Modulation of autoantibody production by mycophenolate mofetil: effects on the development of SLE in (NZB×NZW)F₁ mice

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Abstract

Background. Mycophenolate mofetil (MMF) has been successfully used to improve or prevent the development of systemic lupus erythematosus (SLE) in both humans and in several lupus-prone mice. In the present study, we evaluated mechanisms through which MMF may exert its therapeutic effect on the development of systemic autoimmunity.

Methods. (NZB×NZW)F₁ female mice were continuously treated with 100 mg/kg/day (high dose) or 30 mg/kg/day (low dose) MMF beginning at 3 months of age. The development of an autoimmune syndrome was evaluated by measuring immunoglobulin (Ig) isotypes of autoantibodies and their levels, as well as by evaluating immunopathological kidney abnormalities and mortality curves.

Results. At both doses, MMF efficiently modulated the development of SLE. Although the higher dose of MMF directly inhibited the production of autoantibodies, 30 mg/kg/day MMF promoted qualitative but not quantitative changes in autoantibodies in (NZB×NZW)F₁ female mice. These qualitative changes were manifested as a selective reduction in total or antigen-specific IgG2a antibody levels.

Conclusions. The mechanisms through which MMF controls the development of SLE in (NZB×NZW)F₁ females is highly dependent upon immunosuppressor dose. Interestingly, lower dose MMF selectively reduced IgG2a antibody levels, suggesting that this dose may modulate TH₁ CD4⁺ activity.

Keywords: autoantibodies; mycophenolate mofetil; (NZB×NZW)F₁ mice; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a chronic and progressive autoimmune syndrome characterized by the production of multiple autoantibodies (autoAbs), resulting in the generation of circulating immune complexes (ICs). The deposition of these ICs leads to the development of tissue lesions, such as glomerulonephritis, which is the major cause of death in these patients. Studies in several mouse strains that spontaneously develop an autoimmune syndrome resembling human SLE have been of considerable value in elucidating the cellular and molecular mechanisms responsible for the disease. These studies have demonstrated that multiple genetic and environmental factors play essential roles in the development of this autoimmune syndrome. In addition, it has been clearly shown that CD4⁺ T-cells, especially those belonging to the TH₁ subset, are major contributors to both autoAb production and tissue damage in these animals [1–3].

It has been clearly demonstrated that autoAbs are the essential factors for many of the clinical manifestations associated with SLE [4]. However, it has never been established whether all of the autoAbs generated during the course of autoimmune diseases are pathogenic. In fact, because of occasional lacking of correlations between serum levels of autoAbs and clinical manifestations, it has long been suggested that qualitative aspects of autoAbs may be important in their ability to induce cellular and tissue injuries. Although the nature of such qualitative pathogenic aspects are largely unknown, it is conceivable that the immunoglobulin (Ig) class switch may be critical for the pathogenic potential of autoAbs, since this process has been shown to accompany changes in antibody effector functions [5,6].

The availability of murine SLE models has also been very useful for exploring the efficacy of various therapies using immunosuppressive drugs. Although several immunosuppressive treatments with steroids,
azathioprine, cyclophosphamide or cyclosporin A [7–9] have been used to inhibit the development of lupus nephritis, these involve a high risk of serious side effects. This problem has stimulated the development of extensive research programmes to find new immunosuppressive drugs with lower toxicity. Mycophenolate mofetil (MMF) is a prodrug of mycophenolic acid (MPA) that was introduced as an effective immunosuppressor for the prevention of allogenic rejection following kidney transplantation [10]. MMF has also been used to successfully prevent SLE in several lupus-prone mice strains, including MRL.lpr and (NZB × NZW)F₁ mice [11–13]. However, the precise mechanism through which MMF modulates the development of SLE in mice remains controversial. In some studies, the therapeutic effect of MMF is secondary to inhibition of autoAb production [11,12], whereas in others, MMF blocks the development of IC-mediated glomerulonephritis but not the production of autoAbs [13]. To gain further insight into the mechanisms responsible for the therapeutic effect of MMF, we treated (NZB × NZW)F₁ female mice with either high or low doses of MMF starting at 3 months of age, which is when increased serum levels of antinuclear autoAbs are already present. Our results demonstrated that treatment with MMF protects these mice against the development of SLE. Interestingly, the mechanism responsible for this protection depended on the dose of MMF. At high doses, MMF directly inhibited the production of autoAbs, whereas at lower doses MMF promoted qualitative but not quantitative changes in the autoAbs of (NZB × NZW)F₁ female mice.

