Mycophenolate Mofetil Prevents the Induction of Active Heymann Nephritis: Association with Th2 Cytokine Inhibition

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Abstract. The effect of mycophenolate mofetil (MMF) was examined in active Heymann nephritis (HN), an animal model of human membranous nephropathy. HN was induced in Lewis rats with Fx1A/complete Freund’s adjuvant (CFA), and controls only received CFA. The induction of HN was prevented by MMF (30 mg/kg per d) from 0 to 4 wk after immunization. Proteinuria was not different in CFA controls up to 16 wk, and was significantly less than in untreated HN from 6 wk onward. Serum anti-Fx1A antibody (Ab) levels and glomerular Ig deposition were suppressed during therapy. The interstitial infiltrate of αβ TCR<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, natural killer cells, and macrophages (mφ) observed in untreated HN at 8 wk was absent from rats treated from 0 to 4 wk with MMF. Semiquantitative reverse transcription-PCR for renal mononuclear cell cytokine mRNA at 8 wk demonstrated that MMF from 0 to 4 wk prevented the increased expression of Th1 (interferon-γ, lymphotoxin), Th2 (interleukin-4), and mφ (tumor necrosis factor-α) cytokines identified in untreated HN. In lymph node draining sites of immunization, MMF limited both enlargement and the increased proportion of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells observed in untreated HN and CFA controls. MMF suppressed Th2 (interleukin-4) but not Th1 (interferon-γ, lymphotoxin) cytokine mRNA expression in lymph nodes. MMF from 4 to 8, 6 to 12, or 10 to 14 wk did not prevent proteinuria, serum anti-Fx1A Ab, or glomerular IgG deposition when compared with untreated HN. This study showed that MMF from 0 to 4 wk prevented the induction of HN and was associated with preferential suppression of Th2 cytokines. This therapy may prove useful in human idiopathic membranous nephropathy.

Active Heymann nephritis (HN) is a rat model of human idiopathic membranous nephropathy (MN). A CD4<sup>+</sup> T cell-dependent humoral response (1) results in glomerular Ig deposition and complement activation, which is thought to mediate glomerular injury and cause proteinuria (2). The CD4<sup>+</sup> help for antibody (Ab) responses is a function of Th2 cells, which produce interleukin-4 (IL-4), IL-5, IL-6, IL-10, and IL-13. It has recently been demonstrated that the onset of HN is associated with a glomerular and interstitial T cell and macrophage infiltrate (3). A direct role for cell-mediated injury, particularly by CD8<sup>+</sup> cytotoxic T cells and macrophages, has been suggested and is supported by the effect of CD8<sup>+</sup> cell depletion (3,4). CD4<sup>+</sup> Th1 cells that produce IL-2, interferon-γ (IFN-γ), and tumor necrosis factor-β (TNF-β)/lymphotoxin (LT) are required for help in activation of CD8<sup>+</sup> T cells. The induction of HN is not totally prevented by the conventional immuno-suppressive drugs currently used to treat MN, including corticosteroids (5) and cyclophosphamide (6). Cyclosporin A (CsA) (4) reduces proteinuria, and FK506 can prevent the induction of disease (7). Anti-T cell monoclonal antibody (mAb) therapy is more effective than immunosuppressive drugs (4) in that anti-CD4 mAb therapy induces tolerance to HN, and anti-CD8 mAb therapy can delay the onset of proteinuria. In this study, we examined the effects of a new immunosuppressive agent, mycophenolate mofetil (MMF), on HN.

MMF, a lymphocyte-specific antiproliferative agent (8), is an orally available prodrug of mycophenolic acid (MPA). MMF and MPA block de novo purine synthesis pathways on which lymphocytes, unlike other eukaryotic cells that also use salvage pathways, are exquisitely dependent. Thus, these agents inhibit T and B lymphocyte proliferation. The immunosuppressive properties of MMF and MPA have been demonstrated in experimental (9,10) and clinical organ transplantation (11,12), and may induce tolerance (13). Less is known of the effect of MMF in autoimmune disease. It is effective in experimental models such as uveoretinitis (14), experimental allergic neuritis (M. Penny, unpublished data), diabetes mellitus (15), and murine lupus nephritis (16). MPA has been used clinically in human rheumatoid arthritis (17) and psoriasis (18). Recent clinical reports suggest that MMF may play a role in the treatment of human systemic lupus erythematosus, antineutrophil cytoplasmic antibody-positive vasculitis, IgA nephropathy, glomerulosclerosis, and MN (19–21).

To examine the effect of MMF therapy on HN, rats were treated during the induction of disease, the evolution of proteinuria, and when proteinuria was established. The effect of MMF on Ab responses and T cell infiltrates was also examined. Because the relative effects of MMF on Th1 (IL-2,
IFN-γ) and Th2 (IL-4, IL-5, IL-10, IL-13) producing T cell subsets are not known, these were specifically examined. MMF was shown to prevent the induction of HN and preferentially inhibited Th2 cytokine production.

