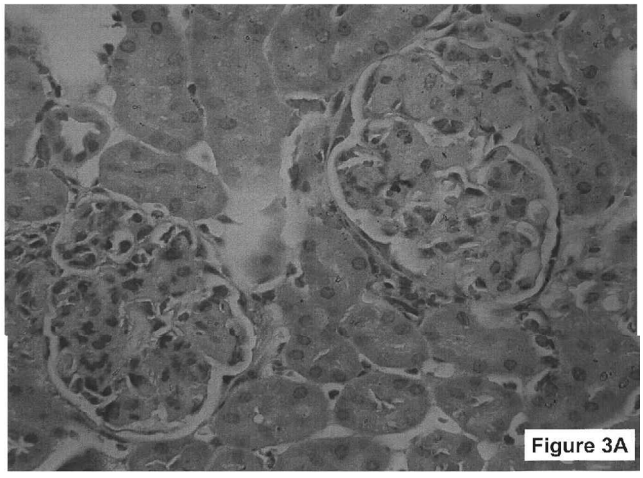


Figure 2 Proteinuria: Effect of MMF treatment on 24-hour urinary protein excretion in MRL/lpr mice (in mg/mouse/24 hours). Data were shown as mean ± S.D., $n = 16$–20. A significant decrease in the amount of proteinuria in mice treated with MMF was observed throughout the treatment period. *$P < 0.05$, **$P < 0.005$ versus vehicle-treated mice.



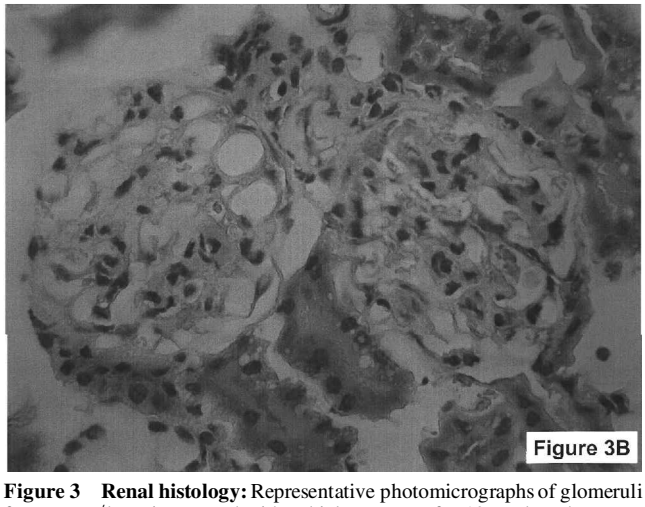


Figure 3 Renal histology: Representative photomicrographs of glomeruli from MRL/lpr mice treated with vehicle or MMF for 12 weeks. There was marked mesangial proliferation and hyaline deposition in the vehicle-treated mice (A). Treatment with MMF resulted in a significant reduction in mesangial proliferation and hyaline deposition (B). (magnification ×400)

Renal histology

The effect of MMF on the histological severity of glomerulonephritis after 12 weeks of treatment is shown in Table 1 and Figure 3. Treatment with MMF significantly reduced the amount of mesangial cell proliferation (score: 1.68 ± 0.58 *vs* 2.4 ± 0.51 for vehicle-treated mice; $P = 0.001$) and hyaline deposition in the glomeruli (score: 0.68 ± 0.85 *vs* 1.53 ± 1.06 for vehicle-treated mice; $P = 0.016$). The severity of leukocyte infiltration in the glomeruli, interstitial infiltration of inflammatory cells and crescent formation were similar between the two treatment groups.

Urinary nitrite/nitrate excretion

Figure 4 shows the effect of MMF treatment on urinary nitrite/nitrate excretion. Treatment with MMF significantly reduced the amount of urinary nitrite/nitrate excretion as compared to treatment with vehicle after 4 weeks (5.3 ± 1.2 *vs* 7.8 ± 1.0 μmol/l, $P < 0.05$) and 8 weeks (4.2 ± 2.0 *vs* 9.5 ± 2.5 μmol/l, $P < 0.005$) of treatment. However, by the end of the 12 weeks' treatment period, the difference in urinary nitrite/nitrate excretion between vehicle and MMF-treated mice was not statistically significant (9.1 ± 4.3 *vs* 7.5 ± 2.9 μmol/l, $P = 0.41$).