**Subjects and methods**

**Mice and treatments**

(NZB × NZW)F₁ female mice and Balb/c mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). MMF (generously provided by Roche Pharmaceuticals, Inc., Palo Alto, CA, USA) was administered i.p. in a suspension made of 0.9% benzyl alcohol, 0.9% sodium chloride, 0.5% carboxymethylcellulose and 0.4% polysorbate 80 in water. Mice received daily i.p. injections of 100 or 30 mg/kg/day MMF starting at 3 months of age until the end of the experiment. Solvent-treated (NZB × NZW)F₁ females and untreated BALB/c mice were used as positive and negative controls, respectively. Random serum samples were obtained 2–4 h after MMF administration and the levels of MPA, the active metabolite of MMF, were measured by EMIT assay (Syva Co., Dade Behring Inc., Cupertino, CA, USA). Serum levels of MPA ranged between 8 and 24 μg/ml. Mice were bled from the retroorbital plexus and serum were stored at −20°C until use. All in vivo experiments with mice were performed in compliance with the Guide for the Care and Use of Laboratory Animals (ILAR, 1985).

In some experiments, (NZB × NZW)F₁ female mice were first treated with 30 mg/kg/day MMF or solvent alone and then immunized with 400 μg i.v. heat-aggregated human gammaglobulin (AHGG) (generously provided by S. Izui, University of Geneva, Switzerland) 15 days after the beginning of treatment. Serum samples were obtained 15 days after immunization.

**Serological assays**

Serum levels of total IgG and IgG subclasses were determined by ELISA, as described elsewhere [3]. Results were expressed as mg/ml in reference to a standard curve obtained with a mouse reference serum (ICN ImmunoBiologicales, Costa Mesa, CA, USA). IgG anti-ssDNA and anti-dsDNA autoAbs were measured in sera by ELISA and results were expressed in titration units (TU) in reference to a standard curve obtained with a serum pool from 6–8-month-old MRL/lpr/lpr mice [3]. Serum levels of total IgG, IgG1 and IgG2a anti-HGG antibodies were measured by ELISA 15 days after immunization [3]. Results are expressed in TU (in the case of IgG anti-HGG) in reference to serum pools from AHGG-immunized mice or in absorbance units at 405 nm (in the case of IgG1 and IgG2a anti-HGG).

**Proteinuria, histopathology and immunofluorescence**

Proteinuria was assessed by determination of urine albumin using reagent strips (Bayer, Spain) and was scored on a 0–4 scale with 0 indicating the absence or traces of proteinuria, 1 indicating proteinuria values between 30 and 100 mg/dl, 2 indicating values between 100 and 300 mg/dl, 3 indicating values between 300 and 1000 mg/dl and 4 indicating values > 1000 mg/dl.

Samples of all major organs were obtained at autopsy, fixed in 4% phosphate buffered formalin and embedded in paraffin. Histological sections (4–6 μm) were stained with haematoxylin eosin (HE) and were examined in a blind fashion for pathology. Glomerulonephritis was scored on a 0–4 scale with 0 indicating the absence of glomerular lesions, 1 indicating 1–10% of glomeruli affected, 2 indicating 11–25% of glomeruli affected, 3 indicating 26–50% of glomeruli affected and 4 indicating > 50% of glomeruli affected.

Tissue-bound IgM and IgG antibodies were studied by immunofluorescence on kidney sections using a fluorescein-iodinated rabbit anti-mouse IgM or rabbit anti-mouse IgG. As a second step, we used a fluorescein isothiocyanate-conjugated goat anti-rabbit Ig. All antibodies were purchased from Tago Inc. (Burlingame, CA, USA).

**Statistical analysis**

Statistical analysis was performed with the Wilcoxon two-sample test. P-values of > 5% were considered insignificant.

**Results**

**Effects of high and low dose MMF on survival rate and on the development of glomerulonephritis in (NZB × NZW)F₁ mice**

To explore the mechanism through which MMF modulates the development of SLE, (NZB × NZW)F₁ female mice were continuously treated beginning at 3 months of age with either high (100 mg/kg/day) or low (30 mg/kg/day) doses of MMF. As previously reported [11], MMF had a therapeutic effect on SLE development in (NZB × NZW)F₁ females (Figures 1
and 2). Thus, while untreated (NZB × NZW)F1 females showed a marked proteinuria at 8 months of age (mean score of proteinuria: 3.1 ± 1.2), mice treated with low (mean score of proteinuria: 1.2 ± 1.1) or high (mean score of proteinuria: 1.3 ± 0.8) doses of MMF had lower proteinuria values that were comparable to values observed in normal Balb/c mice (mean score of proteinuria: 0.7 ± 0.4). Furthermore, untreated (NZB × NZW)F1 female mice developed lethal glomerulonephritis (mean of glomerular score: 3.5 ± 0.3), characterized by increased mesangial and glomerular cellularities, obliteration of glomerular architecture and the presence of tubular cast formation (Figure 1B).