Materials and Methods

Experimental Protocol

In separate sets of experiments, groups of Lewis rats immunized with Fx1A and complete Freund's adjuvant (CFA) were treated with MMF at various intervals after immunization, each with its own untreated HN (Fx1A and CFA) and CFA control groups. Within each of these separate experiments, the same batch was used for all groups as the nephritogenic potential of Fx1A can vary between batches. Therapy from 0 to 4 wk after immunization examined the effect of MMF on the preproteinuric induction phase of HN, during which time anti-Fx1A Ab responses and glomerular immune deposits develop. Therapy with MMF from 4 to 8 and 6 to 12 wk examined the effect of MMF on the phase of HN with established anti-Fx1A Ab responses and glomerular immune deposition, but evolving proteinuria and progressive cellular infiltrates. The phase of HN with well established proteinuria was examined by therapy with MMF from 10 to 14 wk. The effects of MMF on the activated T cell and macrophage infiltrate in renal cortex was also examined by both immunocytochemistry and reverse transcription (RT)-PCR for cytokine mRNA expression.

To examine the mechanism of action of MMF during induction of HN, popliteal lymph nodes (LN) draining the sites of immunization were weighed, their lymphocyte subsets were determined by fluorescence-activated cell sorter (FACS) analysis, and their cytokine profiles were examined by RT-PCR. The effect of MMF on immune events within LN draining sites of primary immunization was examined in a separate experiment. In this study, LN were taken from a group of rats 2 wk after immunization and compared with those from its own untreated HN, CFA control, and normal (nonimmunized) groups.

MMF was given as a daily oral dose of 30 mg/kg sonicated in vehicle containing benzyl alcohol, 5% l-Dextrose, 0.9% NaCl, 0.5% carboxymethylcellulose, 0.4% polysorbate 80, and 88.7% water, pH 3.5. This dose for 4 wk induces reversible anemia, as well as thymic and secondary lymphoid tissue atrophy which recovers to normal after another 4 wk (personal communication, Dr. E. Eugui, Research Syntex, Palo Alto, CA). Similar doses of MMF are known to prevent heart and pancreatic transplant rejection in the rat, and are close to the maximum tolerated by rats.

Experimental Animals and Operative Procedures

Inbred male Lewis rats (Lew/ssn) from the animal facility at the University of New South Wales, were used. Water and standard chow were available ad libitum. All operative procedures have been described previously (3), and were performed under ether anesthesia. Animal experimental protocols were approved by the Animal Care and Ethics Committee, University of New South Wales.

Monoclonal Antibodies

Monoclonal antibodies are described elsewhere and include: MRC OX-12, which recognizes rat K light chains; R73, which recognizes the αβ TCR receptor on T cells; W3/25, which recognizes CD4+ T cells and some macrophages; and MRC OX-8, which recognizes CD8+ T cytotoxic and natural killer (NK) cells. These were produced from clones in the laboratory as described (24). 3.2.3, which recognizes NK cells, and ED-1, which recognizes most macrophages and some dendritic cells, were obtained from Serotec (Oxford, United Kingdom). FITC or phycoerythrin-conjugated mAb: G4.18, which recognizes CD3+ T cells; MRC OX-35, which recognizes CD4+ T cells and some macrophages; MRC OX-8; and MRC OX-33, which recognizes the CD45A isofrom found only on B cells, were obtained from Pharmingen (San Diego, CA).

Induction of HN and Assessment of Disease Activity

Fx1A was prepared from outbred Sprague Dawley rat kidneys as described (25). Experimental rats were immunized subcutaneously in their hind footpads with a total of 200 μl of emulsion composed of 15 mg of Fx1A, 1.0 mg of Mycobacterium tuberculosis H37RA (DIFCO Laboratories, Detroit, MI), 100 μl of incomplete Freund's adjuvant (Sigma, St. Louis, MO), and 100 μl of phosphate-buffered saline (PBS). A booster immunization of 7.5 mg of Fx1A, 50 μl of incomplete Freund adjuvant, and 50 μl of PBS was given subcutaneously at the back of the neck 2 wk later. CFA control groups were immunized using emulsion prepared without Fx1A.

Assessment of Disease Activity

Twenty-four-hour urine samples were collected in metabolic cages (Nalgene, Rochester, NY) during which time rats had access to water ad libitum. Urine protein concentrations were determined by colorimetric assay (BioRad, Oakland, CA) and expressed as mg/100 g body wt per 24 h.

Anti-Fx1A Ab titers were determined by enzyme-linked immunosorbent assay, as described (26). Fx1A for enzyme-linked immunosorbent assay was solubilized and purified by fractional salt precipitation (27). Triplicate sample absorbances were read at 405 nm, corrected for a control sample of known strongly positive serum, and expressed as a percentage of sample absorbance/positive serum absorbance. The Fx1A Ab titer of the control positive serum was 1:250.