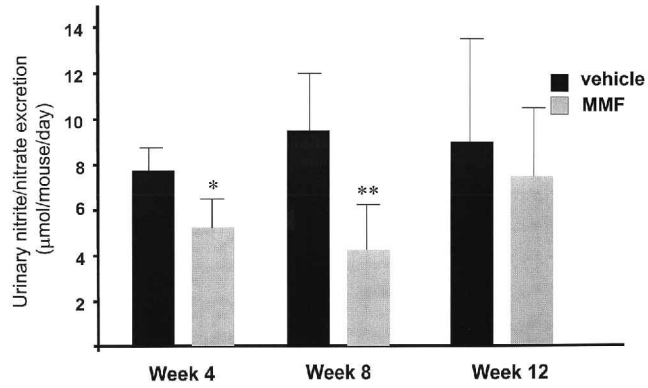


Figure 4 Urinary nitrite/nitrate excretion: Urinary nitrite/nitrate excretion of MRL/lpr mice treated with vehicle or MMF, expressed as micromoles per mouse per 24 hours. Data were shown as mean ± S.D., $n = 16$–20. Treatment with MMF significantly reduced the amount of urinary nitrite excretion during the first 8 weeks of treatment. $^{*}P < 0.05$, $^{**}P < 0.005$ versus vehicle-treated mice.

NO production in the kidney

The *in vivo* production of NO in the kidney tissues of the mice after 12 weeks of treatment (i.e. at the age of 20 weeks when they were sacrificed), as assessed by EPR spectroscopy is shown in Figure 5. The relative amplitudes of the NO-Fe-DETC EPR spectra of the two experimental groups did not differ significantly ($P = 0.3$).

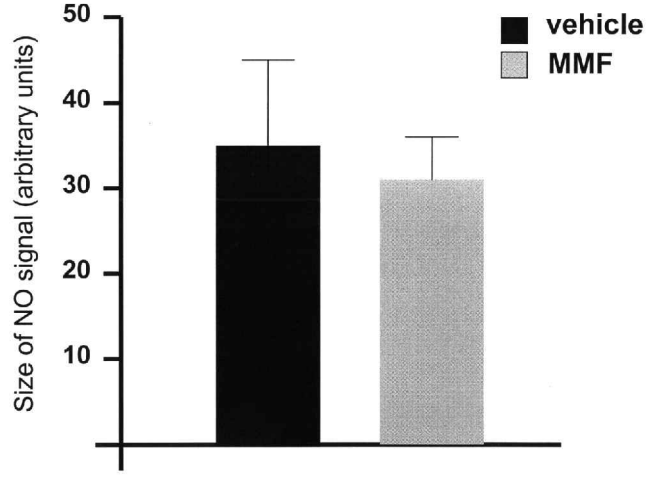


Figure 5 Intra-renal NO production: Relative amplitude of the NO-Fe-DETC signal in the kidney from MRL/lpr mice treated with vehicle or MMF for 12 weeks. Data were shown as the mean ± S.D., $n = 6$. There was no statistically significant difference in the size of the NO signal between vehicle and MMF-treated mice.

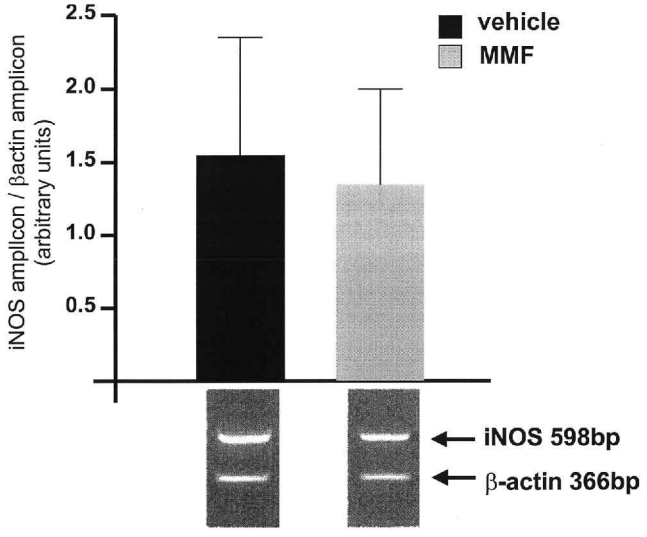


Figure 6 Intra-renal expression of iNOS mRNA: Effect of 12 weeks' of vehicle of MMF treatment on iNOS mRNA expression in the kidneys of MRL/lpr mice was assessed by semi-quantitative RT-PCR. The amount of iNOS mRNA present in the kidney tissue was expressed as a ratio calculated from the integrated signal of the iNOS amplicon over that of the β-actin amplicon. Data were shown as the mean $\pm$ S.D., $n = 16$–20. The difference is iNOS mRNA expression in the 2 treatment groups was not statistically significant.