The presence of IgM and IgG deposits in the glomeruli of untreated and MMF-treated mice (low and high dose-treated mice) was evaluated in (NZB × NZW)F1 mice at the end of treatment by immunofluorescence on kidney cryosections. The intensity of fluorescence is indicated (0 to +++).

Using immunofluorescence, large amounts of IgM and IgG deposits were observed as accumulations in both mesangial areas and in peripheral capillary loops (Table 1). Again, treatment with low or high doses of MMF significantly reduced or inhibited the development of glomerular lesions (mean of glomerular score in low-dose treated mice: 1.7 ± 0.5, Figure 1C; mean of glomerular score in high-dose treated mice: 0.9 ± 0.3; Figure 1D) compared with untreated mice.

Immuno-fluorescence studies on kidney cryosections revealed the presence of IgM but not IgG deposits in mice treated with 30 mg/kg/day MMF, but the absence of Ig deposits of both isotypes in mice treated with 100 mg/kg/day (Table 1). In agreement with the glomerulonephritis data, 50% of untreated (NZB × NZW)F1 females died at 42 weeks of age, whereas > 90% of both low and high dose MMF-treated (NZB × NZW)F1 mice were still alive at this age.

**Fig. 1.** Representative histological appearance of glomeruli from 8-month-old Balb/c mice (A) and from 8-month-old (NZB × NZW)F1 females treated from 3 months of age with either solvent (B), 30 mg/kg/day MMF (C) or 100 mg/kg/day MMF (D). HE staining. Original magnification: ×40.

**Fig. 2.** Cumulative mortality in (NZB × NZW)F1 females treated from 3 months of age with solvent (circles), 30 mg/kg/day MMF (triangles) or 100 mg/kg/day MMF (squares).

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**Table 1.** Effects of low-dose and high-dose MMF on IgM and IgG glomerular deposits in (NZB × NZW)F1 female mice.

880 Ma Angeles Ramos et al.

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In addition, both groups of MMF-treated (NZB×NZW)F_{1} mice showed an equivalent mortality rate.

Effect of high and low dose MMF on autoAb production in (NZB×NZW)F_{1} mice

In the present studies, effects of low or high dose MMF on autoAb production in (NZB×NZW)F_{1} mice were evaluated. At 6 months of age, treatment with 100 mg/kg/day MMF significantly decreased levels of serum IgG (Figure 3A) compared with untreated (NZB×NZW)F_{1} females and completely inhibited the production of IgG anti-dsDNA (Figure 3B) and IgG anti-ssDNA (Figure 3C) autoAbs ($P<0.002$ in all cases). However, treatment with 30 mg/kg/day MMF had no effect on hypergammaglobulinaemia development or anti-DNA production. Indeed, autoAb production was similar in these animals and untreated (NZB×NZW)F_{1} female mice.

Our present results clearly indicate that inhibition of autoAb production is the mechanism by which high dose MMF modulates the development of SLE in (NZB×NZW)F_{1} female mice. However, mice treated with low dose MMF failed to develop histopathological kidney lesions despite elevated production of autoAbs. We postulate that the protective effect of low dose MMF in (NZB×NZW)F_{1} mice may be related to qualitative changes in autoAbs produced in this strain of mice. To explore this possibility more directly, the levels of different subclasses of IgG were compared in untreated and low dose MMF-treated (NZB×NZW)F_{1} mice (Figure 4). A small but non-significant ($P>0.5$) increase in IgG1 antibody levels was observed in low dose MMF-treated animals (mean serum levels of IgG1 in untreated (NZB×NZW)F_{1} female mice: $2.3 \pm 1.9$ mg/ml vs $3.8 \pm 3.7$ mg/ml in mice treated with 30 mg/kg/day MMF), whereas serum levels of total IgG2b and IgG3 antibodies were essentially identical in untreated and low dose MMF-treated (NZB×NZW)F_{1} mice. Interestingly, the levels of IgG2a antibodies were significantly decreased after treatment with 30 mg/kg/day MMF ($P<0.02$).

To further address whether low dose MMF promoted qualitative changes in humoral immune-responses, 3-month-old (NZB×NZW)F_{1} female mice

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**Fig. 3.** Serum levels of total IgG (A), IgG anti-dsDNA (B) and IgG anti-ssDNA (C) in 6-month-old (NZB×NZW)F_{1} females treated from 3 months of age with solvent, 30 mg/kg/day MMF or 100 mg/kg/day MMF and in 6-month-old Balb/c mice.