Glomerular Ig deposition was assessed with MRC OX-12 and immunoperoxidase cytochemistry. The degree of glomerular Ig staining was scored as absent, weak, moderate, and intense.

Immunoperoxidase Cytochemistry

Immunoperoxidase cytochemistry was performed as described (4). Briefly, renal cortical wedge biopsies were embedded in OCT, snap-frozen in liquid nitrogen, and stored at −80°C. Five-micrometer tissue cryosections were air-dried, fixed in acetone, and washed in PBS. The renal cortex was stained for mononuclear cell subsets using the above-described mouse anti-rat mAb and an indirect peroxidase-antiperoxidase complex technique, as previously described (4). The second step Ab for immunoperoxidase cytochemistry was a goat anti-rat IgG alkaline phosphatase, affinity-purified (DAKO, Copenhagen, Denmark). Stained cells in sections were counted on ×400 high-power fields.

Semiqualitative RT-PCR

Total RNA was extracted from BN or renal cortex (perfused in vivo with PBS to remove circulating mononuclear cells) as described (3). Samples of frozen cortex were crushed and further dissociated at 4°C in RNAzol B (Cinna/Biotech, Houston, TX). RNA extraction was then performed following the standard protocol for RNAzol B. The final product was air-dried, dissolved in diethyl pyrocarbonate-treated water, and stored at −80°C. Sample RNA levels were quantified by reading the absorbance at 260 nm. First-strand complementary DNA (cDNA) was synthesized using the Moloney murine leukemia virus.
reverse transcriptase kit (Life Technologies, Grand Island, NY) following the standard protocol. One microgram of total RNA and Primer dT(12) (Boehringer Mannheim) was used to prime the reaction. Controls included samples with no RT enzyme, and reaction mixture without RNA.

Primers were designed from rat mRNA sequences where available, or from regions of homology in murine and human mRNA sequences. The primer sets and their original references for IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, TNF-α, LT/INF-β, perforin/cytolysin, granzyme A, and GAPDH are described elsewhere (3). The primer set for granzyme B was as follows: Gr B, 5'-GAC TGT GTG ACT GCT GCT CAC T-3'; Gr B r, 5'-CCT CCT GTA CTG TTA GCT CAA CCT-3' (27). Amplification of the housekeeping gene GAPDH was used as a positive control for intact RNA and efficiency of RT. Reaction and amplification conditions were optimized for each primer set. Corbett Research FTS-960 thermal sequencers (Corbett Research, Sydney, NSW, Australia) were used to amplify product, which was then analyzed by molecular weight on 6% polyacrylamide gels and stained with ethidium bromide. Specificity of the products was verified by Southern transfer and hybridization with digoxigenin (DIG) 3' end labeled oligonucleotide probes. Hybridized probe was detected using the DIG luminescence detection kit (Boehringer Mannheim). The semiquantitative RT-PCR method modified from Dablman and Porter (28) involved a cycle titration at five-cycle intervals. The cycle number used was that at which bands of PCR product were first identified. Duplicate amplifications were done at each cycle. This technique was reproducible, and within experimental groups similar results were obtained for individual rats. Negative controls without cDNA and positive controls of cDNA from concanavalin A-stimulated rat lymphocytes were included in all experiments. Levels of mRNA expression were compared using the cycle of first appearance of PCR product.

**Staining of LN Cell Subpopulations**

Analysis of lymphocyte subsets in LN was performed by FACS analysis (FACScan, Becton Dickinson, San Jose, CA). LN draining sites of immunization were harvested, and a single cell suspension of lymphocytes in PBS/2% bovine serum albumin was prepared by centrifugation at 300 × g on a Ficoll-Hypaque gradient (specific gravity 1.096) (29). Direct staining was performed, as described (29), with a combination of FITC and phycoerythrin-conjugated mouse anti-rat mAb to cell surface antigens to identify CD4+/CD3+ T cells (MRC OX-35+/G4.18+), CD8+/CD3+ T cells (MRC OX-8+/G4.18+), and B cells (MRC OX-33+/G4.18+).

**Statistical Analyses**

Urine protein estimations, anti-Fx1A Ab levels, leukocyte counts, LN weight, and lymphocyte FACS analysis for each group were expressed as mean ± SEM. Comparisons between groups were made by ANOVA, and when significant were examined by a Bonferroni–Dunn multiple comparisons post hoc test. Semiquantitative RT-PCR results were treated as parametric data with comparisons between groups made by ANOVA and a Bonferroni–Dunn post hoc test, and expressed graphically as the median PCR cycle number at which PCR product was first identified. This is an intentionally conservative analysis of the data, as differences in expression of PCR product are exponential. P ≤ 0.05 was considered significant, unless reduced by Bonferroni–Dunn corrections. Data were analyzed using StatView for Macintosh (Abacus Concepts, Berkeley, CA).