iNOS expression in the kidneys

After 12 weeks of treatment, in both the vehicle and the MMF-treated mice, positive staining for iNOS protein was observed in the glomerular mesangium, the tubular cells and the cellular crescents. There was no statistically significant difference in the intensity of iNOS staining between the two groups of mice (score: 2.3 ± 0.77 *vs* 2.56 ± 0.51 for vehicle-treated mice; $P = $ NS).

iNOS mRNA expression in the kidney

The effect of MMF treatment on the expression of iNOS mRNA in the kidney tissue after 12 weeks of treatment was studied by semi-quantitative RT-PCR. The amount of iNOS mRNA present in the kidney tissue was expressed as a ratio calculated from the integrated signal of the iNOS amplicon over that of the β-actin amplicon. A statistically significant difference in iNOS mRNA expression in the kidney tissues of mice treated with vehicle or MMF was not observed (Figure 6).

Discussion

This study shows that in MRL/lpr mice, treatment with MMF for 12 weeks, starting from 8 weeks of age, significantly prolonged survival, reduced the amount of proteinuria and decreased the histological severity of glomerulonephritis. Moreover, we found that MMF treatment significantly reduced urinary excretion of NO metabolites during the first 8 weeks of treatment. However, by the end of the 12 weeks' treatment period, we did not observe a significant difference between mice treated with vehicle or MMF in terms of urinary excretion of NO metabolites, intra-renal generation of NO, expression of iNOS protein and induction of iNOS mRNA.

Our data confirms the results of previous studies that in lupus-prone MRL/lpr mice, MMF is effective in attenuating the severity of nephritis when administered as a preventive treatment.[5,6] However, in this murine model of lupus nephritis, MMF treatment could not completely prevent the development of nephritis. In addition, when MMF was given to MRL/lpr mice with established glomerulonephritis, it was not effective in inducing disease remission.[6] These observations indicate that MMF treatment alone is only partially effective in suppressing disease activity in these lupus-prone mice. This is not entirely unexpected as multiple immunological and inflammatory pathways are likely to be involved in the pathogenesis of nephritis in these mice, and not all of these pathways are affected by MMF. Combination therapy of MMF and other immunosuppressive or anti-inflammatory agents with different modes of action might therefore confer better control of disease activity. This is supported by a recent study in which combination of MMF and a cyclooxygenase-2 inhibitor was found to be significantly more effective than either agent alone in ameliorating murine lupus nephritis.[28]

Although the beneficial effects of MMF on disease activity in murine models of lupus nephritis have been established by several studies, including ours, the exact mechanism of action of MMF in the attenuation of lupus activity remains incompletely understood. It has been shown that treatment with MMF decreased the deposition of immunoglobulin and C3 in the glomeruli of MRL/lpr mice.[6] As MMF is a lymphocyte-selective anti-proliferative agent, one would expect a parallel reduction in the production of autoantibodies in these mice. However, a significant effect of MMF on autoantibody formation and lymphocyte proliferation *in vivo* was not observed and it was suggested that the reduction of glomerular immunoglobulin deposition in the MMF-treated mice was due to decreased binding of immune complex in the glomerular capillary wall rather than an absolute decrease in immune complex formation.[6]

Another possible mechanism of action of MMF in attenuating lupus activity may be mediated through the inhibition of NO production. It has been recognized that iNOS generated NO plays a significant role in the pathogenesis of lupus nephritis[12,13] and that inhibition of iNOS activity reduces the severity of murine lupus nephritis.[14,15] Mycophenolate mofetil

has been shown to inhibit NO formation both *in vitro*[20] and *in vivo*.[21] In this study, we found that mice treated with MMF had significantly reduced urinary nitrite/nitrate excretion during the first 8 weeks of treatment, which was paralleled by a significant reduction of proteinuria. It has been shown in an animal model of nephrotoxic nephritis that urinary nitrite excretion correlates well with renal NO production.[29] Our observation thus provides indirect evidence that MMF treatment reduces the production of NO in the kidney of these lupus-prone mice. It is therefore plausible to postulate that the protective effect of MMF on disease activity in the MRL/lpr mice during the first 8 weeks of treatment is due, at least partly, to the inhibitory effect of MMF on NO generation. Our postulation would be further strengthened if we had corresponding data on the intra-renal production of NO and expression of iNOS of the mice during the first 8 weeks of treatment. However, such analysis had not been performed by virtue of the original design of the study.