**Fig. 4.** Serum levels of total IgG1 (A), IgG2a (B), IgG2b (C) and IgG3 (D) in 6-month-old (NZB×NZW)F_{1} females treated from 3 months of age with solvent or 30 mg/kg/day MMF. Results are expressed in mg/ml.
Discussion

In the present study, we analysed potential mechanisms through which MMF may modulate the development of systemic autoimmune diseases. In agreement with previous reports [11], we demonstrated that continuous treatment with MMF in (NZB × NZW)F1 female mice inhibited the development of SLE. However, the mechanism involved in this protection depended upon the MMF dose. At the higher dose (100 mg/kg/day), MMF caused a direct and near complete inhibition of autoAb production that correlated with the absence of kidney immunopathology and with prolonged survival. Since the development of SLE in these animals is primarily mediated by the deposition of autoAb-containing ICs in affected organs, it is possible that inhibition of autoAb production is the mechanism through which high dose MMF blocks the development of SLE in (NZB × NZW)F1 female mice.

At lower doses of MMF (30 mg/kg/day), a different mechanism was involved in protection against SLE. Indeed, (NZB × NZW)F1 female mice treated with 30 mg/kg/day MMF produced essentially identical levels of autoAbs as solvent-treated controls, despite prolonged survival rates and reduced kidney disease. Nevertheless, the decreased severity of SLE in female mice treated with 30 mg/kg/day MMF was associated with qualitative changes in circulating antibodies, manifested by a significant reduction in IgG2a antibody levels and a concomitant slight, but non-significant, increase in serum IgG1 antibodies. A similar effect was observed when we analysed immune responses against exogenous T-cell-dependent antigen HGG. Again, treatment with low doses of MMF promoted a selective reduction in IgG2a anti-HGG antibody levels. As stated above, autoAbs are essential factors for many of the clinical manifestations of SLE [4]. However, not all autoAbs produced in the course of an autoimmune reaction may be pathogenic, and their ability to promote tissue damage can be influenced by both Fab region-dependent properties (specificity and affinity) and effector functions associated with the Fc region of different Ig isotypes. In this regard, the Fc region of immunoglobulins influences the ability of the different Ig isotypes to activate the complement cascade [5] and alters their ability to interact with Fcγ receptors (FcγR) to promote antibody-dependent cellular cytotoxicity [6]. This latter mechanism seems to play an essential role in the development of nephritis in (NZB × NZW)F1 females treated with either solvent or 30 mg/kg/day MMF at 15 days after i.v. immunization with 400 μg AHGG. Results are expressed as optical density with units set at 405 nm.

Fig. 5. Serum levels of total IgG (A), IgG1 (B) and IgG2a (C) anti-HGG antibodies in (NZB × NZW)F1 females treated with either solvent or 30 mg/kg/day MMF at 15 days after i.v. immunization with 400 μg AHGG. Results are expressed as optical density with units set at 405 nm.
mice having prolonged survival, a reduced severity of the disease correlated with a decrease in IgG2a and IgG3 antibody levels and an increase of IgG1 antibodies [17]. In accordance with the selective reduction in serum IgG2a antibody levels, (NZB × NZW)F1 mice treated with 30 mg/kg/day MMF that showed increased production of IgG autoAbs also showed IgM but not IgG glomerular deposits. These results further emphasize the particular pathogenic potential of the IgG2a isotype, which is by far the predominant IgG subclass detected in glomeruli of untreated (NZB × NZW)F1 females [18].

Several studies have demonstrated that the development of SLE in lupus-prone mice is dependent upon the activity of CD4+ T-cells belonging to the T(H)1 subpopulation [1–3]. Thus, repeated injections of recombinant interferon-γ (IFNγ) promote an acceleration of the disease in (NZB × NZW)F1 mice, and treatment in these mice with anti-IFNγ monoclonal antibodies completely blocks the development of SLE [1]. In addition, the acceleration of SLE found in MRL mice bearing the Yaa gene (Y-linked autoimmune acceleration) is associated with an increased T(H)1 activity [3], whereas interleukin-4 transgenic (NZW × C57BL/6)YaaF1 mice, having an imbalance towards T(H)2 predominance, fail to develop an autoimmune syndrome [2]. Since IFNγ, produced by CD4+ T(H)1 cells, is known to positively regulate IgG2a and IgG3 isotype antibody production [19], it is tempting to speculate that the changes in the IgG isotype profile observed in low dose MMF-treated (NZB × NZW)F1 female mice may be due to an effect of this drug on the activity of T(H)1 CD4+ cells. The downregulation of T(H)1 activity observed in our study is in contrast with the protective effect of MMF in other types of experimental nephritis, such as in Heyman nephritis in rats where MMF-induced protection is associated with inhibition of T(H)2 activity [20].

These differences may be due to variations in the treatment regimen employed in these studies, to the animal species used (rat or mouse), to the type of pathology studied or to a combination of these factors. Further investigations will help to elucidate these important questions.

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