**Results**

**Effect of MMF on the Induction of HN**

Treatment of the induction phase of HN with MMF from 0 to 4 wk prevented disease, with no significant proteinuria at any time point up to 16 wk (Figure 1). MMF inhibited serum anti-Fx1A Ab responses (Figure 2A) and glomerular Ig deposition (Figure 2B) during the 4 wk of therapy, but both appeared after the drug was ceased. At 4 wk, anti-Fx1A Ab titers in 0- to 4-wk MMF were not significantly elevated compared with CFA controls (P = NS), but were significantly lower than untreated HN (P < 0.0001). At 6 wk, the anti-Fx1A Ab titers in 0- to 4-wk MMF were significantly greater than CFA controls (P < 0.01), but significantly lower than untreated HN (P < 0.0001). Similar results were observed at 8 wk, with anti-Fx1A Ab levels in 0- to 4-wk MMF significantly higher than CFA controls (P < 0.01), and significantly lower than untreated HN (P < 0.01). By 12 wk, anti-Fx1A Ab titers in 0- to 4-wk MMF had fallen to a similar level as untreated HN. Glomerular IgG deposition in all tissue samples from 0- to 4-wk MMF was weak to moderate at 4 wk and intense from 8 wk. This was delayed when compared with the deposits in untreated HN, which were intense from 4 wk onward. Deposits were absent in all CFA controls at 4 and 8 wk. At 4 wk, there was no difference in the weight of rats in 0- to 4-wk MMF (256 ± 5 g, mean ± SEM) compared with those in untreated HN (251 ± 3 g); however, rats in both groups weighed less than CFA controls (277 ± 6 g; P < 0.05). There was no significant difference in weight between the three groups by 8 wk.

Proteinuria was not detected in 0- to 4-wk MMF at 4, 6, 8,
**Figure 2.** Anti-Fx1A antibody (Ab) levels and glomerular Ig deposition in 0- to 4-wk MMF compared with untreated HN and CFA control groups. (A) Anti-Fx1A Ab levels were significantly lower in 0- to 4-wk MMF (○) at 4 to 8 wk, but not at 12 wk, compared with untreated HN (□). Fx1A Ab levels in 0- to 4-wk MMF were significantly higher than in CFA controls (■) at 6 and 12 wk, but not at 4 wk. Untreated HN had significantly higher anti-Fx1A Ab levels than in CFA controls at all time points. Anti-Fx1A Ab levels are expressed as percentage binding (compared with known positive serum, 1:250 titer) mean ± SEM, n = 5 per group. *P < 0.01 and **P < 0.0001 for comparisons with 0- to 4-wk MMF. (B) There was significantly less glomerular IgG deposition at 4 wk in 0- to 4-wk MMF compared with untreated HN (P = 0.0051), but not at 8 wk. Glomerular IgG deposition was scored as follows: 0, absent; 1, minimal; 2, moderate; 3, intense. Results are expressed as mean ± SEM, n = 4 per group.

and 12 wk, with levels at the background of CFA controls. A progressive increase in proteinuria was detected in untreated HN at 6, 8, 12, and 16 wk compared with both 0- to 4-wk MMF and CFA controls. At 16 wk, proteinuria in 0- to 4-wk MMF did approach significance when compared with CFA controls. Three rats had virtually no proteinuria (13.1, 9.9, and 9.8 mg/100 g body wt per 24 h), and two had moderate proteinuria of 61.8 and 62.6 mg/100 g body wt per 24 h. Thus, two of five rats had late-onset mild disease, but not of the severity of untreated HN (263 ± 25.3 mg/100 g body wt per 24 h). This group was not studied any further to determine whether the remainder eventually developed proteinuria.

**Examination of Mononuclear Cell Infiltrates in Renal Cortex**

MMF therapy of HN from 0 to 4 wk prevented the development of a renal cortical cellular infiltrate. At 4 wk, small numbers of cells from all populations were identified in renal cortex but the only difference between groups was for less R73+ (αβTCR+) cells in 0- to 4-wk MMF when compared with untreated HN (P = 0.0009) and CFA controls (P = 0.0021) (Figure 3A). This was not reflected in any changes in CD4+ or CD8+ T cell populations.

At 8 wk, there was a prominent interstitial infiltrate in renal cortex from untreated HN that was absent in 0- to 4-wk MMF and CFA controls (Figure 3B). When compared with CFA controls and 0- to 4-wk MMF, there were increased numbers of cells in untreated HN stained by: R73 (αβTCR+), W3/25 (CD4+), 3.2.3 (NK cell), and ED-1 (macrophage). The W3/25+ population in untreated HN exceeded the T cell infiltrate because many of these cells were macrophages, which were also stained by W3/25. ED-1 only stains a portion of infiltrating macrophages, which explains why the W3/25 count is higher than the combined T cell and ED-1 count. MRC OX-8 stains both CD8+ T cells and NK cells, but there was a trivial increase in 3.2.3+ NK cells in untreated HN compared with the MRC OX-8+ population. Thus, the increase in MRC OX-8+ cells in untreated HN was due to CD8+ T cells and not NK cells.