The exact mechanism whereby MMF suppresses NO production *in vivo* has not been completely elucidated. Previous studies have suggested that MMF might reduce NO production *via* the inhibition of the biosynthesis of tetrahydrobiopterin, an essential co-factor of NOS.[20] We have recently shown in a mouse model of ischemia-reperfusion injury that MMF reduces the production of NO directly by suppressing the induction of iNOS at the transcription level.[21] The induction of iNOS is dependent on the transcription factor NFκB, which is inducible by various pro-inflammatory cytokines, such as tumor necrosis factor, interleukin-1 and interferon-γ. Although MMF does not have a direct effect on cytokine production on a per cell basis,[1] it may limit the total amount of cytokine produced by limiting clonal expansion of the triggered lymphocytes.[30] This effect may also contribute to the overall reduction in NO production. It thus appears that the suppression of NO production is a specific effect of MMF rather than a non-specific effect related to the inhibition of inflammation.

Our findings that MMF did not reduce the generation of NO and the induction of iNOS by the end of the 12 weeks' treatment period indicate that MMF is unable to persistently suppress the generation of NO in the MRL/lpr mice. As the disease progresses, the inhibitory effect of MMF on NO production becomes diminished. It is worthwhile to note that the disease activity of the MMF-treated mice had increased substantially towards the end of the treatment period, although it was still significantly less than that of the vehicle-treated mice. The reason for the loss of inhibitory effect of MMF on NO production with time is not clear. One possible explanation is that as the lupus nephritis progresses, alternative pathways in the generation of NO that are not suppressed by MMF might become activated, thus leading to the enhanced production of NO. It is worth noting that the MRL/lpr model of lupus is in general more refractory to treatment than other murine models such as the NZB/NZW mice.[14] It would be interesting to see if the suppressive effect of MMF on NO production might be more marked and persistent in the latter model.

The continued suppression of disease activity by MMF in the MRL/lpr mice despite a lack of effect on NO generation by the end of the treatment period, suggests that MMF probably has other modes of action in suppressing lupus activity. Mycophenolate mofetil has been shown to inhibit the glycosylation of adhesion molecules such as selections through depletion of guanosine nucleotides, thereby preventing the adhesion molecules from proper functioning.[31] Altered function of the adhesion molecules might in turn reduce the recruitment of lymphocytes, neutrophils and monocytes to the site of inflammation. In animal model of renal transplantation, it has been shown that MMF inhibits the upregulation of adhesion molecules and reduces the recruitment of inflammatory cells to the allograft.[32] Mycophenolate mofetil has also been shown to suppress perivascular T cell infiltration in the kidneys of MRL/lpr mice.[33] Nevertheless, the contribution of altered adhesion molecule function to the immunosuppressive effect of MMF in lupus nephritis remains to be confirmed.

The suppression of mesangial cell proliferation by MMF might also contribute to its beneficial effect on lupus nephritis. Mesangial cells have been recognized as an active player of glomerulonephritis.[34] In experimental model of mesangial proliferative glomerulonephritis, there are both *in vitro* and *in vivo* evidence to show that MMF has a direct inhibitory effect on mesangial cell proliferation.[35] In our study, we have also observed a significant reduction in mesangial cell proliferation in the MMF-treated mice, raising the possibility that the beneficial effect of MMF may be mediated partly through inhibition of mesangial cell proliferation.

In conclusion, MMF is effective in attenuating disease activity in the MRL/lpr mouse model of lupus nephritis. In this model, the beneficial effects of MMF on lupus disease activity during the early phase of the disease might be attributed partly to a reduced production of NO. However, as the disease progresses, the effect of MMF on NO production diminishes. It appears that our current knowledge on the mechanism of action of MMF could not adequately explain the beneficial effects of MMF on lupus nephritis. We speculate that additional mechanisms of action might exist to fully explain the beneficial effects of MMF on

lupus activity in this model. Further studies are warranted to uncover these mechanisms of action.