**Examination of Mononuclear Cell Cytokine mRNA in Renal Cortex**

The increased expression of mononuclear cell cytokine mRNA, for Th1 and Th2 cells and macrophages, in the renal cortex of untreated HN at 8 wk was not present in 0- to 4-wk MMF or controls (Figure 4). A lower cycle number indicates earlier amplification of cDNA to detectable levels, and hence more mRNA in the original specimen. Expression of the housekeeping gene GAPDH was identical at 15 cycles in all samples, confirming that similar amounts of cDNA were used in all PCR reactions.

As described previously (3), there is increased expression of cytokines in the renal cortex of rats with HN produced by Th1 (IL-2, IFN-γ, and LT) and Th2 cells (IL-4), and macrophages (TNF-α and IL-10). IL-2, IFN-γ, LT, and IL-4 are specific cytokines for T cells, whereas TNF-α and IL-10 expression may have been upregulated by resident mesangial cells (30), as well as by infiltrating macrophages. MMF therapy prevented
MMF therapy (0- to 2-wk MMF). The weight of popliteal LN (n = 4 per group) from untreated HN, 0- to 2-wk MMF, and CFA controls was increased when compared with unimmunized normal animals. Untreated HN popliteal LN weighed significantly more than both those treated with MMF from 0 to 2 wk and CFA controls (Figure 5A). The weight of LN in 0- to 2-wk MMF was not significantly different from that of CFA controls, but was more than normal LN.

LN from 0- to 2-wk MMF, when compared with unimmunized normal animals, only had an increase in the proportion of CD8+ T cells, but not of CD3+ and CD4+ T cells, and a decrease in the proportion of B cells. Popliteal LN from CFA controls and untreated HN had a significant increase in the proportion of CD3+, CD4+, and CD8+ T cells, and a decrease in the proportion of B cells, compared with the cells from unimmunized normals (Figure 5B).

Semi quantitative RT-PCR of mononuclear cell cytokine mRNA in popliteal LN (Figure 6, A and B) demonstrated less Th2 cytokine mRNA expression (IL-4, IL-5, and IL-6, but not IL-10) in 0- to 2-wk MMF than in untreated HN. MMF did not reduce cytokine mRNA expression for Th1 cells (IL-2, IFN-γ, and LT) and cytotoxic cells (granzyme B and perforin) compared with untreated HN, except for granzyme A mRNA, which was undetectable. In all specimens, GAPDH mRNA was first detected at 20 cycles. There was significantly increased cytokine mRNA expression in LN from the untreated HN group when compared with unimmunized normals, for Th1 cells (IL-2, IFN-γ, and LT), Th2 cells (IL-4, IL-5, and IL-6, but not IL-10), and cytotoxic cells (granzyme A and perforin, but not granzyme B). The increase in cytokine mRNA expression in CFA controls compared with unimmunized normals was only significant for IL-6.

Effect of MMF on Evolving HN

MMF therapy of HN from 4 to 8 wk did not ameliorate evolving disease. Table 1 summarizes data from three separate experiments using different batches of Fx1A, and the degree of proteinuria in each separate control group differs because the severity of disease varies with the batch of antigen used. During this phase, anti-Fx1A Ab responses and glomerular IgG deposition are established, there is a progressive glomerular cellular infiltrate, and proteinuria develops. Urine protein excretion levels in 4- to 8-wk MMF and untreated HN were not different at 4 wk (40.8 ± 9.0 compared with [c/w]25.0 ± 8.6 mg/100 g body wt per d, respectively, P = NS). Despite 4 wk of MMF treatment, proteinuria continued to increase at 8 wk with no significant difference between the groups (307.4 ± 22.8 c/w 314.8 ± 18.0 mg/100 g body wt per d, P = NS). Serum anti-Fx1A Ab levels, expressed as percentage of positive binding, were also not different between 4- to 8-wk MMF and untreated HN, being 79.9 ± 5.1% c/w 70.7 ± 5.9%, respectively (P = NS) at 4 wk, and 68.7 ± 5.8% c/w 88.4 ± 13.6% (P = NS) at 8 wk. There was no difference in glomerular IgG deposition between 4- to 8-wk MMF and untreated HN, with glomeruli from both groups staining intensely at 8 wk.

the increased expression of all of these cytokines except IL-2. IL-6 was not detected in any group. Granzyme A and perforin mRNA, effector CD8+ T cell, and NK cell molecules had high background levels, and increases were not readily detected in HN.

Examination of LN Draining Sites of Primary Immunization

The effect of MMF on initial immune events was examined in LN draining sites of primary immunization after 2 wk of

Figure 3. Cellular infiltrates in renal cortex at 4 and 8 wk in 0- to 4-wk MMF compared with untreated HN and CFA control groups. (A) At 4 wk, significantly less R73+ (αβ TCR) cells were identified in 0- to 4-wk MMF (■) than in untreated HN (□) or CFA controls (■). There were no other significant differences between groups at 4 wk. (B) At 8 wk, a significant cellular infiltrate present in untreated HN was absent in 0- to 4-wk MMF and CFA controls. This infiltrate comprised all cell subsets examined. There were no differences between 0- to 4-wk MMF and CFA controls at 8 wk, other than significantly less OX-8+ (CD8) cells in 0- to 4-wk MMF. Results are expressed as cells/high-power field (HPF), 10 HPF per specimen, n = 4 per group. *P < 0.005; **P < 0.001; ***P < 0.0001.
Figure 4. Mononuclear cell cytokine mRNA expression in renal cortex at 8 wk in 0- to 4-wk MMF compared with untreated HN and CFA control groups. (A) Summary of reverse transcription-PCR results shows increased cytokine mRNA in untreated HN (n = 4) for cytokines of Th1 (interferon-γ [IFN-γ], lymphotoxin [LT], but not interleukin-2 [IL-2]), Th2 (IL-4, but not IL-6), and macrophage (tumor necrosis factor-α [TNF-α], but not IL-10) subsets, compared with 0- to 4-wk MMF (n = 3) and CFA controls (n = 3). No significant difference in mRNA expression between 0- to 4-wk MMF and CFA controls was detected for any cytokine. There was no significant difference between groups for cytotoxic cell cytokines (granzyme A [GrA], perforin [Perf]). PCR results expressed as median cycle number at which product was first detected. *P < 0.01, **P < 0.001, and ***P < 0.0001 for comparisons with untreated HN. U, PCR product undetectable. (B) Cytokine mRNA profiles of representative rats for each group. Results for each cytokine are shown at five-cycle intervals from 20 to 35 cycles. GAPDH is shown at 10 to 25 cycles.
Figure 5. Weight and lymphocyte subset analysis at 2 wk in popliteal lymph node (LN) draining sites of immunization in 0- to 2-wk MMF compared with untreated HN, CFA control, and normal unimmunized groups. (A) All immunized LN weighed significantly more than normal unimmunized (■), with untreated HN (▲) weighing significantly more than 0- to 2-wk MMF (■) and CFA controls (■). There was no significant difference in weight between 0- to 2-wk MMF and CFA controls. Weight is expressed in milligrams, mean ± SEM, n = 4 per group. (B) The proportion of B cells (MRC OX-33*/G4.18*) in LN from all immunized groups was significantly lower than in unimmunized normals, and there was no significant difference between CFA controls, untreated HN, and 0- to 2-wk MMF. There was a significant increase in both CD3+ T cells (G4.18*/MRC OX-33*) and in CD4+ T cells (MRC OX-35*/G4.18*) in CFA controls and untreated HN, compared with unimmunized normals and 0- to 2-wk MMF. There was an increase in CD8+ T cells (MRC OX-8*/G4.18*) in CFA controls, untreated HN, and 0- to 2-wk MMF compared with unimmunized normals. Results are expressed as percentage of total lymphocytes, mean ± SEM, n = 4 per group. *P < 0.01; **P < 0.0005; ***P < 0.0001.

Proteinuria did not improve in rats with evolving HN treated with MMF for a more extended time period of 6 to 12 wk (Table 1). At 6 wk, proteinuria was 27.1 ± 5.3 and 31.5 ± 12.2 mg/100 g body wt per d in 6- to 12-wk MMF and untreated HN, respectively (P = NS), and at 12 wk had respectively increased to 135.9 ± 45.1 and 145.6 ± 23.9 mg/100 g body wt per d (P = NS). Fx1A Ab levels at 6 wk in 6- to 12-wk MMF and untreated HN were 104.7 ± 19.4% and 118.1 ± 18.8% of positive binding, respectively (P = NS), and at 12 wk were 69.7 ± 6.4% and 74.4 ± 11.5% (P = NS). Glomerular IgG deposition was not studied in this group.

Treatment of HN with MMF from 4 to 8 wk did not prevent the development of renal cortical infiltrates (Table 1). There was no difference in the size of the mononuclear cell infiltrate in 4- to 8-wk MMF and untreated HN at 8 wk when stained for R73+ (αβTCR+, P = NS) and ED-1+ cells (macrophage, P = NS).

**Effect of MMF on Established HN**

Treatment of established HN with MMF did not reduce proteinuria (Table 1). Proteinuria in 10- to 14-wk MMF when compared with untreated HN was not significantly different at 10 and 14 wk. Anti-Fx1A Ab levels and glomerular Ig deposition were not assessed in this group.