Acknowledgements

This study was supported by grants from the Hong Kong Society of Nephrology and the Department of Medicine, the University of Hong Kong. The authors would like to thank Hoffmann-La Roche Inc. for providing the MMF powder for our study. We are also grateful to Dr HY Lan, Dr S Tam, Dr KS Lo and Mr JCK Leung for their excellent technical assistance and valuable advice.

References

1 Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology* 2000; **47**: 85–118.
2 Halloran P, Mathew T, Tomlanovich S, Groth C, Hooftman L, Barker C. Mycophenolate mofetil in renal allograft recipients: a pooled efficacy analysis of three randomized, double-blind, clinical studies in prevention of rejection. The International Mycophenolate Mofetil Renal Transplant Study Groups. *Transplantation* 1997; **63**: 39–47.
3 Kobashigawa J, Miller L, Renlund D *et al.* A randomized active-controlled trial of mycophenolate mofetil in heart transplant recipients. Mycophenolate Mofetil Investigators. *Transplantation* 1998; **66**: 507–515.
4 Fisher RA, Ham JM, Marcos A *et al.* A prospective randomized trial of mycophenolate mofetil with neoral or tacrolimus after orthotopic liver transplantation. *Transplantation* 1998; **66**: 1616–1621.
5 Corna D, Morigi M, Facchinetti D, Bertani T, Zoja C, Remuzzi G. Mycophenolate mofetil limits renal damage and prolongs life in murine lupus autoimmune disease. *Kidney Int* 1997; **51**: 1583–1589.
6 Van Bruggen MC, Walgreen B, Rijke TP, Berden JH. Attenuation of murine lupus nephritis by mycophenolate mofetil. *J Am Soc Nephrol* 1998; **9**: 1407–1415.
7 McMurray RW, Elbourne KB, Lagoo A, Lal S. Mycophenolate mofetil suppresses autoimmunity and mortality in the female NZB×NZW F1 mouse model of systemic lupus erythematosus. *J Rheumatol* 1998; **25**: 2364–2370.
8 Jonsson CA, Svensson L, Carlsten H. Beneficial effect of the inosine monophosphate dehydrogenase inhibitor mycophenolate mofetil on survival and severity of glomerulonephritis in systemic lupus erythematosus (SLE)-prone MRL lpr/lpr mice. *Clin Exp Immunol* 1999; **116**: 534–541.
9 Chan TM, Li FK, Tang CS *et al.* Efficacy of mycophenolate mofetil in patients with diffuse proliferative lupus nephritis. Hong Kong-Guangzhou Nephrology Study Group. *New Engl J Med* 2000; **343**: 1156–1162.
10 Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *New Engl J Med* 1993; **329**: 2002–2012.
11 Kone BC. Nitric oxide in renal health and disease. *Am J Kidney Dis* 1997; **30**: 311–333.
12 Weinberg JB. Nitric oxide as an inflammatory mediator in autoimmune MRL-lpr/lpr mice. *Environ Health Perspect* 1998; **106**(Suppl 5): 1131–1137.
13 Weinberg JB, Granger DL, Pisetsky DS *et al.* The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: increased nitric oxide production and nitric oxide synthase expression in MRL-*lpr*/*lpr* mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N^G-monomethyl-L-arginine. *J Exp Med* 1994; **179**: 651–660.
14 Oates JC, Ruiz P, Alexander A, Pippen AM, Gilkeson GS. Effect of late modulation of nitric oxide production on murine lupus. *Clin Immunol Immunopathol* 1997; **83**: 86–92.
15 Yang CW, Yu CC, Ko YC, Huang CC. Aminoguanidine reduces glomerular inducible nitric oxide sythase (iNOS) and transforming growth factor-beta 1 (TGF-beta 1) mRNA expression and diminishes glomerulosclerosis in NZB/W F1 mice. *Clin Exp Immunol* 1998; **113**: 258–264.
16 Wanchu A, Khullar M, Deodhar SD, Bambery P, Sud A. Nitric oxide synthesis is increased in patients with systemic lupus erythematosus. *Rheumatol Int* 1998; **18**: 41–43.