**Discussion**

Therapy with MMF for 4 wk from the time of disease induction prevented significant proteinuria for up to 16 wk. No serum anti-Fx1A Ab was detected during the 4 wk of treatment, but after this there was a limited response. Glomerular Ig deposition was only slightly attenuated by MMF at 4 wk, and was similar to that in untreated HN by 8 wk. However, MMF completely prevented the renal cortical mononuclear cell infiltrate of T cells, NK cells, and macrophages observed in untreated HN. Furthermore, in the absence of this infiltrate the cytokine profile of activated Th1 cells and macrophages was not detected by RT-PCR. Later treatment with MMF, during evolution of or during established HN, had no effect on any disease parameter even though the dose used in our study approached the maximum that rats tolerate for extended therapy (31) (E. Eugui, personal communication).

MMF limited enlargement of the popliteal LN draining sites of immunization by limiting the increase in CD3+ and CD4+ T cells that occurred in HN. This may have been due to MMF impairing recruitment of lymphocytes into LN by altering fructose and mannose glycoproteins on the lymphocyte surface so that they were not recognized by selectins (32). MMF would also have prevented T cell proliferation within the node. An
unexpected effect was that MMF prevented the induction of Th2 cytokine mRNA, in that IL-4, IL-5, and IL-6 mRNA was less than in normal LN, and was not increased as in untreated HN LN. The increase in IL-10 mRNA may have been due to production by activated mφ, as well as Th2 cells. Th1 and cytotoxic cell cytokine mRNA expression in LN, except for granzyme A, was not inhibited by MMF. The sparing of Th1 cytokines was consistent with the reports that MMF does not inhibit early cytokine production (33–35) such as IL-2, or the expression of IL-2 receptors by T cells (CD4+ and CD8+) stimulated by mitogens or mixed lymphocyte reactions (36,37). MMF blocks T cells after the initial phase of cytokine induction in that it impairs cell division by limiting the availability of purines. Our results showing that MMF had a preferential effect on the activation of Th2 rather than Th1 cells in LN draining sites of immunization have not been reported previously. The failure to identify Th1 and macrophage cytokines in renal cortex in MMF-treated rats reflects the lack of an infil-

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**Figure 6.** Mononuclear cell cytokine mRNA expression at 2 wk in popliteal LN draining sites of immunization in 0- to 2-wk MMF (■) compared with normal unimmunized (■), CFA control (■), and untreated HN (■) groups. (A) Summary of reverse transcription-PCR results shows preferential suppression of Th2 cell cytokine mRNA (IL-4, IL-5, IL-6, but not IL-10) in 0- to 2-wk MMF LN compared with untreated HN. MMF did not suppress cytokine mRNA for Th1 cells (IL-2, IFN-γ, LT). Cytotoxic T cell cytokine mRNA for granzyme B (GrB) and perforin (Perf) was unaffected by MMF; however, granzyme A (GrA) was suppressed. PCR results are expressed as median cycle number at which PCR product was first detected, n = 4 per group. (B) Representative cytokine mRNA profiles of LN. Results for each cytokine are shown at five-cycle intervals from 35 to 20 cycles. GAPDH was assayed from 10 to 25 cycles. *P ≤ 0.01; **P ≤ 0.001; ***P ≤ 0.0001. U, PCR product undetectable.
trate and the T effector mechanisms that mediate HN. Th2 cytokines are not identified in HN kidneys (3). A preferential effect of MMF or MPA on activated Th2 cells has not been described in vivo. There is indirect in vitro evidence suggesting that T cell-dependent B cell responses (Th2) are suppressed with a lower concentration of MMF or MPA than is required for suppression of cell-mediated responses (Th1) (36). MMF is also known to be effective at inhibiting both Th2 cell-mediated B cell proliferation and preventing the production of Ab to foreign antigens by B cells (36). The relative inhibition of Th2 responses with MMF therapy did not totally prevent the development of serum anti-Fx1A Ab, and hence glomerular immune deposits which further increased after cessation of the drug. Thus, MMF only partially suppresses the B cell response in HN and did not induce tolerance.

The injury resulting in proteinuria is mediated by both anti-Fx1A Ab and activated Th1 and cytotoxic cells (3,4). This study showed that MMF therapy had no effect on Fx1A Ab titers, glomerular Ig deposits, or mononuclear cell infiltration into the kidneys of rats with evolving or established HN. MMF has previously been shown not to suppress Ab production in already activated B cells (9). MMF and MPA also do not impair the function of activated cytotoxic T cells (33,36,37). Thus, MMF would not be expected to affect either Ab or T cell-mediated glomerular injury, once these responses are established. The action of MMF on adhesion molecules such as selectins and vascular cell adhesion molecule-1 may prevent the glomerular recruitment of mononuclear cells, which have been suggested to mediate injury in HN (3,4). However, MMF therapy of evolving HN, when glomerular infiltrates are developing but before the development of interstitial infiltrates (3), did not modify disease. The resistance of evolving and established disease is unlikely to be due to inadequate dosing of MMF as the same dose prevented the induction of HN, and is the maximum tolerated by rats.

Drug therapy of active HN, from time of induction of disease, has met with varying success. Glucocorticoid monotherapy reduces the severity of HN (5), as does ACTH and nitrogen mustard (5), but these therapies do not prevent the eventual development of proteinuria. Combination chemotherapy with prednisolone, cyclophosphamide (CP), and azathioprine (AZA) prevented the development of HN (6), including complete suppression of the Fx1A Ab response. Treatment of active HN from induction of disease with CsA, which principally impairs T cell mechanisms, reduced Ab production, glomerular Ig deposition, and proteinuria during the course of therapy. Disease developed and progressed with severity equivalent to untreated HN after cessation of CsA (4,38). In contrast, treatment of active HN from induction of disease with FK-506 suppressed Fx1A Ab responses and glomerular IgG deposition, and prevented proteinuria (7). MMF monotherapy during the induction of HN appears as effective as anti-CD4 mAb therapy (4). Our results with MMF are superior to those reported with other immunosuppressive agents, which either have no effect or only attenuate disease (such as CsA or ATG). FK-506 (7) or prolonged triple therapy with prednisolone, CP, and AZA (6) from the time of immunization are the only reported drug therapies reported to be as effective as MMF in preventing the induction of HN.

Evolving HN is unresponsive to all therapies including methylprednisone, CP, or AZA monotherapy (39), but triple drug therapy (6) or FK506 (7) delayed and reduced disease severity. Treatment of established active HN with glucocorticoids (40), CP or 6-mercaptopurine (40), a combination of prednisolone, CP, and AZA (6) or CsA (38) did not reverse proteinuria.

No immunosuppressive agent or mAb given alone or in combination has been shown to be effective in reversing established disease. The resistance of established disease to MMF and other therapies may be due to a threshold level of

### Table 1. Proteinuria and anti-Fx1A Ab levels in 4- to 8-wk MMF, 6- to 12-wk MMF, and 10- to 14-wk MMF, compared with untreated HN and CFA control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Proteinuriaa</th>
<th>Anti-Fx1A Ab Levelsc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 wk</td>
<td>8 wk</td>
</tr>
<tr>
<td>Untreated HN</td>
<td>5</td>
<td>25.0 ± 8.6</td>
</tr>
<tr>
<td>4- to 8-wk MMF</td>
<td>5</td>
<td>40.8 ± 9.0</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>12 wk</td>
</tr>
<tr>
<td>Untreated HN</td>
<td>5</td>
<td>31.5 ± 12.2</td>
</tr>
<tr>
<td>6- to 12-wk MMF</td>
<td>5</td>
<td>27.1 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>12 wk</td>
</tr>
<tr>
<td>Untreated HN</td>
<td>5</td>
<td>310.1 ± 26.8</td>
</tr>
<tr>
<td>10- to 14-wk MMF</td>
<td>5</td>
<td>295.9 ± 25.0</td>
</tr>
</tbody>
</table>

a MMF therapy did not alter the course of evolving or established HN. Ab, antibody; MMF, mycophenolate mofetil; HN, Heymann nephritis; CFA, complete Freund’s adjuvant; NA, not available.

b Proteinuria expressed as mean ± SEM mg/100 g body wt per 24 h, n = 5 per group.

c Anti-Fx1A Ab levels expressed as percentage binding (compared with known positive serum, 1:250 titer) mean ± SEM, n = 5 per group.
damage to the filtration barrier beyond which repair is not possible. It should be noted that the relative severity of the nephrotic syndrome in HN rats in this study is more than an order of magnitude greater than that in human disease. MMF may be of benefit in the less severe clinical situation. However, established proteinuria in active HN can be reversed by total lymphoid irradiation (41) and transplantation of active HN kidneys into normal hosts (42), both resulting in partial to complete abrogation of proteinuria in the presence of persistent glomerular IgG deposits.

The optimal treatment for human idiopathic MN is unresolved due to the variable natural history of the disease and the limitations of clinical trials (43). Steroid therapy alone is not consistently beneficial (44,45). Cytotoxic therapy improves the chance of complete remission of proteinuria (46) and may preserve renal function (47). In idiopathic MN, CsA can reduce proteinuria, but relapses occur after cessation of therapy (48,49). CsA has been shown to benefit long-term renal function in this disease (50). Both cytotoxic agents and CsA are associated with significant risks of long-term toxicity. As MMF appears superior to CsA and cytotoxic agents and similar to FK506 in the treatment of active HN, and its clinical toxicity profile is less than those of the other drugs (11), it may have a therapeutic role in human idiopathic MN.

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References