17 Gonzalez-Crespo MR, Navarro JA, Arenas J, Martin-Mola E, De La Cruz J, Gomez-Reino JJ. Prospective study of serum and urinary nitrate levels in patients with systemic lupus erythematosus. *Br J Rheumatol* 1998; **37**: 972–977.
18 Gilkeson G, Cannon C, Oates J, Reilly C, Goldman D, Petri M. Correlation of serum measures of nitric oxide production with lupus disease activity. *J Rheumatol* 1999; **26**: 318–324.
19 Lieberthal W. Biology of ischemic and toxic renal tubular cell injury: role of nitric oxide and the inflammatory response. *Curr Opin Nephrol Hypertens* 1998; **7**: 289–295.
20 Senda M, Delustro B, Eugui E, Natsumeda Y. Mycophenolic acid, an inhibitor of IMP dehydrogenase that is also an immunosuppressive agent, suppresses the cytokine-induced nitric oxide production in mouse and rat vascular endothelial cells. *Transplantation* 1995; **60**: 1143–1148.
21 Lui SL, Chan LY, Zhang XH *et al.* Effect of mycophenolate mofetil on nitric oxide production and inducible nitric oxide synthase gene expression during renal ischaemia-reperfusion injury. *Nephrol Dial Transplant* 2001; **16**: 1577–1582.
22 Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Adv Immunol* 1985; **37**: 269–390.
23 Moshage H, Kok B, Huizenga JR, Jansen PL. Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin Chem* 1995; **41**: 892–896.
24 Zweier JL, Wang P, Kuppusamy P. Direct demonstration of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. *J Biol Chem* 1995; **270**: 304–307.
25 Chan KL, Zhang XH, Fung PCW, Guo WH, Tam PKH. Role of nitric oxide in intestinal ischaemia-reperfusion injury studied using electron paramagnetic resonance. *Br J Surg* 1999; **86**: 1427–1432.
26 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
27 Leung JC, Tsang AWL, Chan TM, Lai KN. Absence of CD89, polymeric immunoglobulin receptor and asialoglycoprotein receptor on human mesangial cell. *J Am Soc Nephrol* 2000; **11**: 241–249.
28 Zoja C, Benigni A, Noris M *et al.* Mycophenolate mofetil combined with a cyclooxygenase-2 inhibitor ameliorates murine lupus nephritis. *Kidney Int* 2001; **60**: 653–663.
29 Bremer V, Tojo A, Kimura K *et al.* Role of nitric oxide in rat nephrotoxic nephritis: comparison between inducible and constitutive nitric oxide synthase. *J Am Soc Nephrol* 1997; **8**: 1712–1721.
30 Lui SL, Ramassar V, Urmson J, Halloran PF. Mycophenolate mofetil reduces production of interferon-dependent major histocompatibility complex induction during allograft rejection, probably by limiting clonal expansion. *Transplant Immunol* 1998; **6**: 23–32.
31 Allison AC, Kowalski WJ, Muller CJ, Waters RV, Eugui EM. MMF and brequinar, inhibitors of purine and pyrimidine synthesis, block the glycolysation of adhesion molecules. *Transplant Proc* 1993; **25**(Suppl 3): 67–70.
32 Heemann U, Azuma H, Hamar P, Schmid C, Tilney N, Philipp T. Mycophenolate mofetil inhibits lymphocyte binding and the upregulation of adhesion molecules in acute rejection of rat kidney allografts. *Transplant Immunol* 1996; **4**: 64–67.
33 Jonsson CA, Erlandsson M. Svensson L, Molne J, Carlsten H. Mycophenolate mofetil ameliorates perivascular T lymphocyte inflammation and reduces the double-negative T cell population in SLE-prone MRLlpr/lpr mice. *Cell Immunol* 1999; **197**: 136–144.
34 Johnson RJ, Iida H, Alpers CE *et al.* Expression of smooth muscle cell pheonotype by rat mesangial cells in immune complex nephritis. Alpha-smooth muscle actin is a marker of mesangial proliferation. *J Clin Invest* 1991; **87**: 847–858.
35 Ziswiler R, Steinmann-Niggli K, Kappeler A, Daniel C, Marti HP. Mycophenolic acid: a new approach to the therapy of experimental mesangial proliferative glomerulonephritis. *J Am Soc Nephrol* 1998; **9**: 2055–2066.